UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Composición bioactiva de materiales lignocelulósicos: Determinación de compuestos fenólicos y elagitaninos con propiedades antioxidantes y anticancerígenas mediante técnicas analíticas diversas

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Resumen

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Se han desarrollado métodos analíticos que permiten la caracterización y cuantificación de compuestos bioactivos presentes en el corcho procedente del alcornoque *Quercus suber* L.

Se han aplicado técnicas de HPLC desarrollando nuevos métodos para el análisis de compuestos fenólicos de bajo peso molecular y elagitaninos, así como el aislamiento de elagitaninos mediante cromatografía líquida preparativa.

Se han estudiado diferentes procesos de extracción de compuestos fenólicos de bajo peso molecular y de elagitaninos en el corcho. Se han caracterizado dichos compuestos mediante la determinación de fenoles totales, taninos totales, actividad antioxidante, compuestos fenólicos de bajo peso molecular y elagitaninos.

Se han propuesto métodos para determinar el contenido de fenoles totales de bajo potencial de oxidación en aguas de cocido de corcho mediante técnicas electroanalíticas utilizando diferentes tipos de electrodos, como carbón vitrificado y electrodos impresos modificados con grafeno. Por otro lado, se ha propuesto un método para la determinación del contenido total de compuestos fenólicos en aguas de cocido de corcho mediante análisis de inyección de flujo con detección amperométrica, utilizando electros impresos modificados con grafeno.

Índice

1. Introducción

1.1. El corcho

Los materiales lignocelulósicos son los materiales más abundantes que se encuentran en la naturaleza. Están compuestos principalmente de celulosa, hemicelulosa y lignina. Dentro de este tipo de materiales encontramos el corcho, que se extrae del alcornoque (*Quercus suber* L.).

El corcho está formado por células muertas que protegen las paredes vivas del tronco y ramas del alcornoque. Se forma como consecuencia del crecimiento en grosor del árbol, o crecimiento secundario. La zona donde se produce está formada por un conjunto o camada de células que se mantienen vivas hasta que, con la "*saca*" (proceso de extracción del corcho del alcornoque), mueren. Esta camada recibe el nombre de *felógeno*.

En una plancha de corcho se pueden distinguir la *espalda* y el *vientre* o *barriga*. La *espalda* es la parte que está, antes de la *saca*, en contacto con el aire y en ella se encuentra una zona leñosa, dura, oscura y frágil que se denomina *raspa*. El *vientre* o *barriga* es la parte que en el momento de la *saca* está en contacto con los tejidos vivos del árbol.

Figura 1.1.1. Barriga (B) y espalda (E) del corcho.

En el corte transversal del tronco del alcornoque, el anillo situado entre el corcho y la madera recibe el nombre de *casca* o *capa madre*, por donde circula la savia elaborada. En la *capa madre* hay dos zonas que están produciendo células, y lo hacen en sentido opuesto. Así, el *felógeno* produce corcho hacia el exterior y, en la parte interna, el cambium vascular produce madera o xilema hacia el interior.

En el costado de la plancha se verifica el grosor, que recibe el nombre de *calibre*. También se aprecian unas líneas paralelas conocidas como *líneas de crecimiento*.

Atravesando el corcho de *espalda* a *barriga* se encuentran unos poros de tamaño y dimensiones variables, conocidos como *lenticelas* o *lentículas*, rellenos de un polvillo marrón-rojizo cuya finalidad es comunicar las zonas vivas del árbol con el exterior.

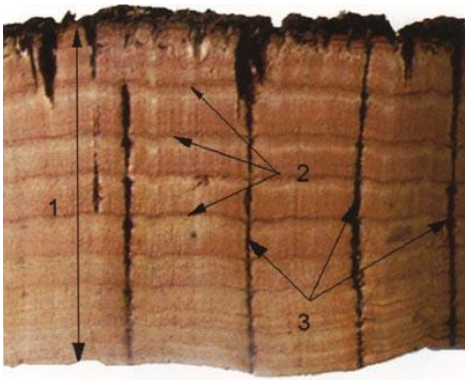


Figura 1.1.2. Sección transversal de una plancha de corcho, en la que se distinguen el calibre (1), líneas de crecimiento (2) y poros o lenticelas (3).

El primer tejido vegetal que se examinó al microscopio fue el corcho, siendo descrito y dibujado por Robert Hooke en 1665. Hooke indicó que estaba formado por pequeñas cavidades a las que denominó células, por el parecido con las celdas de un panal de abejas.

La disposición de las células sigue un orden riguroso de hileras radiales sin que existan huecos entre ellas.

La longitud de las células de corcho varía entre 10 y 70 micras, aceptándose como valor medio 40 micras. El número de celdas de corcho por centímetro cúbico se aproxima a 35 millones (Del Pozo et al., 1999).

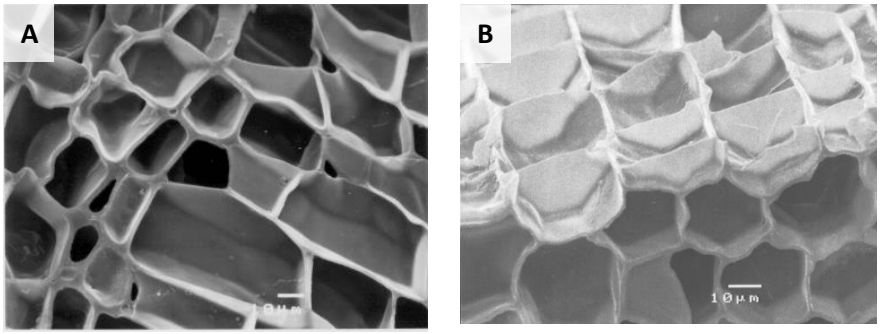


Figura 1.1.3. Estructrura del corcho. Corte axial (A). Corte radial-tangencial (B).

Portugal, España, Argelia, Marruecos, Francia, Túnez e Italia son los países con mayor superficie alcornocal en el mundo. El corcho se extrae del árbol de forma periódica para su comercialización, cada 9 años en Extremadura. La comunidad autónoma de Extremadura presenta una de las mayores producciones de corcho en España (casi el 40% de la producción española y más del 10% de la producción mundial, Martínez Cañas et al., 2012).

Las características únicas que posee el corcho, tales como baja densidad aparente (de 0.13 a 0.25 $Kg \cdot dm^{-3}$), alta compresibilidad, gran impermeabilidad a líquidos y gases, elasticidad, alto coeficiente de fricción, estabilidad química y durabilidad lo convierten en una de las materias primas naturales más versátil (Rosa y Fortes, 1988; Vaz y Fortes, 1998; Anjos et al., 2014).

El corcho ha sido utilizado por el hombre desde tiempo inmemorial, existiendo pruebas de su empleo en el taponamiento de vasijas y ánforas y como material de flotación en artes de pesca, por parte de egipcios, griegos y romanos. Además de para estos usos, los árabes lo utilizaron para el aislamiento térmico de viviendas, así como para trabajos de ornamentación y utensilios domésticos, y los chinos para la elaboración de zapatos. No obstante, su aceptación y uso industrial fue a raíz del descubrimiento del vino de Champagne en la segunda mitad del siglo XVII por el monje benedictino Dom Pierre Perignon (Del Pozo et al., 1999).

Algunos de estos usos han permanecido prácticamente inalterados a través de los años hasta la actualidad. Fue sólo con el auge de la industria química cuando los polímeros sintéticos sustituyeron el corcho en algunas aplicaciones, ya sea totalmente, como en los

dispositivos de pesca y equipos de flotación, o en gran medida, como en el aislamiento de frío y de calor.

Sin embargo, el corcho, como sellador de recipiente de líquidos, se ha mantenido en su esencia prácticamente sin cambios, aun con la automatización e innovación tecnológica introducida en los procesos industriales. El corcho es el material en el que se piensa cuando se quiere abrir y cerrar un recipiente, y los tapones de corcho son el símbolo de una botella de vino. Aunque el uso de tapones de plástico y tapones de rosca de aluminio se inició en algunas bodegas, el tapón de corcho natural sigue siendo, sin duda, el cierre idóneo de los vinos de calidad.

La innovación también se produjo en el desarrollo de nuevos materiales de corcho, es decir, en materiales compuestos y en aislantes de alto rendimiento. Vehículos espaciales o estructuras complejas bajo vibraciones y cargas dinámicas son ejemplos de sus aplicaciones de alta tecnología (Pereira, 2007).

1.1.1. Tipos de corcho

Teniendo en cuenta su procedencia y aplicación se pueden distinguir en campo los siguientes tipos de corcho (Del Pozo et al., 1999).

a) Bornizo

Es el corcho que se produce en troncos y ramas en la primera extracción. Su espalda es muy rugosa y presenta grandes colenas (grietas profundas), lo que hace que no tenga un calibre homogéneo. Su vientre es irregular y se traba con el tronco. No sirve para tapón. Existen distintos tipos de bornizo, entre ellos se encuentran:

- Bornizo de verano: es el resultado del primer aprovechamiento corchero. Su destino es el granulado, el aglomerado o la decoración. La primera saca suele realizarse cuando el perímetro del tronco, a la altura de 130 cm, alcanza los 65 cm. Esto suele suceder cuando el árbol alcanza una edad de 25 ó 30 años.

- Bornizo de invierno: es el que se obtiene fuera de la época de descorche, procedente de podas de ramas o de árboles derribados. Suele contener trozos de capa madre.

b) Corcho de reproducción

Se forman en las sucesivas sacas tras la extracción del bornizo. Sus superficies son más regulares que las del bornizo y ya puede destinarse al taponamiento y a otros usos. En este tipo de corcho se puede distinguir:

- Corcho segundero: Es el que se obtiene tras el bornizo. Su espalda aún es agrietada, pero menos que la del bornizo. Su color es más oscuro que el del bornizo. Su vientre es liso, lo que indica que la espalda del próximo corcho de reproducción también lo será. Este tipo de corcho, salvo excepciones, aún no es útil para tapón natural de una pieza, por lo que suele dedicarse a trituración.

- Corcho de fábrica: Es el corcho propiamente de reproducción. Su espalda y su vientre son prácticamente lisos y su calibre homogéneo. Es apto para la industria del taponamiento. Se produce en la tercera y sucesivas sacas.

- Pedazos: Son trozos de corcho con una superficie inferior a 400 cm^2. Se producen por rupturas en la saca. Tradicionalmente no se apila y se junta con el bornizo y el corcho segundero.

- Refugo: Corchos de reproducción con grandes defectos, que se separan por ello del corcho en plancha.

- Zapatas: Corcho procedente de la base de los árboles en contacto con el suelo, incluso enterrado. No ha de juntarse con la pila de planchas aptas para la fabricación de tapones.

1.1.2. Composición del corcho

Las propiedades de un material dependen de las características físico-químicas del mismo, de sus componentes, de la cantidad relativa y de la distribución de los mismos. En

el corcho, como en otros materiales celulares, los componentes químicos se encuentran en las paredes celulares así como en los espacios intercelulares, lo que representa una red tridimensional sólida que rodea células muertas, huecas y llenas de aire. Muchas de las propiedades específicas del corcho, como la inercia química y biológica, así como su durabilidad, están en relación directa con su composición química, mientras que otras propiedades, tales como el comportamiento mecánico y la interacción con fluidos, son el resultado tanto de las características estructurales a nivel celular como a la estructura química de la pared celular.

La constitución química del corcho se ha examinado extensamente, encontrando que depende de factores como el origen geográfico, las condiciones del clima y del suelo, origen genético, dimensiones del árbol, edad del árbol y condiciones de crecimiento (Pereira, 2007).

La composición química del corcho es sustancialmente diferente del resto de las partes del árbol, como la madera. Los componentes del corcho son, por orden de importancia relativa, suberina (alrededor del 50% del total), lignina (20 - 25 %), polisacáridos, como celulosa y hemicelulosa (aproximadamente un 20%), sustancias extraíbles, que incluyen mayoritariamente sustancias lipídicas y fenólicas (14 - 18%), y por último componentes inorgánicos (1 - 2%) (Silva et al., 2005; Pereira, 2007). Las proporciones de esta composición varían en función de los métodos empleados para su determinación, así como del corcho utilizado (Del Pozo, 1999).

El corcho posee algunos componentes “libres”, no vinculados químicamente a la estructura principal y, por lo tanto, extraíbles con disolventes, ya sean orgánicos, tanto polares como no polares, o inorgánicos. Algunos de estos componentes son responsables de las propiedades organolépticas del vino, pues también se encuentran en los hollejos de la uva.

Los componentes extraíbles más importantes son las ceras y los compuestos fenólicos. Las ceras son extraídas por disolventes no polares o con baja polaridad, como el benceno, cloroformo, acetato etilo, hexano y éter; son consideradas como las responsables de la impermeabilidad del corcho, y están compuestas de diversos compuestos alifáticos y aromáticos. Por otra parte, los compuestos fenólicos son extraídos con disolventes polares como agua o metanol, además son compuestos como ácidos fenólicos, aldehídos fenólicos

y cumarinas, también incluye compuestos pertenecientes a las familias químicas de flavonoides y taninos (Silva et al., 2005).

Se ha identificado (Conde et al., 1997) mediante HPLC en corcho de reproducción los siguientes compuestos fenólicos de bajo peso molecular: ácidos gálico, protocatéquico, vainíllico, cafeíco, ferúlico y elágico; aldehídos protocatéquico, vainíllico, coniferálico y sinápico; y esculetina y escopoletina. La composición de algunos de ellos presentaba marcadas diferencias entre los diferentes árboles o en relación con las etapas de procesamiento industrial de la primera transformación.

Análogamente, se han llevado a cabo una serie de estudios para determinar la composición fenólica de corcho, mostrando la presencia de diferentes grupos de compuestos que pueden ser extraídos del corcho mediante una disolución hidroalcohólica (Conde et al., 1998).

Por otro lado, en diferentes extracciones sucesivas con diferentes disolventes, se han identificado como componentes mayoritarios el ácido elágico, seguido de los ácidos gálico, protocatéquico y cafeíco; y esculetina. En otro extracto diferente se identificaron pequeñas cantidades de vainillina, ácidos vainíllico, cumárico y ferúlico, y ácido *p*-hidroxibenzoico en otro extracto distinto. Otros compuestos que también fueron identificados son ácido salicílico, eriodictiol y naringenina en los diferentes extractos mencionados anteriormente, además de ácido quínico y ácido hidroxifenilláctico (Santos et al., 2010).

Respecto a taninos derivados del ácido elágico, Conde et al. (1998) se han identificado en corcho: roburina A y E, grandinina, vescalagina y castalagina, además de otros elagitaninos con estructuras relacionadas. Estos investigadores apreciaron diferencias notables tanto en el contenido global de los diferentes grupos de taninos como en el contenido de cada uno de los elagitaninos entre los diferentes árboles e incluso entre las muestras analizadas de un mismo árbol.

1.1.3. Transformación del corcho

El descorche del árbol se lleva a cabo en los meses de mayo a agosto, y se realiza de forma manual, utilizando fundamentalmente el *hacha corchera*, adaptada al trabajo de descorche, además de la *burja*, una palanca de madera que ayuda a descorchar las partes altas del árbol y a sacar las zapatas (corcho de la base del árbol); la *escalera*, escala simple ligera que sirve para trabajar en las partes altas del árbol; y la *navaja de rajar*, que permite cortar las planchas de corcho para facilitar su transporte.

Figura 1.1.4. Herramientas utilizadas en la saca del corcho. Escala simple (A), burja (B), hacha corchera (C) y navaja o cuchilla de rajar (D).

La corteza se extrae del tronco y de las ramas más robustas teniendo cuidado de no dañar la corteza interna, de lo contrario se abren heridas por las que pueden producirse infecciones que se extenderían por el árbol a través de la savia. Este proceso lo realiza el sacador, el cual con el hacha traza marcas muy precisas horizontales y verticales para señalar las planchas de corcho que van a ser extraídas. Después se ahueca el corcho, despegándolo del felógeno para así despegar con facilidad el corcho del tronco (Del Pozo et al., 1999).

La primera transformación a la que tiene que someterse el corcho para posteriormente poder fabricar tapones es la preparación del corcho en plancha, cuya finalidad es poner en

las condiciones adecuadas el corcho que se ha extraído de los árboles. El proceso de preparación consta de diversas etapas que se detallan a continuación.

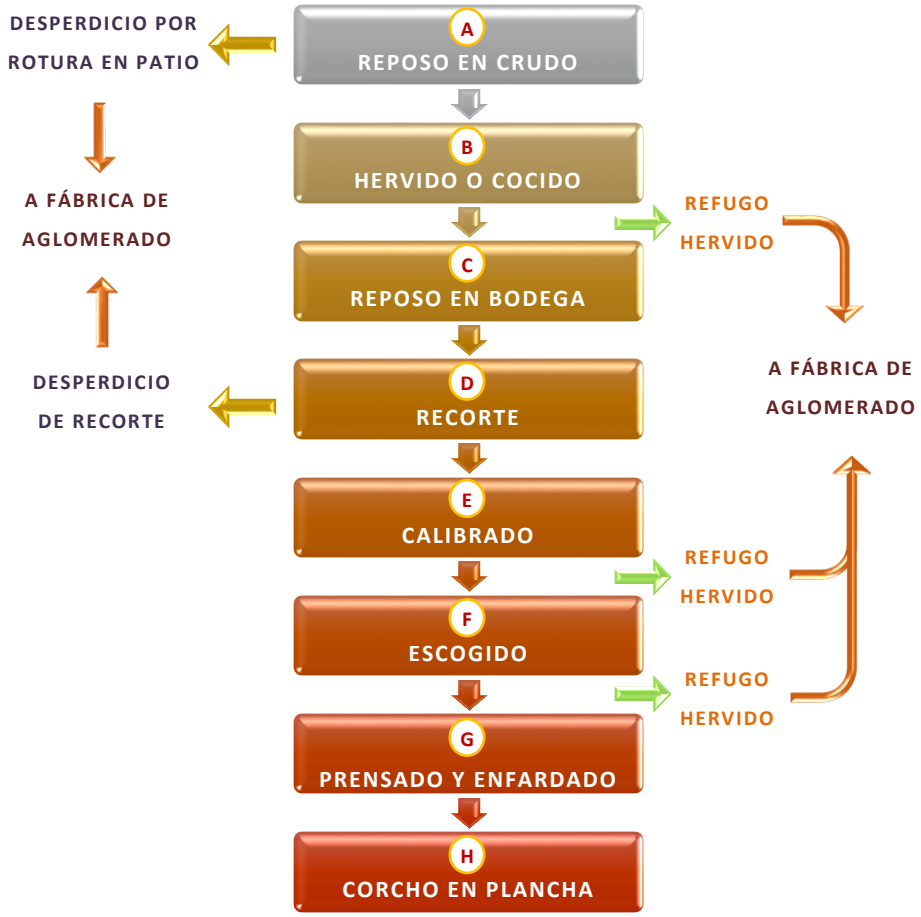


Figura 1.1.5. Esquema del proceso de preparación de corcho en plancha (Ávila et al., 1998).

Una vez descorchados los árboles, se reúnen todas las planchas en forma de pila. Al cabo de cierto tiempo el corcho se pesa, antes de ser transportado a la industria preparadora.

En dicha industria comienza el proceso de transformación del corcho, iniciado por una primera fase de reposo del corcho en un patio de almacenamiento durante 6 meses como mínimo, después de la cual se procede al hervido en agua durante una hora en una caldera abierta de acero inoxidable. Esta primera etapa busca que la tensión que tienen las planchas de corcho, por la curvatura a la que han estado sometidas en el árbol, comiencen

a desaparecer. De este modo, se produce una contracción de las fibras de celulosa y hemicelulosa que hacen que el material se estabilice. De no proceder de este modo, podría darse el caso que, una vez fabricado el tapón, se produjese una desestabilización, provocando torsiones y variaciones en las dimensiones del tapón. La finalidad del cocido de corcho es proceder a su limpieza, eliminar sustancias hidrosolubles o posibles microorganismos, aumentar el espesor, reducir la densidad, mejorar la flexibilidad y aplanar las planchas que tienen todavía la curvatura propia del tronco.

Después del hervido, el corcho pasa por un período de estabilización (de 2 a 4 semanas), durante el cual se aplana la plancha y se seca hasta alcanzar la consistencia adecuada para el recorte. Tras estos primeros pasos se realiza una clasificación por grosores y calidades, un escogido (clasificación según la calidad) y por último se realiza un prensado y enfardado del corcho (Ávila et al., 1998).

El cocido del corcho se realiza en agua hirviendo a una temperatura cercana a 100ºC durante al menos una hora. Esta práctica es obligatoria y recogida por el Código Internacional de Prácticas Taponeras (C.E. Liège, 2015). Además, por regla general, antes de comenzar el proceso de fabricación de tapones, el corcho debe pasar un segundo hervido que dura al menos 30 minutos y se realiza a una temperatura cercana a los 100ºC y que tiene como finalidad aumentar el contenido de agua en el corcho facilitando las siguientes etapas.

El proceso de cocido de corcho genera normalmente alrededor de 400 litros de agua por tonelada de corcho tratado (Mendoça et al., 2004). Las empresas de preparación habitualmente realizan tareas de hervido de corcho entre 4 y 5 días a la semana y cambiando el agua de la caldera por agua limpia entre una y dos veces por semana. El volumen de agua residual generado por este tipo de empresas no es constante a lo largo de todo el año debido a que su actividad se concentra en los meses comprendidos entre octubre y mayo. Según un estudio realizado por la empresa de consultoría ECA (Entidad Colaboradora de la Administración, S.L.), (ECA, 2006) se estima que en San Vicente de Alcántara, zona de mayor concentración de industrias corcheras en Extremadura, las empresas preparadoras de corcho en plancha y las de transformación de corcho natural generan alrededor de 21000 m^3 anuales de aguas residuales.

Durante el cocido del corcho muchas de las sustancias contenidas en él se disuelven en el agua, dando lugar a un efluente con una alta concentración de materia orgánica (compuestos fenólicos principalmente), así como pH ácido, materiales en suspensión y baja biodegradabilidad. Es por ello que estas aguas se caracterizan por altos valores de demanda química de oxígeno (DQO), demanda biológica de oxígeno (DBO) y contenido de polifenoles totales (PT). Algunos de los compuestos fenólicos que se encuentran en este tipo de aguas son: ácidos gálico, protocatéquico, vainíllico, siríngico, ferúlico, tánico y elágico; vainillina, siringaldehído, coniferaldehído y sinapaldehído (Minhalma y de Pinho, 2001; Santos et al., 2013). No se puede hablar de valores concretos de contaminación, por lo que en la tabla 1.1.1 se muestran algunos de los parámetros de caracterización de este tipo de aguas obtenidos por distintos autores.

Tabla 1.1.1. Caracterización de aguas de caldera de cocido de corcho.

Referencia	pH	DBO_5[a], mg $O_2 \cdot L^{-1}$	DQO, mg $O_2 \cdot L^{-1}$	PT[b], mg$\cdot L^{-1}$	STS[c], mg$\cdot L^{-1}$
Benítez et al., 2003	5.4	1150	1900	290	-
Peres et al., 2004	5	802	4250	994	-
Silva et al., 2004	4.5	1950	5100	-	100
Benítez et al., 2006	4.7	1750	4290	-	280
Domínguez et al., 2007	4.6	1035	3047	381	2890
Sánchez, 2007	5.5	1376	3242	567	4000
Vilar et al., 2009	7.5	750	1748	740	124
De Torres-Socías et al., 2013	7.2	161	1240	-	290

[a]DBO_5 (demanda biológica de oxígeno disuelto consumido en cinco días)

[b]PT (polifenoles totales), expresado en mg$\cdot L^{-1}$ de ác. Cafeíco

[c]STS (sólidos totales en suspensión)

Numerosos estudios realizados en aguas de caldera de cocido de corcho demuestran el aumento progresivo de los parámetros tales como DQO, DBO, sólidos totales en suspensión

y fenoles totales con la cantidad de corcho cocido (Domínguez et al., 2007; Sánchez, 2007; Jiménez, 2011).

1.2. Compuestos fenólicos

Los compuestos fenólicos son sustancias químicas en cuya estructura existe un anillo aromático con al menos un sustituyente hidroxílico libre o sustituido. Los compuestos fenólicos tienen su procedencia en el mundo vegetal.

Las plantas sintetizan distintos compuestos químicos durante y después de la fotosíntesis, por medio de un conjunto de reacciones químicas y enzimáticas que se generan de manera secuencial y ordenada llamadas rutas o vías metabólicas (ver Figura 1.2.1. donde se puede visualizar un esquema de las rutas). Esta secuencia lógica de reacciones genera metabolitos o productos en la planta necesarios para todas las funciones básicas de los vegetales, y son considerados como *metabolitos primarios*. Se denominan *metabolismos secundarios* a aquellos compuestos o moléculas orgánicas que no parecen tener una función directa en procesos fotosintéticos, respiratorios, asimilación de nutrientes, transporte de solutos o síntesis de proteínas, carbohidratos o lípidos (Trejo et al., 2007).

Los metabolitos secundarios además de no presentar una función definida en los procesos mencionados, difieren también de los metabolitos primarios en que no todos los metabolitos secundarios se encuentran en todos los grupos de plantas (Ávaloz, 2009). Se sintetizan en pequeñas cantidades y no de forma generalizada, estando a menudo su producción restringida a un determinado género de plantas, a una familia, o incluso a algunas especies.

Generalmente todos los vegetales, como producto de su metabolismo secundario en estado normal, son capaces de biosintetizar un elevado número de compuestos fenólicos, algunos de los cuales son indispensables para sus funciones fisiológicas y otros son de utilidad para defenderse ante situaciones de estrés que pueden ser producidas por animales, condiciones climáticas (hídrico, luminoso), etc.

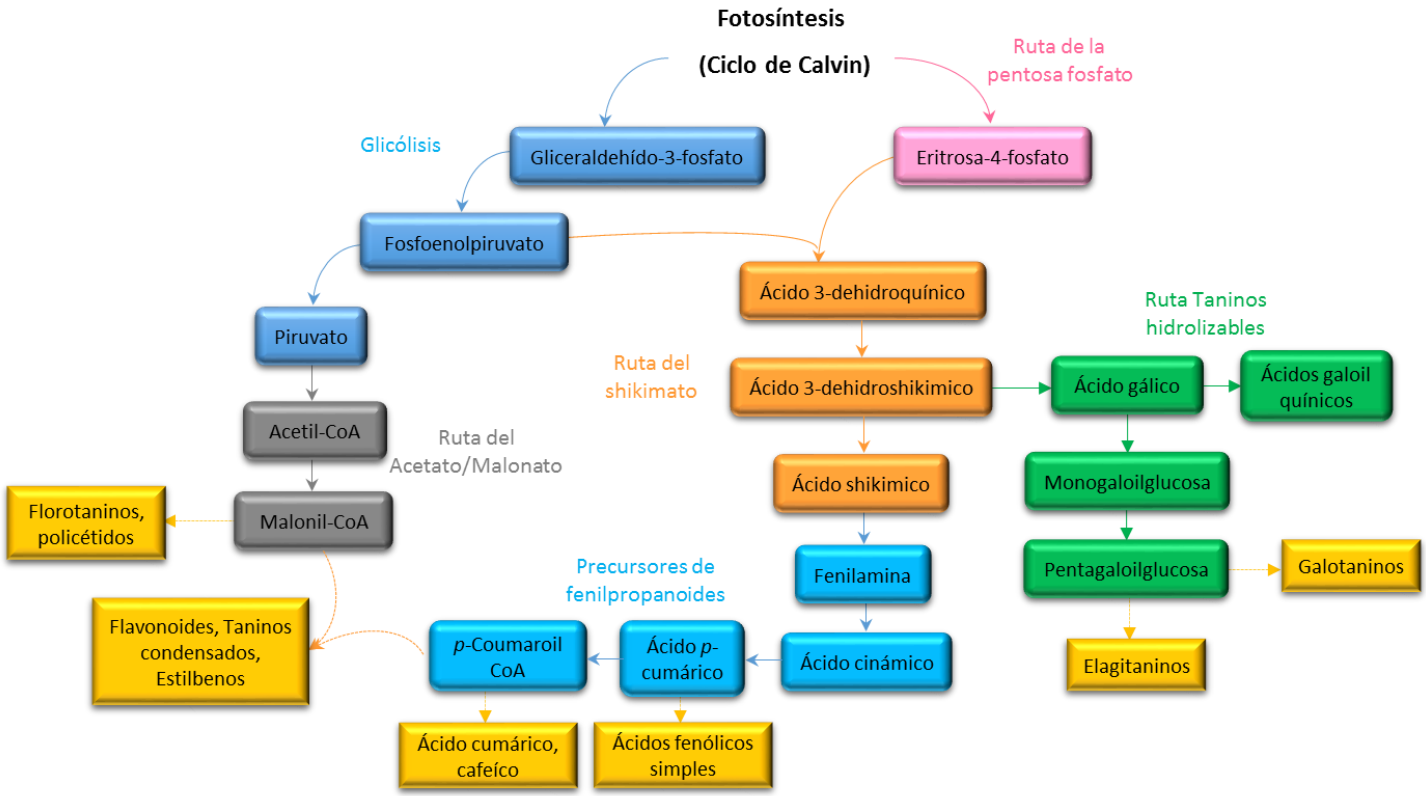


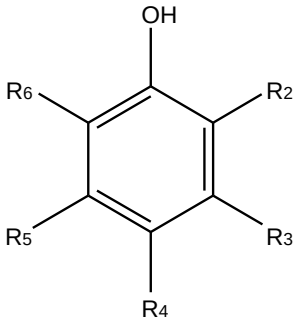
Figura 1.2.1. Esquema de las rutas de biosíntesis de diferentes compuestos fenólicos (Salminen and Karonen, 2011).

1.2.1. Clasificación

Se han llegado a identificar más de 8000 compuestos fenólicos con estructura muy variada, por lo que su clasificación es una tarea compleja. Los compuestos fenólicos se pueden clasificar atendiendo a diferentes criterios. Así, teniendo en cuenta su complejidad química, en la Figura 1.2.2. se muestra un esquema de un tipo de clasificación.

- Fenoles simples: Este grupo de compuestos se caracteriza por poseer en su estructura química un anillo aromático y grupos hidroxílicos (C_6) (Tabla 1.2.1.). La presencia de fenoles en el medio ambiente es consecuencia tanto de acciones naturales (son productos intermedios de degradación de sustancias proteícas) como del aporte antropogénico de carácter industrial. La mayoría de los compuestos fenólicos que se pueden encontrar en las aguas residuales son el resultado de diversas actividades industriales y agrícolas.

Tabla 1.2.1. Estructura química de los fenoles simples.

Fenoles simples C_6	R_2	R_3	R_4	R_5	R_6	Compuesto
	H	H	H	H	H	Fenol
	H	Cl	H	H	H	3-clorofenol
	H	H	Cl	H	H	4-clorofenol
	H	CH_3	H	H	H	*m*-cresol
	H	H	CH_3	H	H	*p*-cresol
	Cl	H	Cl	H	H	2,4-diclorofenol
	NH_2	H	H	H	H	2-nitrofenol
	H	H	NH_2	H	H	4-nitrofenol

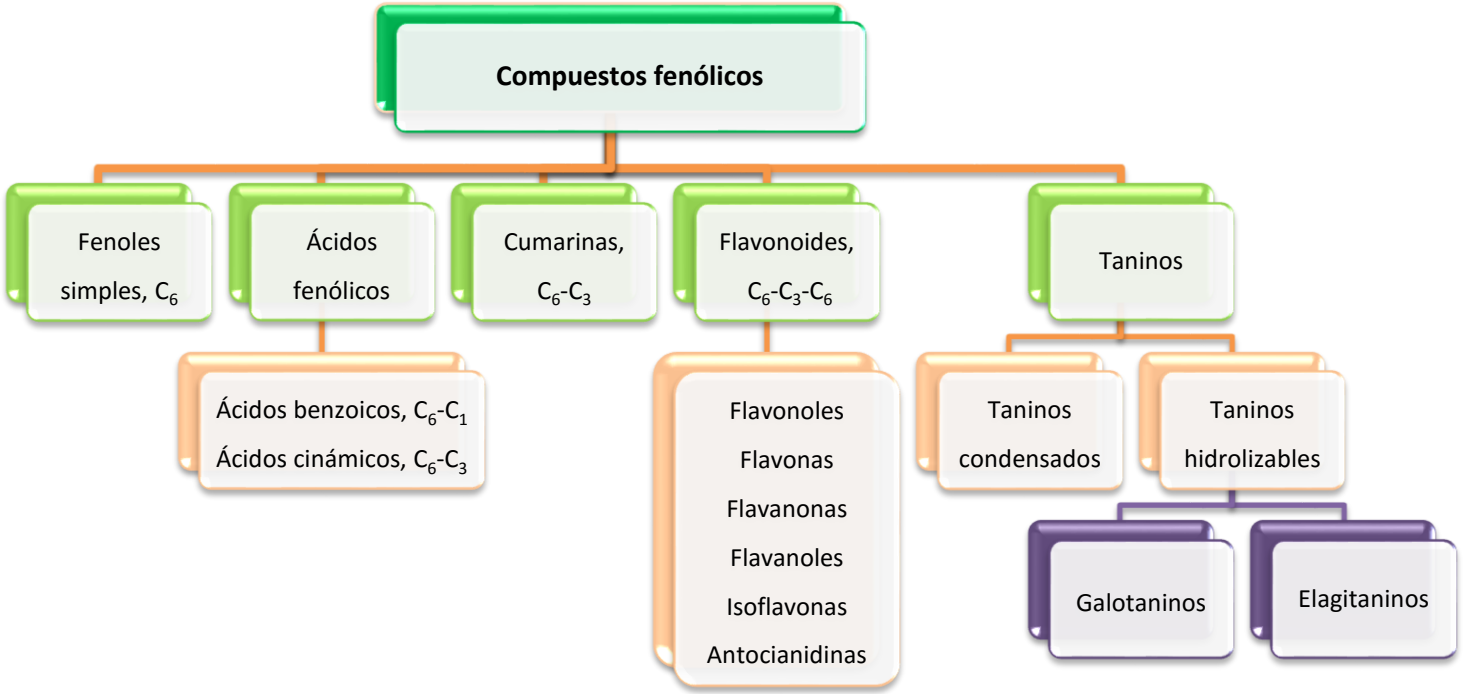
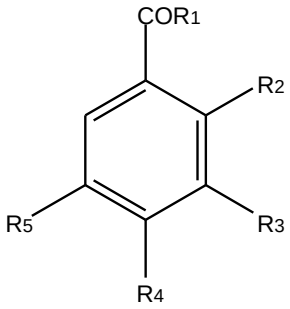


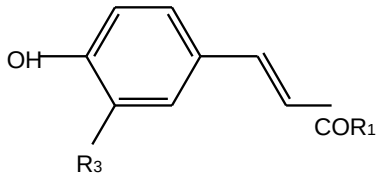
Figura 1.2.2. Esquema de la clasificación de compuestos fenólicos.

Ácidos fenólicos: La estructura química de este grupo de compuestos fenólicos consta de un anillo aromático y un grupo hidroxílico comunes a todos los compuestos fenólicos y de un grupo carboxílico (Tabla 1.2.2.). Los ácidos fenólicos constituyen aproximadamente un tercio de los fenoles dietéticos, que pueden estar presentes en las plantas tanto de forma libre como ligada (Robbins, 2003). Los compuestos fenólicos ligados pueden estarlo a varios componentes de la planta a través de enlaces éster, éter o acetal (Zadernowski et al., 2009). Dentro de los ácidos fenólicos se pueden encontrar los derivados del ácido benzoico (C_6-C_1) y del ácido cinámico (C_6-C_3). Los derivados del ácido benzoico son abundantes en la naturaleza fundamentalmente en forma libre, como ácidos (ácido vaníllico, ácido gálico, ácido protocatéquico, etc.) o aldehídos (vanillina, anisaldehído) (Bravo, 1998). Los derivados del ácido cinámico, tales como los ácidos *p*-cumárico, cafeíco, ferúlico y sinápico desempeñan un papel importante en la naturaleza. De hecho, su amplia distribución y alta concentración les proporciona un papel clave en la biosíntesis de sistemas fenólicos más complicados. Los ácidos cinámicos se encuentran también en varias formas conjugadas, incluyendo amidas, ésteres (principalmente ésteres de ácidos hidroxílicos, tales como ácido tartárico y derivados de azúcar), y glicósidos (Teixeira et al., 2013).

Tabla 1.2.2. Estructura química de los ácidos fenólicos.

Ácidos benzoicos C_6-C_1	R_1	R_2	R_3	R_4	R_5	Compuesto
	OH	H	OH	OH	OH	Ácido gálico
	OH	OH	H	H	H	Ácido salicílico
	OH	H	OH	OH	H	Ácido protocatéquico
	OH	H	OCH_3	OH	H	Ácido vainíllico
	OH	H	OCH_3	OH	OCH_3	Ácido siríngico
	H	H	OCH_3	OH	H	Vainillina
	H	H	H	OCH_3	H	Anisaldehído

Cont. Tabla 1.2.2. Estructura química de los ácidos fenólicos.

Ácidos cinámicos C_6-C_3	R_1	R_3	Compuesto
	OH	OH	Ácido cafeíco
	OH	H	Ácido p-cumárico
	OH	OCH_3	Ácido ferúlico
	Ácido tartárico	OH	Ácido cafeil-tartárico
	Ácido quínico	OH	Ácido clorogénico

Los ácidos fenólicos son metabolitos secundarios de las plantas y constituyen una de las vías metabólicas más estudiadas y ampliamente explotadas en la investigación vegetal (Whiting, 2001; Boudet, 2007). Los metabolitos secundarios juegan un papel importante ya sea como factores de resistencia locales o sistémicos en la protección frente a diversos agentes patógenos (Chester, 1993; Metraux, 2001; Redman et al., 2003).

- Cumarinas: Este grupo se encuentran ampliamente distribuidos en el mundo vegetal, y muchos exhiben actividades biológicas útiles y diversas (Egan et al., 1990; Borges et al., 2005), y tienen en común una estructura química de 2H-1-benzopiran-2-ona, (C_6-C_3), denominada cumarina.

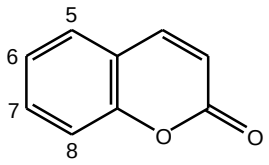


Figura 1.2.3. Estructura de la cumarina.

En la práctica, todas las cumarinas, a excepción de la cumarina propiamente dicha, poseen un sustituyente hidroxílico en la posición 7 libre, como sucede en la umbeliferona, esculetina y escopoletina. Es frecuente que sobre los carbonos 6 u 8 se sitúen radicales isoprenílicos de 5, 10, o con menor frecuencia, 15 átomos de carbono, que por su alta reactividad pueden originar anillos adicionales de tipo furánico o piránico. A este grupo de cumarinas isopreniladas se les conoce en conjunto como cumarinas complejas debido a la gran variabilidad química de sus estructuras (furano y piranocumarinas).

- Flavonoides: Los flavonoides son compuestos de bajo peso molecular, que contienen quince átomos de carbono, dispuestos en una configuración C_6-C_3-C_6. Esencialmente la estructura consta de dos anillos aromáticos, A y B, unidos por un puente de 3 carbonos, usualmente en forma de un anillo heterocíclico, C. El anillo aromático A se deriva a través del acetato/malonato, mientras que el anillo B a través de la fenilalanina del shikimato (Merken et al., 2000).

Las variaciones en los patrones de sustitución del anillo C provocan la mayor clase de flavonoides, es decir, flavonoles, flavonas, flavanonas, flavanoles (o catequinas), isoflavonas, flavanonoles y antocianidinas (Hollman et al., 1999), de los cuales las flavonas y los flavonoles son los que tiene el mayor número de compuestos y unas estructuras más diversas (Harborne et al., 1999). Las sustituciones a los anillos A y B dan lugar a compuestos dentro de cada clase de flavonoides (Pietta, 2000). Estas sustituciones pueden incluir oxigenación, alquilación, glicosilación, acilación y sulfonación (Balasundram et al., 2006).

- Taninos: Los taninos son compuestos de alto peso molecular que constituyen el tercer grupo más importante de compuestos fenólicos; éstos pueden subdividirse en taninos hidrolizables y condensados (Porter, 1989).

Los taninos hidrolizables son, como su nombre indica, posibles de ser degradados por hidrólisis química o enzimática en varias unidades estructurales que los componen. Son constituidos por una parte polialcohólica (normalmente glucosa, pero también puede ser ácido quínico, fenoles o glicósidos) y una parte fenólica (por ejemplo, ácido gálico) unidos a través de un éster (Hagerman, 2010). Los taninos hidrolizables pueden ser divididos en galotaninos (su parte fenólica es el ácido gálico) y elagitaninos (su parte fenólica es el ácido hexahidroxidifénico, HHDP) (Quideau et al., 1996). Una característica de los elagitaninos es su capacidad para liberar ácido elágico tras la hidrólisis de los grupos ésteres del HHDP, que experimentan una lactonización rápida, fácil e inevitable. Los galotaninos también pueden transformarse en elagitaninos mediante el acoplamiento C-C oxidativo entre los grupos galilo espacialmente adyacentes para formar grupos HHDP (Seeram et al., 2004). La pentagaloilglucosa (PGG) y la vescalagina son ejemplos de galotaninos y elagitaninos, respectivamente.

Los taninos condensados son polímeros constituidos por dos o más unidades de flavan-3-ol. Cuando estos compuestos se encuentran en medio ácido originan antocianidinas (reacción de Bate-Smith), de ahí que también sean conocidos como proantocianidinas (Cheynier, 2005). La estructura de los principales flavan-3-ols presentes en la naturaleza varia con el número de grupos hidroxilo del anillo B y con la estequiometría del carbono 3 del heterocíclico C. Así, dependiendo de la estequiometría del carbono 3, podemos tener, por ejemplo (+)-catequina o (-)-epicatequina, y dependiendo del grado de hidroxilación del anillo B podemos tener (epi)afzelequina (monohidroxilado), (epi)catequina (dihidroxilado) o (epi)galocatequina (trihidroxilado) (Hagerman, 2002).

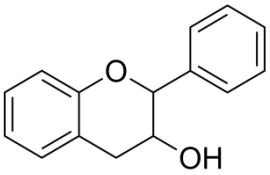


Figura 1.2.4. Estructura del flavan-3-ol.

1.2.2. Actividad biológica

La búsqueda de un estilo de vida natural y saludable ha aumentado el interés por los compuestos naturales bioactivos que pueden ser introducidos en la dieta o ser usados como medicamentos naturales (Silva et al., 2007). Los compuestos fenólicos son sustancias con un creciente interés debido a sus propiedades biológicas, tales como actividad antioxidante, antitrombótica, antibacteriana, antialérgica, anticancerígena y antiinflamatoria. Como consecuencia de sus efectos beneficiosos en la salud humana, la ingesta de alimentos ricos en compuestos fenólicos es recomendado en la dieta alimentaria (Sánchez et al., 2013). El interés en compuestos fenólicos naturales para aplicaciones alimentarias, cosméticas y nutracéuticas se ha incrementado considerablemente en los últimos años, además de por sus propiedades, indicadas anteriormente, por no presentar efectos adversos como frecuentemente ocurre con sus homólogos sintéticos (Santos et al., 2010).

Las plantas aromáticas y medicinales poseen numerosos compuestos bioactivos, que pueden ser de estructura fenólica, y que presentan actividad antioxidante. Una fuente

potencial de compuestos antioxidantes son las raíces, tallos y cortezas de distintos arboles (Chung et al., 1999; Siddhuraju et al., 2002; Moure et al., 2005) y de estos una parte de alto interés son sus hojas (Mohd Zin et al, 2002; Zainol et al., 2003).

El interés por los flavonoides se debe a la protección que ejercen en enfermedades como cáncer (Ramanathan et al., 1993) y enfermedades cardiovasculares (Padilla et al., 2005). Además, poseen propiedades antioxidantes y antimicrobianas (Tim et al., 2005). Los flavonoides son antioxidantes especialmente importantes debido a su alto potencial redox, lo que les permite actuar como agentes reductores, dadores de hidrógeno, además de tener un alto potencial quelante con los metales (Tsao et al., 2003). También son los fitoquímicos más comunes, normalmente estos productos químicos ayudan a proteger la planta contra la luz UV, parásitos fúngicos, herbívoros, patógenos y lesiones celulares oxidativas (Cook et al., 1996). El consumo regular de flavonoides por el ser humano se ha asociado con una reducción en la incidencia de enfermedades como el cáncer y las cardiovasculares (Beecher, 2003; Cook et al., 1996; Liu et al., 2008).

Además de las propiedades antioxidantes de los compuestos fenólicos, se ha visto que diversos elagitaninos presentan propiedades antisecretoras y antiulcerogénicas. Los extractos obtenidos de diversas plantas medicinales ricas en taninos, han sido tradicionalmente usados en todo el mundo para combatir úlceras gástricas. Además de estos efectos, se ha visto que rebajan los niveles de urea en sangre, inhiben la actividad de la enzima transformadora de Angiotensina (con la inhibición de esta enzima se impide la transformación de la Angiotensina tipo I en Angiotensina tipo II, involucrada en enfermedades como la hipertensión arterial, infarto de miocardio, nefropatías debidas a diabetes, así como la propia diabetes tipo II), poseen actividad antiviral y anti-VIH e inhiben la peroxidación de lípidos (con la consecuente disminución de radicales libres que destruirían las células) (Okuda et al., 1992).

El notable efecto antitumoral de estos compuestos en varios modelos animales y en líneas celulares permitió postular que estos compuestos fenólicos son agentes antitumorales universales (Gali-Muhtasib et al., 1999).

Las especies del género Quercus son de las especies vegetales que presentan mayor contenido en taninos (Scalbert et al., 1990). En relación con los elagitaninos del corcho, éstos migran del tapón al vino que tapa. Una vez allí, reaccionan con otros componentes

del vino (catequinas) mediante reacciones de condensación, favorecidas por el pH ácido del vino y la presencia del etanol. Los compuestos a los que dan lugar estas reacciones son la Acutissimina A y la Acutissimina B, sustancias que muestran propiedades anticancerígenas muy potentes. Estos compuestos son inhibidores de la enzima humana Topoisomerasa II, siendo la actividad 250 veces mayor (in vitro) que la que presenta el medicamento Etopósido (VP-16), actualmente administrado clínicamente como anticancerígeno en carcinoma microcítico de pulmón, enfermedad de Hodgkin, linfomas malignos, leucemia aguda monocítica y mieolomonocítica, y en tumores testiculares (Castonguay et al., 1997; www.vademecum.es).

1.3. Extracción de compuestos fenólicos. Antecedentes bibliográficos

La extracción de compuestos fenólicos de matrices vegetales se realiza por técnicas de extracción convencionales, tales como extracción sólido-líquido, con disolventes polares: agua, metanol, etanol o acetona, entre otros disolventes. En base a esto, son varios los autores que han propuesto diferentes métodos de extracción de compuestos fenólicos tanto en corcho como en otras matrices. En la Tabla 1.3.1 se recogen los métodos más significativos que aparecen en la bibliografía.

Tabla 1.3.1. Métodos de extracción recogidos en bibliografía para diferentes matrices.

Disolvente	Analitos[a]	Matriz	Comentarios	Referencia
Metanol/Agua	AG, AV, AS, AF, AE, V, SIR, ESC, CON, SIN, ESCU	Madera de roble	10g muestra/ 300 mL MeOH:H_2O (1:1) T ambiente / 24 horas	Fernández et al., 1996
Etanol	AS, AC, ACl, AV, flavonoles, flavones.	Hojas de olivilla	Extraído 3 veces 80mL EtOH 80% con bisulfato sódico al 2%	Romani et al., 1996
Metanol/Agua	AG, AP, AV, AC, AF, AlP, ESCU, V, ESC, CON, SIN, ELAG	Corcho	2g muestra/150mL MeOH:H_2O (80:20) Extracción posterior con dietil éter	Conde et al., 1997,1998
Fluido supercrítico CO_2	AG, AP, AC, AF, V, AlP, trans-reveratrol, catequina	Uvas	Estudios preliminares para la extracción de fenoles de uvas y mosto	Palma y Taylor, 1999
Acetona/Agua Metanol/Agua	Vescalagina y Castalagina	Duramen de roble blanco	Oscuridad Atmósfera de N_2 Acetona: H_2O (70:30) o MeOH:H_2O (70:30) Mayores rendimientos con Acetona/agua	Zhentian et al., 1999
Etanol/Agua a altas presiones	AmHb, AG, AP, ApHb, AV, AC, AS, ACu, AF, AHc; V, CON, SIN, ESC, SIR	Madera de roble	1.5g muestra/H_2O:EtOH (sobrecalentada)	González-Rodríguez et al., 2004

Cont. Tabla 1.3.1. Métodos de extracción recogidos en bibliografía para diferentes matrices.

Disolvente	Analitos[a]	Matriz	Comentarios	Referencia
Acetona/Agua	Hidroxicinamatos, flavanoles, antocianidinas, dihidrochalcones, flavonoles	Manzanas	90g muestra / 375mL Acetona:H_2O (70:30 v/v)	Vrhovsek et al., 2004
CH_2Cl_2 Diferentes procesos de extracción	AG, AP, AV, AC, AF, Acu, ApHb, ESCU, V, ELAG	Corcho	Matriz/CH_2Cl_2 Fracción I: MeOH:H_2O (80:20) Extracción posterior con dietil éter Fracción II (secuencial): MeOH H_2O	Santos et al., 2010
Varios disolventes	Ácidos fenólicos, catequinas, flavonoides y antocianidinas	Brotes grosellas negras	50mg (liofilizada) / 1.5mL EtOH: H_2O(0.05M H_3PO_4) (1:1)	Vagiri et al., 2012
		Hojas grosellas negras	400mg (liofilizada) / 14mL EtOH: H_2O(0.05M H_3PO_4) (1:1)	
		Frutos grosellas negras	50mg (liofilizada) / 1.5mL HCOOH:AcN (10:5, v/v)	

Cont. Tabla 1.3.1. Métodos de extracción recogidos en bibliografía para diferentes matrices.

Disolvente	Analitos[a]	Matriz	Comentarios	Referencia
Acetona	ACu, ApHb, AC, ACl, AF, AN, flavonoles	Bayas de Camarina	1g (deshidratada-congelada)/ 25mL Acetona:H_2O:HCOOH (70:29.5:0.5, v/v/v) Analizado por LC–DAD–MS/MS	León-González et al., 2013
	AB, ASal, AG, AP, AV, ACi, AF, ApHb, AS, AC, ACu, ASin		Bayas deshidratadas-congeladas Acetona:H_2O: HCOOH (70:29.5:0.5, v/v/v) Ratio 1:1 (w/v) Analizado por GC/MS	

[a]Analitos: AB (ác. benzoico), AC (ác. cafeíco), ACi (ác. cinámico), ACl (ác. clorogénico), ACu (ác. cumárico), AE (ác. elágico), AF (ác. ferúlico), AG (ác. gálico), AHc (ác. hidroxicinámico), AlP (aldehído protocatéquico); AmHb (ác. m-hidroxibenzoico), AN (ác. noeclorogénico), AP (ác. protocatéquico), ApHb (ác. p-hidroxibenzoico), AS (ác. siríngico), ASal (ác. salicílico), ASin (ác. sinápico), AV (ác. vainíllico), CON (coniferaldehído), ELAG (elagitaninos), ESC (escopoletina), ESCU (esculetina), SIN (sinapaldehíso), SIR (siringaldehído) y V (vainillina).

1.4. Cuantificación y separación de compuestos fenólicos. Antecendentes bibliográficos

Los métodos desarrollados para la determinación de compuestos fenólicos requiere de una mayor sensibilidad y selectividad en los métodos analíticos (Liu et al., 2008).

La espectrofotometría UV-Vis es de las técnicas de análisis más utilizadas, publicándose numerosos métodos para la cuantificación de compuestos fenólicos. Estos métodos se basan en diferentes principios y se han utilizado para determinar diferentes grupos estructurales presentes en los compuestos fenólicos.

Así, el método de Folin-Ciocalteau (Singleton y Rossi, 1965) es el más extensamente utilizado para la determinación de fenoles totales. El método se basa en la oxidación total de los compuestos fenólicos presentes en las muestras por la acción del reactivo Folin-Ciocalteau. El reactivo de Folin-Ciocalteau contiene molibdato y tungstato sódico, que reaccionan con cualquier tipo de fenol, formando complejos fosfomolíbdico-fosfotúngstico (Peterson, 1979). La transferencia de electrones a pH básico reduce los complejos fosfomolíbdico-fosfotúngstico en óxidos, cromógenos de color azul intenso, de tungsteno (W_8O_{23}) y molibdeno (Mo_8O_{23}), siendo proporcional la intensidad de este color al número de grupos hidroxilo de la molécula (Julkunen-Tiito, 1985).

Respecto a los taninos totales se determinan por la reacción del reactivo Folin-Denis (compuesto de tugstato de sodio, ácido fosfomolibdico y ácido fosfórico) con la capacidad reductora de los taninos en solución alcalina (Schanderl, 1970). Por otro parte, para la estimación de proantocinidinas totales se utiliza los ensayos de vainillina y proantocinidina (Naczk et al., 2006).

Por otro lado, el contenido total de flavonoides puede ser determinado utilizando un método colorimétrico basado en el complejo de los compuestos fenólicos con Al (III) (Naczk et al., 2006; Huang et al., 2009).

Los métodos espectrofotométricos para la cuantificación de antocianinos totales utilizando el método de diferencia de pH se basan en su comportamiento característico en condiciones ácidas. (Lapornik et al., 2005).

Estos métodos proporcionan información cualitativa y cuantitativa muy útil; actualmente, la espectroscopia es la principal técnica utilizada para la cuantificación de diferentes clases de compuestos fenólicos debido a su simplicidad y bajo coste. La gran desventaja de estos métodos espectrofotométricos es que sólo proporcionan una estimación del contenido fenólico total, además no separan ni aportan medida cuantitativa de compuestos individualmente.

La cromatografía líquida de alta eficacia es ampliamente conocida y en la bibliografía se encuentra extensamente descrita. A pesar de ello, y por la gran versatilidad y facilidad que ésta presenta, se ha realizado una revisión bibliográfica acerca de la determinación de compuestos fenólicos en diferentes materiales lignocelulósicos y en frutas haciendo uso de la misma, encontrándose numerosas publicaciones. En la Tabla 1.4.1. se recogen algunos de los métodos cromatográficos más relevantes.

Tabla 1.4.1. Métodos cromatográficos recogidos en la bibliografía para la determinación de compuestos fenólicos.

Analitos	Matriz	Fase estacionaria	Fase móvil[a] A	Fase móvil[a] B	T, °C	Flujo, mL·min^{-1}	Referencia
Elagitaninos	Madera de alcornoque	Lichrospher RP 18 250 x 4 mm, 5µm	H_2O (0.1% HCOOH)	MeOH Gradiente: 0-8% B, 45min; 8-100% B, 49min; 100-0% B, 53min	28	0.8	Masson et al., 1995
Ácidos fenólicos y cumarinas	Madera de alcornoque	Hypersil ODS C18 200 x 4 mm, 5µm	H_2O/H_3PO_4 (999:1)	$MeOH/H_3PO_4$ (999:1) Gradiente: 20-100% B, 40min	30	1.0	Fernández et al., 1996
Ácidos fenólicos y flavonoides	Hojas de olivilla	Lichrosorb RP 18 250 x 4.6 mm, 5µm Lichrosorb RP 18 precol. 10 x 4.0 mm	H_2O pH 3.2 con H_3PO_4	CH_3CN Gradiente: 0-45% B, 106 min	26	1.0	Romani et al., 1996
Ácidos fenólicos y cumarinas	Corcho	Hypersil ODS C18 200 x 4 mm, 5µm	$MeOH/H_3PO_4$ (999:1)	H_2O/H_3PO_4 (999:1) Gradiente: 20-100% A, 40min; 100% A, 45min	30	1.0	Conde et al., 1997, 1998

Cont. Tabla 1.4.1. Métodos cromatográficos recogidos en la bibliografía para la determinación de compuestos fenólicos.

Analitos	Matriz	Fase estacionaria	Fase móvil[a] A	Fase móvil[a] B	T, °C	Flujo, $mL \cdot min^{-1}$	Referencia
Elagitaninos	Corcho	Hypersil ODS C18 200 x 4 mm, 5µm	$MeOH/H_3PO_4$ (999:1)	H_2O/H_3PO_4 (999:1) Gradiente: 0-10% A, 40min; 30% A, 70min; 100% A, 90min	30	1.0	Conde et al., 1998
Ácidos fenólicos	Corcho	Supelcosil LC-18 250 x 4.6 mm, 5µm	H_2O/Ácido acético/alcohol isopropílico/MeOH (87:2:2:9) (v/v)	Gradiente: 0.9 $mL \cdot min^{-1}$, 12 min; 1.4 $mL \cdot min^{-1}$, 28 min	25	0.9-1.4	Mazzoleni et al., 1998
Ácidos fenólicos y catequinas	Uvas	Luna C18 150 x 2 mm, 5µm	2% ácido acético/H_2O	MeOH Gradiente: 0% B, 0min; 8% B, 5min; 15% B, 8min; 15% B, 12min; 50% B, 19min; 100% B, 22min	-	0.5	Palma et al., 1999
Elagitaninos	Duramen de roble	Lichrospher RP 18 250 x 4 mm, 5µm	MeOH	H_2O (0.2% TFA) Gradiente: 0-10% A, 40min; 100% A, 45min; 100% A, 50min; 0% A, 10min; 0% A, 5min	-	0.75	Zhentian et al., 1999

Cont. Tabla 1.4.1. Métodos cromatográficos recogidos en la bibliografía para la determinación de compuestos fenólicos.

Analitos	Matriz	Fase estacionaria	Fase móvil[a] A	Fase móvil[a] B	T, °C	Flujo, $mL \cdot min^{-1}$	Referencia
Ácidos fenólicos y cumarinas	Madera de alcornoque	Hypersil ODS C18 200 x 4 mm, 5µm	H_2O/H_3PO_4 (999:1)	$MeOH/H_3PO_4$ (999:1) Gradiente: 0-100% B, 70min	30	1.0	Cadahía et al., 2001
Ácidos fenólicos, flavonoides, catequinas y antociani-dinas	Manzana, cereza, frambuesa y uva	Luna C18 250 x 4.6 mm, 5µm	6% Ácido acético en 2mM de AcNa	ACN Gradiente: 0-10% B, 45min; 30% B, 60min; 50% B; 65min; 100% B, 70min	-	1.0	Tsao et al., 2003
Ácidos fenólicos y cumarinas	Madera de alcornoque	Hypersil ODS C18 250 x 4.6 mm, 5µm	$MeOH/H_2O$ (10:90)	$MeOH/H_2O$ (1:1) pH 3 con ácido acético Gradiente: 5% B, 1mL·min^{-1}, 10min; 10% B, 20min; 15% B, 0.4 mL·min^{-1}, 30min; 20% B, 60min; 30% B, 1.0 mL·min^{-1}, 70min; 55% B, 0.8 mL·min^{-1}, 90min	-	0.4-1.0	González et al., 2004

Cont. Tabla 1.4.1. Métodos cromatográficos recogidos en la bibliografía para la determinación de compuestos fenólicos.

Analitos	Matriz	Fase estacionaria	Fase móvil[a] A	Fase móvil[a] B	T, °C	Flujo, mL·min^{-1}	Referencia
Proantocia-nidinas	Manzana	Luna Silica-2 Phenomenex 250 x 4.6 mm, 5µm	1% HCOOH en cloruro de metileno	1% HCOOH en MeOH Gradiente: 8-50% B, 60min; 8% B, 65min	20	1	Vrhovsek et al., 2004
Ácidos fenólicos y catequinas		Hypersil ODS RP18 250 x 2.1 mm, 5µm Hypersil ODS RP18 Precol. 20 x 2.1 mm, 5µm	0.5% HCOOH en H_2O	2% HCOOH en MeOH Gradiente: 8-22% B, 14.3min; 27.8% B, 19.8min; 100% B, 20.8min; 100% B, 22.8min; 8% B, 24.8min	40	0.4	
Antociani-dinas		Xterra MS C18 150 x 2.1 mm, 3.5µm Xterra MS C18 Precol. 10 x 2.1 mm, 3.5µm	5% HCOOH en H_2O	5% HCOOH en MeOH Gradiente: 10% B, 0min; 30% B, 10min; 40% B, 17min; 51.2% B, 21min; 64% B, 26min; 90% B, 30min	40	0.2	
Flavonoides		Xterra MS C18 150 x 2.1 mm, 3.5µm Xterra MS C18 Precol. 10 x 2.1 mm, 3.5µm	1% HCOOH en H_2O	ACN Gradiente: 15% B, 0min; 17.4% B, 16min; 100% B, 30min	40	0.2	

Cont. Tabla 1.4.1. Métodos cromatográficos recogidos en la bibliografía para la determinación de compuestos fenólicos.

Analitos	Matriz	Fase estacionaria	Fase móvil[a] A	Fase móvil[a] B	T, ºC	Flujo, $mL \cdot min^{-1}$	Referencia
Antociani-dinas	Grosella y uva	Superspher 100 RP 250 x 4.6 mm, 18.5µm	10% HCCOH en H_2O	MeOH/H_2O/HCOOH (45:45:10)(v/v) Gradiente: 35% B, 0min; 95% B, 20min; 100% B, 25min; 100% B, 30min	30	0.8	Lapornik et al., 2005
Ácidos fenólicos	Espino	Lichrocart Hypersil ODS 250 x 4 mm, 5µm	2.5% Ácido acético	ACN/2.5% Ácido acético (80:20)(v/v) Gradiente: 7-40% B, 50min; 100% B, 55min	-	1.0	Svedström et al., 2006
Flavonoides y catequinas				Gradiente: 7-20% B, 50min; 100% B, 60min			
Proantocia-nidinas				Gradiente: 5-50% B, 35min; 100% B, 40min			
Antociani-dinas	Zumo	Diamonsil C18 250 x 4.6 mm, 5µm	0.1% HCOOH/H_2O	80% ACN/H_2O Gradiente: 66% B, 40min	-	1.0	Fang et al., 2009
Flavonoides			1% HCOOH	ACN Gradiente: 70% B, 10min; 70% B, 14min; 10% B, 15min; 10% B, 20min	-	0.5	
Ácidos fenólicos			4% Ácido acético	MeOH Isocrático A/B (80/20)	-	1.0	

Cont. Tabla 1.4.1. Métodos cromatográficos recogidos en la bibliografía para la determinación de compuestos fenólicos.

Analitos	Matriz	Fase estacionaria	Fase móvil[a] A	Fase móvil[a] B	T, ^{o}C	Flujo, $mL \cdot min^{-1}$	Referencia
Ácidos fenólicos	Judías	C18 Phenomenex 150 x 4.6 mm, 5µm C18 Phenomenex Precol. 4.0 x 3.0 mm	0.1% HCOOH	MeOH Gradiente: 15-80% B, 55min; 15% B, 57min; 15% B, 62min	25	0.7	Ross et al., 2009
Ácidos fenólicos, cumarinas y elagitaninos	Corcho	Discovery C18 150 x 2.1 mm, 5µm	0.1% HCOOH en H_2O	0.1% HCOOH en ACN Gradiente: 10% B, 0min; 100% B, 80min; 100%, 110min	-	0.2	Santos et al., 2010
Ácidos fenólicos, catequinas, flavonoides y antocianidinas	Grosella	Synergie hydro RP-80A 250 x 4.6 mm, 4µm	$HCOOH/H_2O$ (7:93)(v/v)	$ACN/MeOH/H_2O$ (90:5:5)(v/v) Gradiente: 8% B, 2min; 16% B, 21.5min; 23% B, 51.5min; 40% B, 56.5min; 8% B, 61.5min	24	1.2	Vagiri et al., 2012
Ácidos fenólicos y flavonoides	Bayas de camarina	Lichrocart C18 RP 250 x 4 mm, 5µm	H_2O/HCOOH (99:1)(v/v)	MeOH Gradiente: 5% B, 0min; 8% B, 10min; 13% B, 15min; 40% B, 47min; 65% B, 64min; 98% B, 65min; 98% B, 70min	-	1.0	León-González et al., 2013

[a]Fase móvil: ACN (acetonitrilo), AcNa (acetato de sodio) y CH_3CN (acetonitrilo)

1.5. Cuantificación de compuestos fenólicos mediante técnicas electroanalíticas. Antecendentes bibliográficos

Los sensores químicos tienen hoy en día un interés renovado debido a la necesidad actual de disponer de una mayor información analítica en unas condiciones no convencionales. Estos sensores presentan una nueva clase de instrumentación analítica, caracterizada por pequeñas dimensiones, un bajo coste, una utilización amigable y una generación de la información en tiempo real (Alegret et al., 2004).

Las técnicas electroanalíticas han utilizado diferentes tipos de electrodos de trabajo: gota de mercurio, film de mercurio tanto en estático como en dinámico, electrodos de carbono vitrificado, pasta de carbono, Au, Pt, así como la modificación de los mismos, y utilizando diferentes estrategias para conferir, al nuevo dispositivo, mayor sensibilidad, selectividad, mejor transferencia de carga, etc. y utilizando además diferentes geometrías. Recientemente, se ha incorporado la utilización de los electrodos impresos a los que se dedicará un apartado específico más adelante.

Uno de los principales cambios a los que se enfrenta la Química Analítica es al desarrollo de métodos que respondan a la creciente necesidad de realizar análisis rápidos in situ. Estos métodos deben ser sensibles y precisos, y ser capaces de determinar diversas sustancias con diferentes propiedades en muestras reales. En la actualidad, muchos de los métodos desarrollados con este fin se han basado en el uso de técnicas electroquímicas debido a su alta sensibilidad y selectividad, su fácil transporte y bajo coste.

Para fabricar los sensores químicos en estado sólido existen tres formas:

- Manual, es la tecnología más versátil y necesita una menor infraestructura para su desarrollo, pero presenta el inconveniente de poseer una baja reproducibilidad.

- Película fina (*thin film*), que se basa en la aplicación de un conjunto de técnicas de deposición y grabado que permiten la deposición secuencial de capas con espesores del orden de 1 a 10 micrómetros.

- Película gruesa (*thick film*), que es una tecnología intermedia entre las dos anteriores, la cual consiste en la utilización de una serie de técnicas que permiten

construir sensores planos de estado sólido, aplicando pastas o tintas sobre soportes o sustratos.

Los electrodos impresos son de reciente aplicación en electroanálisis. Desde los años 90, la tecnología screen-printing (impresión de tinta), adaptada desde la industria microelectrónica, ha ofrecido un alto volumen de producción extremadamente barato, y sin embargo con una alta reproducibilidad y fiabilidad en sensores (electrodos) de un solo uso; una técnica que es una gran promesa para el seguimiento in-situ. Sin embargo, el uso de tecnología screen-printing en la producción en serie de electrodos desechables de bajo coste para la determinación electroquímica de una amplia gama de sustancias está actualmente en fase de crecimiento (Domínguez et al., 2007).

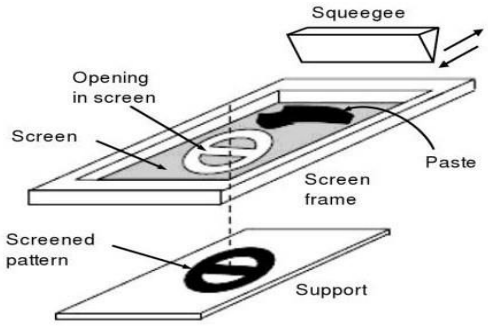


Figura 1.4.1. El proceso screen-printing (Bănică, 2012).

Los electrodos screen-printed (electrodos impresos) se encuentran dentro de los fabricados mediante la tecnología *thick film*. El proceso screen-printing se realiza mediante el recubrimiento de una pasta de diferentes tipos (carbono, pinturas de plata, etc) en un soporte plano a través de aberturas en una pantalla. La pasta es presionada sobre la pantalla y repartida con la ayuda de una espátula (squeegee), y la pantalla determina el patrón de la capa depositada sobre el soporte (Figura 1.4.1.). La pasta es una mezcla de los materiales sensores que se aplican, un aditivo orgánico y un disolvente. Después de la impresión, la capa se deja secar y posteriormente se somete a un tratamiento térmico. Durante esta etapa, el portador orgánico se quema, y otros procesos químicos y físicos producen la adhesión (Bănică, 2012).

Los electrodos modificados son aquellos a los que su superficie se altera deliberadamente, se trata químicamente, se funcionaliza, etc., con el fin de modificar sus propiedades electroquímicas o mejorar su sensibilidad y selectividad en una determinación analítica. La modificación de un electrodo de trabajo puede llevarse a cabo, por ejemplo, fijando reactivos en la superficie del electrodo.

Se suele utilizar como material base de los electrodos el vidrio, platino, oro y carbono. En numerosas aplicaciones electroanalíticas, la base de electrodo que suele ser modificada es la de carbono. Una de las principales razones para el desarrollo de electrodos modificados es para reemplazar los electrodos de mercurio (Thomas y Henze, 2001).

Los electrodos impresos son comercializados por diferentes empresas. Los que se han utilizado para la realización de esta Tesis Doctoral han sido suministrados por la casa comercial DropSens.

Se comercializan diferentes tipos de electrodos basados en carbono, oro, platino, plata y modificados con nanotubos de carbono, grafeno, etc. Los electrodos screen-printed de carbono (SPCEs) modificados también los comercializa DropSens.

A continuación se mencionan alguna de las modificaciones de los electrodos impresos:

- SPCEs modificado con mesoporos ordenados de carbono.
- SPCEs modificados con estreptavidina.
- SPCEs modificados con nanomateriales de carbono/nanopartículas de oro.
- SPCEs modificados con polianilina.
- SPCEs modificados con nanotubos de carbono.
- SPCEs modificado con nanofibras de carbono, diseñados para el desarrollo de biosensores con un área activa electroquímica mayor.
- SPCEs que incluyen Co-Ftalocianina o azul de Prusia, recomendados para el desarrollo de biosensores enzimáticos basados en oxidasas.

- SPCEs modificados con ferrocianuro desarrollados para trabajar con microvolúmenes.

- SPCEs modificados con óxido de bismuto ideales para la determinación de metales pesados a niveles ppb.

- SPCEs modificado con óxido de niquel diseñados para la oxidación electrocatalítica de moléculas orgánicas pequeñas.

- SPCEs modificados con nanopartículas de oro diseñados para el desarrollo de biosensores con propiedades de transferencia electrónica mejoradas.

- SPCEs modificados con grafeno.

Estos últimos SPCEs son uno de los electrodos utilizados para la realización de esta Tesis.

El grafeno es un firme candidato para su uso enaplicaciones potenciales en numerosos campos tales como la nanoelectrónica, dispositivos nanoelectromecánicos, nanocompuestos, sensores, ultracondensadores, células solares, dispositivos de cristal líquido, etc. Para aplicaciones en dispositivos electroquímicos, el grafeno posee excelentes propiedades, como alta conductividad electrónica, velocidad de transferencia de electrones heterogénea rápida en los bordes de los hojas de grafeno y en los sitios de defectos del plano basal, elevada superficie específica, y buena biocompatibilidad.

Los electrodos de pasta de carbono modificados con grafeno, película de la superficie del electrodo modificado con grafeno y micro/nanoelectrodos de grafeno se han desarrollado para determinar diversas especies como glucosa, ácido ascórbico, dopamina o peróxido de hidrógeno entre otras (Ping et al., 2012). Sin embargo, los trabajos publicados en los que se utilizan electrodos impresos de carbón modificados con grafeno para la determinación de diferentes compuestos son escasos (Eissa et al., 2012; Ping et al., 2012).

En la revisión bibliografía efectuada no se ha encontrado antecedentes acerca de la utilización de técnicas electroanalíticas para la determinación del contenido de compuestos fenólicos en matrices de corcho o en el agua de cocido. Si bien, un capítulo de esta Tesis ya ha sido publicado en la revista Electroanalysis, en el que se ha propuesto métodos para la determinación del contenido total de fenoles de bajo potencial de oxidación en aguas de

cocido de corcho. No obstante, sí hay antecedentes bibliográficos en los que se utilizan métodos electroanaliticos para el estudio y determinación de diferentes compuestos fenólicos, así como el contenido de fenoles totales en diversas matrices tales como vino, frutas, zumos, algodón, etc.

En la Tabla 1.4.2. se recogen los trabajos más relevantes que aparecen en la bibliografía en relación con la utilización de métodos electroanalíticos para el estudio de fenoles presentes en diferentes matrices (vinos, frutas, algodón, etc), recogiéndose las características más importantes de los métodos propuestos. Dobes et al. (2013) han publicado un review acerca de métodos electroanalíticos para la determinación de fenoles en alimentos.

En algunos estudios electroanalíticos, Blasco et al. 2005, proponen un "índice electroquímico" para la determinación de la capacidad antioxidante para la determinación de fenoles totales presentes en la muestra, como alternativa al método espectrofotométrico de Folin-Ciocalteau. La alternativa proporciona, de una forma más real, el contenido total de fenoles presentes en las muestras, ya que en el método de Folin-Ciocalteau es poco selectivo.

En general, el contenido de fenoles totales obtenido utilizando las técnicas electroanalíticas se compara con el fotométrico de Singleton y Rossi (1965), estableciendo correlaciones entre ambos métodos. También se utiliza en algunos casos HPLC para cuantificar cada uno de los fenoles presentes en las muestras (Blasco et al., 2005; Cuartero et al., 2011; Šeruga et al., 2011; Makhotkina y Kilmartin, 2012; Filik et al., 2013).

Así pues, el contenido de fenoles totales, tanto por el método espectrofotométrico de Folin-Ciocalteau como por el electroquímico, se suele expresar como equivalentes en ácido gálico, elágico o cafeíco, etc. Por ello, es de interés el estudio del comportamiento electroanalítico de estos compuestos ya que la capacidad antioxidante, se expresa en relación a algunos de los compuestos anteriormente citados.

Tabla 1.4.2. Revisión bibliográfica de técnicas voltamperométricas para la determinación de compuestos fenólicos.

Compuesto fenólico	Electrodo de trabajo	Técnica	Muestras	Condiciones	Comentarios	Referencia
Fenoles totales	Carbón vitrificado	CV	Vinos modelo	pH 3.6	Diferentes tipos de vino El método propuesto (en vino), se compara con el método FC El contenido de polifenoles por FC proporciona valores más altos que CV	Kilmartin et al., 2001
Fenoles totales	Carbón vitrificado	CV	Vinos blancos y tintos	Preparación de patrones en vino modelo: etanol 12%, ácido tartárico 0.033 M y NaOH hasta pH 3.6	Correlación entre composición fenólica en vinos y la respuesta en CV El método se compara con el métodoFC Contenido de polifenoles por FC más altos que CV Se comparan resultados por HPLC	Kilmartin et al., 2002.
Fenoles totales	Carbón vitrificado	CV	Té verde, oolong y negro; y café	Tampón fosfato pH 7.0 Extracción con agua a diferentes Tª (10-100^{o}C) Dilución 1/50	Comparación con HPLC El nivel de componentes fenólicos aumenta con la temperatura de extracción La adicción de leche disminuye la respuesta más en el té que en el café	Kilmartin y Hsu, 2003

Cont. Tabla 1.4.2. Revisión bibliográfica de técnicas voltamperométricas para la determinación de compuestos fenólicos.

Compuesto fenólico	Electrodo de trabajo	Técnica	Muestras	Condiciones	Comentarios	Referencia
Ácidos: cafeico, sinápico, ferúlico y *p*-cumárico	Carbón vitrificado y carbón vitrificado modificado	CV	Zumo de naranja	Tampón acetato pH 5.6	Linealidad: 10^{-4} -10^{-3} M	Sousa et al., 2004
Fenoles totales	Carbón vitrificado	FIA	Alimentos	E 0.8 V pH 7.5	Patrón estandar (+)-catequina Limpieza del electrodo: física, química y electroquímica Propuesta de un índice electroquímico de fenoles como método de screening de PT Resultados en concordancia con los obtenidos en HPLC-DAD Más bajos que con FC	Blasco et al., 2005
Fenoles totales	Biosensor de Tyrosinasa modificado con nanopartí-cula de oro (Tyr-nAu-GCE)	Ampero-metría	Vinos	Tampón fosfato 0.1M pH 7.4. Medida a 100mV	Método de adicción estándar Sin limpieza del electrodo Se comparan resultados utilizando el método de FC Resultados: expresado como equivalentes de ácido cafeico, obteniéndose valores más bajos que con FC pero con buena correlación entre ambos métodos	Carralero et al., 2005

Cont. Tabla 1.4.2. Revisión bibliográfica de técnicas voltamperométricas para la determinación de compuestos fenólicos.

Compuesto fenólico	Electrodo de trabajo	Técnica	Muestras	Condiciones	Comentarios	Referencia
Ácido cafeíco	Carbón vitrificado polimérico	DPV	Vino tinto	Atmósfera de N_2 Tampón acetato pH 3.5	Linealidad: $9.65 \cdot 10^{-7}$ -$1.10 \cdot 10^{-5}$ M; LOD=$2.0 \cdot 10^{-6}$ M	da Silva et al., 2008
Ácido gálico, epicatequina y galato de epicate-quina (ECG)	Carbón vitrificado	CV, SWV			Estudio de mecanismos $LOD_{ECG}=3.26 \cdot 10^{-7}$ M	Novak et al., 2009
	Polipirrol	CV	Vinos		Clasificación vinos	Arrieta et al., 2010
Fenoles y flavonoides totales	Carbón vitrificado	CV	Vinos	pH 3.0 Vinos libres de SO_2 (adicionando acetaldehído)	Caracterización de vino y polifenoles en vino por medida electroquímica Se estima el nivel de SO_2 comparando las señales CV antes y después de la adición	Makhotkina, y Kilmartin, 2010
Índice antioxidante	Carbón vitrificado	CV	Infusiones	Tampón acetato	Determinación de la capacidad antioxidante de las muestras	Piljac-Žegarac et al., 2010

Cont. Tabla 1.4.2. Revisión bibliográfica de técnicas voltamperométricas para la determinación de compuestos fenólicos.

Compuesto fenólico	Electrodo de trabajo	Técnica	Muestras	Condiciones	Comentarios	Referencia
Ácido gálico	Gota de mercurio	AdCSV	Plasma, orina y té	E_{ac} 50mV; t_{ac} 100s Tampón borax 0.1M pH 7.53	La sensibilidad del método es más alta que en otros métodos existentes Linealidad: 0.1-600 $ng \cdot L^{-1}$; LOD: 0.05 $ng \cdot L^{-1}$	Abbasi et al., 2011
Ácido gálico	Carbón vitrificado	AdSV	Té negro	Tampón fosfato 0.2M	Estudio mecanismo electroquímico	Abdel-Hamid y Newair, 2011
Ácido elágico libre y total	Carbón vitrificado	SWV, CV, DPV	Alimentos	Tampón 0.2 M AcH/AcONa pH 5.5 Hidrólisis de la muestra en HCl 2 M y extracción en dietil éter	Sin limpieza del electrodo entre muestras Linealidad: 10^{-7}-$1.5 \cdot 10^{-6}$ M LOD: 10^{-8} M (S/N=3) Se comparan resultados obtenidos para el AE total en distintos alimentos mediante SWV y HPLC, con buena concordancia	Cuartero et al., 2011

Cont. Tabla 1.4.2. Revisión bibliográfica de técnicas voltamperométricas para la determinación de compuestos fenólicos.

Compuesto fenólico	Electrodo de trabajo	Técnica	Muestras	Condiciones	Comentarios	Referencia
Ácido elágico y ácido gálico	Pasta de carbono modificado con nanotubos de carbono	DPV	Cáscara de granada, hojas de mirto e hierbas	Tampón B-R pH 2.0	Método adicción estándar El procedimiento está libre de interferencias de la matriz para el AE en cáscara de granada Las interferencias de la matriz no son significativas en el caso de determinación de AG en hierbas LOD_{AG} = 0.2 µM; LOD_{AE} = 0.21 nM	Ghoreishi et al., 2011
Fenoles totales	Carbón vitrificado	DPV	Vinos tintos	Tampón AcH/AcONa pH 3.6 Linealidad: 1-15 $mg \cdot L^{-1}$; LOD: 0.53 $mg \cdot L^{-1}$	Patrón (+)-catequina HPLC y espectrofotometría. Contenido más bajo en HPLC que con FC Buena correlación DPV-HPLC y DPV-FC	Šeruga et al., 2011
Fenoles totales	Pasta de carbono modificado con nanotubos de carbono (MCPE)	DPV	Vino	Tampón fosfato pH 2.5	Resultados similares a los obtenidos mediante FC Determinación de la capacidad antioxidante de muestras de vino Linealidad: $5.0 \cdot 10^{-7}$-$1.5 \cdot 10^{-5}$ M; LOD= $3.0 \cdot 10^{-7}$ M	Souza et al., 2011

Cont. Tabla 1.4.2. Revisión bibliográfica de técnicas voltamperométricas para la determinación de compuestos fenólicos.

Compuesto fenólico	Electrodo de trabajo	Técnica	Muestras	Condiciones	Comentarios	Referencia
Esculetina	Biosensor basado en nanopartí-culas de Au	SWV	Té negro	Muestras de té negro preparado por infusión en tampón acetato (pH 4.5)	Método de adicción estándar. Método simple y que presenta alta sensibilidad. Linealidad: 0.40-9.86 µM; LOD: 0.11 µM	Zapp et al., 2011
Fenoles totales	Carbono vitrificado	CV	Zumo de uva	pH 3.3 Eliminar el SO_2 (adicionando acetaldehído)	Tres picos en el zumo de uva Confirman cada pico por HPLC-MS a las fracciones previas mediante HPLC semipreparativo Buena correlación de resultados del contenido fenólico total en zumos entre el método FC y el área de la corriente anódica a 700 mV	Makhotkina y Kilmartin, 2012
Ácido gálico	Carbón vitrificado modificado con poliepinefrina	CV, SWV	Té negro	Tampón fosfato 0.2M		Abdel-Hamid y Newair, 2013

Cont. Tabla 1.4.2. Revisión bibliográfica de técnicas voltamperométricas para la determinación de compuestos fenólicos.

Compuesto fenólico	Electrodo de trabajo	Técnica	Muestras	Condiciones	Comentarios	Referencia
Ácido cafeíco	Carbón vitrificado modificado con óxido de grafeno	SW-AdSV	Vino blanco	Tampón acetato 0.1M pH 5.0	Método de adicción estándar Resultados comparados con HPLC con buena concordancia LOD = $9.1 \cdot 10^{-8}$ M	Filik et al., 2013
Ácidos fenólicos totales	Carbón vitrificado	DPV	Hojas de algodón	Tampón fosfato 0.2M pH 3.0	Extracción con etanol. Polifenoles totales expresado como equivalentes de ácido cafeíco Método de alta velocidad, sensibilidad, bajo coste, fácil operación y buena selectividad comparado con otros métodos analíticos Linealidad: $1 \cdot 10^{-7}$-$1 \cdot 10^{-6}$ M; LOD = $6.8 \cdot 10^{-8}$ M	Magarelli et al., 2013
Fenoles totales	Carbón vitrificado modificado con nanotubos de carbono (GC/CNT/PEI)	FIA	Vino	Tampón acetato pH 4.5 Velocidad de flujo 2.5mL·min^{-1}	Resultados más bajos que mediante FC pero con buena correlación entre ambos LOD < 0,1 µM	Sánchez et al., 2013

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2. Objetivos

2. Objetivos

El objetivo principal de esta Tesis Doctoral es el de proponer métodos analíticos que permitan caracterizar y cuantificar compuestos bioactivos presentes en materiales lignocelulósicos, tales como el corcho procedente del alcornoque *Quercus suber* L. u otros materiales susceptibles de contener estos compuestos de interés por presentar propiedades antioxidantes y anticancerígenas.

Como objetivos específicos, se plantean los siguientes:

- ✓ Proponer métodos analíticos para la determinación, por técnicas cromatográficas, de compuestos fenólicos con propiedades antioxidantes y anticancerígenas.

- ✓ Aplicar los métodos propuestos para la cuantificación de compuestos fenólicos presentes en corcho.

- ✓ Optimizar el proceso de extracción de los compuestos fenólicos de bajo peso molecular y de los elagitaninos mayoritarios presentes en muestras de corcho.

- ✓ Aislar los compuestos bioactivos del corcho.

- ✓ Desarrollar métodos para la determinación de compuestos fenólicos presentes en aguas de cocido de corcho, mediante técnicas electroanalíticas utilizando diferentes tipos de electrodos (carbono vitrificado y electrodos impresos modificados con grafeno).

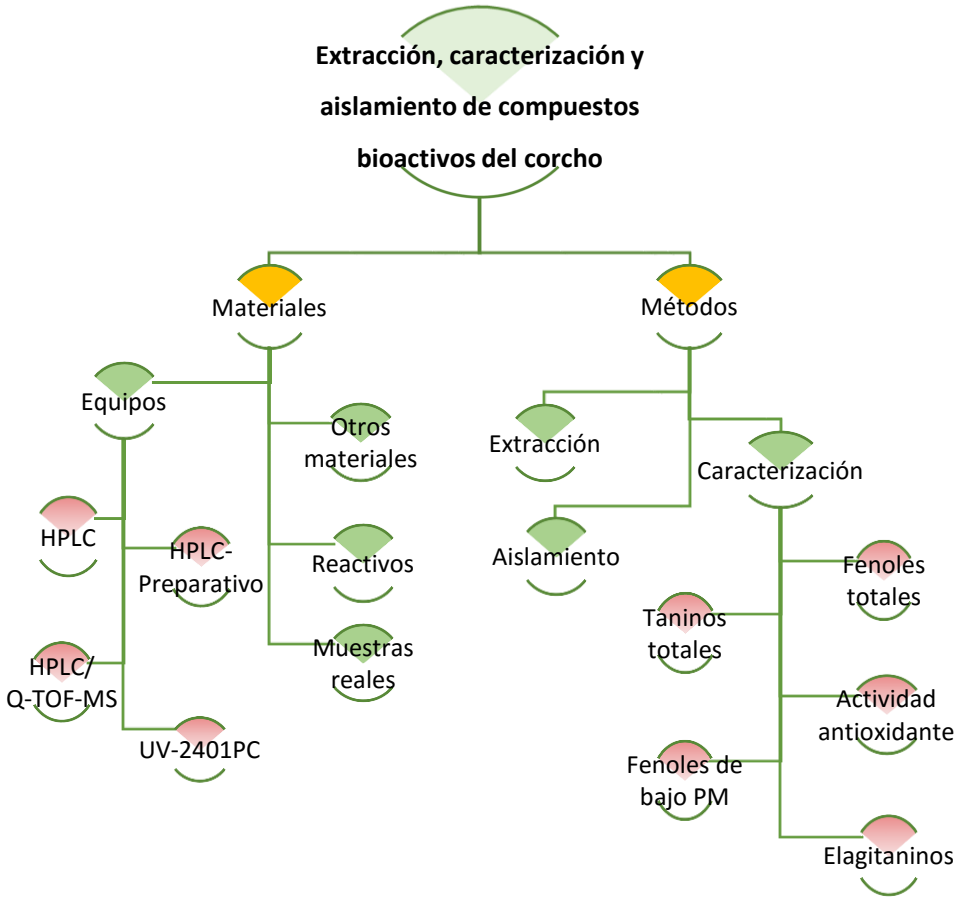
- ✓ Desarrollar métodos en continuo: FIA con detección electroquímica utilizando electrodos impresos modificados con grafeno, para la determinación del contenido total de fenoles presentes en aguas de cocido de corcho.

3. Materiales y métodos

En este capítulo se describen los materiales, así como los métodos utilizados en el desarrollo experimental de esta Tesis Doctoral. El capítulo se ha estructurado en dos partes diferenciadas: extracción, caracterización y aislamiento de compuestos bioactivos del corcho; y análisis del contenido de fenoles totales en aguas de cocido de corcho. En cada una de ellas se incluye el material e instrumentación utilizada y las diferentes metodologías aplicadas.

3.1. Extracción, caracterización y aislamiento de compuestos bioactivos del corcho.

En esta primera parte del capítulo se describe los materiales y métodos utilizados en la presente Tesis Doctoral para la extracción, caracterización y aislamiento de compuestos bioactivos del corcho. En el esquema global siguiente se resumen los principales apartados que se van a desarrollar.



3.1.1. Materiales

3.1.1.1. Equipos

- Cromatógrafo líquido de alta eficacia (HPLC)

La determinación analítica de los compuestos fenólicos de bajo peso molecular y los elagitaninos en extractos de corcho, se ha llevado a cabo en un cromatógrafo líquido de alta eficacia Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA).

El cromatógrafo está equipado con una cabina de disolventes, desgasificador de vacío, bomba cuaternaria que permite flujos comprendidos entre 0.001-10 $mL \cdot min^{-1}$ para análisis en gradiente, muestreador automático para 100 viales, y termostatizador de columna que permite la separación desde temperatura ambiente hasta 80˚C. El cromatógrafo está acoplado a un detector de diodos para análisis ultrarrápidos multi-λ y espectrales con ocho señales y velocidad de adquisición de datos de 80 Hz. El equipo se monitoriza a través del software Chemstation, que permite controlar todos los componentes del sistema para llevar a cabo los análisis de forma automatizada.

El estudio cromatográfico y la separación analítica se ha llevado a cabo utilizando una columna Lichrospher RP-18 (250 mm x 4 mm x 5 µm) de Agilent.

- Cromatógrafo de líquido preparativo (HPLC-Preparativo)

El aislamiento de compuestos bioactivos del corcho se ha realizado con un cromatógrafo de líquidos preparativo Agilent 1260 Infinity Preparative System (Agilent Technologies, Santa Clara, CA, USA).

El sistema cromatográfico está formado por:

- Dos bombas binarias, permiten trabajar con presiones de hasta 50 MPa, proporcionando caudales de hasta 100 $mL \cdot min^{-1}$, pudiendo ser manejado desde el software del equipo o desde el panel de control de la propia bomba, lo que permite modificar puntualmente las condiciones de separación de forma instantánea. La configuración de la bomba permite trabajar en modo isocrático o en gradiente.

- Detector de diodos para análisis ultrarrápidos multi-λ con celda de flujo preparativa, permite realizar espectros en el intervalo de longitud de onda de 180-950 nm de forma online.

- Colector de fracciones automático, con posibilidad de colectar rango de volúmenes desde mililitros hasta litros y permite la acumulación de muestra según tiempo, volumen o pico.

- Columna PrepHT SB-C18 (21.2 x 150 mm, 7µm) (Agilent, USA)

- Software ChemStation

➢ Cromatógrafo de líquidos con detector de masas y tiempo de vuelo (HPLC/Q-TOF-MS)

Para la validación del método cromatográfico en la determinación de compuestos fenólicos de bajo peso molecular en los extractos de corcho, se ha utilizado un equipo de HPLC acoplado a un detector de espectrometría de masa de tiempo de vuelo (HPLC/Q-TOF-MS) Agilent 6530 Series Accurate Mass QTOF-MS (Agilent Technologies, Santa Clara, CA, USA). La validación de los compuestos bioactivos aislados también se ha realizado con este equipo.

➢ Espectrofotómetro UV-Vis

La determinación de fenoles totales en aguas de cocido de corcho se ha llevado a cabo en un espectrofotómetro UV-Vis Varian Cary-50 (Palo Alto, California, USA). Este equipo consta de una lámpara de flash de xenón y un haz concentrado, el cual permite realizar mediciones de muestras de hasta 3 unidades de absorbancia sin dilución. El rango de longitudes de onda que barre el espectrofotómetro está comprendido entre 190-1100 nm y dispone de una resolución < 1.5 nm.

➢ Espectrofotómetro UV-2401PC

La determinación de la actividad antioxidante de los extractos de corcho se ha llevado a cabo con un espectrofotómetro Shimadzu UV-2401PC (Shimadzu Corporation, Tokio, Japón).

Este equipo dispone de un sistema monocromador simple con rejilla holográfica de alto rendimiento. Se pueden realizar estudios de cinética enzimática, análisis de pureza de ADN, citochromo P-450, análisis de color, medición de espesor de films, tests de identidad y uniformidad de contenido en tabletas. En esta Tesis se ha utilizado para el estudio de la cinética en la cuantificación de la actividad antioxidante.

3.1.1.2. Otros materiales y reactivos

- Liofilizador LyoQuest Plus-55, (Telstar, España).
- Rotavapor VV 2001 (Heidolph. Alemania).
- Trituradora Retsch Cross Beater Mill SK1 (Haan, Alemania).
- Trituradora Retsch Mühle (Haan, Alemania).
- Nitrógeno 5.5 Halocarbon Free (Linde, España).

3.1.1.3. Reactivos

Se han utilizado patrones de una pureza superior al 99% de:

- Ácido gálico, ácido protocatéquico, ácido p-hidroxifenil, ácido p-hidroxibenzoico, ácido vainíllico, ácido cafeíco, ácido siríngico, ácido p-cumárico, ácido ferúlico, ácido salicílico, ácido elágico, siringaldehído, coniferaldehído, sinapaldehído, esculetina, vainillina, eriodictiol, naringenina y ácido tánico suministrados por Sigma-Aldrich (Steinheim, Alemania).
- Vescalagina y castalagina, han sido suministrados por el Doctor Stephane Quideau, del Instituto Europeo de Química y Biología de Pessac, Francia.

Además se han utilizado otros reactivos que se relacionan a continuación:

- Carbonato de sodio anhidro, grado PA (Panreac, España).
- Reactivo de Folin-Ciocalteau (Panreac, España).
- Carbonato de sodio decahidratado, 99.5% (Sigma-Aldrich, Alemania).

- Reactivo Folin-Denis (Panreac, España).
- Fosfato monosódico, 98% (Panreac, España).
- Fosfato disódico, 98% (Panreac, España).
- Peroxidasa tipo VI (POD), 250-330 U/mg (Sigma-Aldrich, Alemania).
- 2,2'-azino-bis-(3-etil-benzotiazolin-6-sulfónico) (ABTS), 98% (Fluka, Alemania).
- Ácido 6-hidroxi-2,5,7,8-tetrametilcromano-2-carboxílico (TROLOX), 98% (Sigma-Aldrich, Alemania).
- Ácido ortofosfórico, 85-88% (Panreac, España).
- Lana de vidrio lavada, químicamente pura (Panreac, España).
- Metanol, grado HPLC (Panreac, España).
- Diclorometano, PA (Panreac, España).
- Acetona, grado HPLC (Scharlau, España).
- Dietil éter, PA (Scharlau, España).
- Peróxido de hidrógen, 30% v/v (Sigma-Aldrich, Alemania).
- Ácido fórmico, 98% PA (Panreac, España).
- Ácido trifluoroacético (TFA), RA (Panreac, España).

3.1.1.4. Muestras reales

El corcho utilizado en la investigación de esta Tesis Doctoral ha sido suministrado por empresas corcheras de San Vicente de Alcántara (Badajoz). Las muestras de corcho han sido clasificadas de acuerdo al criterio industrial por el personal técnico del Instituto del Corcho, la Madera y el Carbón Vegetal (Mérida).

3.1.2. Métodos

3.1.2.1. Extracción de compuestos bioactivos del corcho

Para la extracción de compuestos bioactivos del corcho, se ha partido de un corcho crudo con raspa clasificado en calidad de refugo. Con el objetivo de facilitar la extracción de los compuestos bioactivos, las muestras de corcho han sido previamente trituradas y tamizadas, para así trabajar con una granulometría lo más homogénea posible.

Con el fin de optimizar el proceso de aislamiento se ha procedido a realizar varios métodos de extracción, los cuales se describen a continuación.

Método_1

Se pesan aproximadamente unos 10 g de corcho, con un tamaño de grano entre 0.25 y 0.5 mm de diámetro, se realiza una extracción en soxhlet con diclorometano durante 6 horas para eliminar los componentes lipofílicos.

El corcho, una vez lavado y seco (24h a 35˚C), se pone en contacto con una disolución de metanol-agua, 80/20 (v/v), a temperatura ambiente durante 24 h con agitación constante. Se filtra la suspensión y el metanol se evapora en rotapvapor. La solución acuosa, libre de metanol, se extrae tres veces con dietil éter. En la fase orgánica el disolvente se elimina mediante evaporación a vacío, obteniendo así el extracto *A*; y la fase acuosa se procede a liofilizar, dando el extracto *B*. En la Figura 3.1.1. se resume el procedimiento de extracción llevado a cabo.

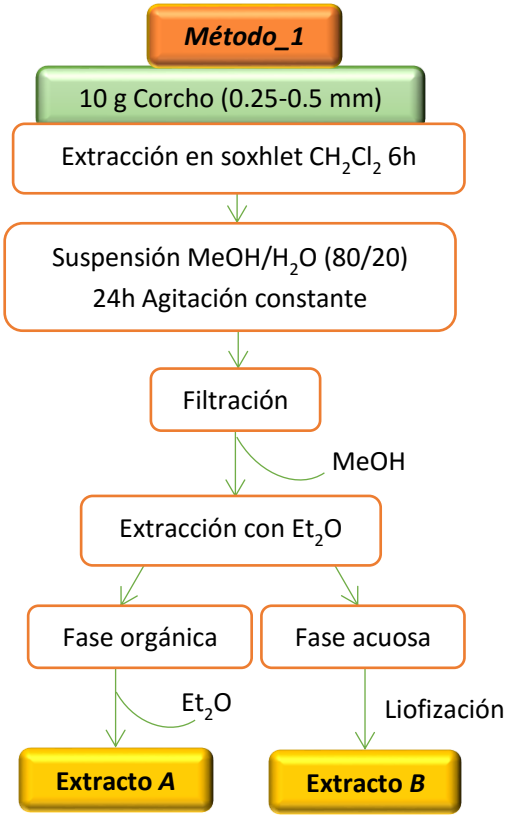


Figura 3.1.1. Esquema del método_1 de extracción.

Método_2

Se toman unos 10 g de corcho, con un tamaño de grano entre 0.25 y 0.5mm de diámetro, se le realiza una extracción en soxhlet con diclorometano durante 6 horas para eliminar los componentes lipofílicos.

El corcho tratado, lavado y seco (24h a 35˚C), se le somete a una extracción en soxhlet con metanol durante 6 horas (extracto *C*), seguido por un reflujo de agua durante 6 horas (extracto *D*). Los solventes se eliminan de los extractos líquidos por evaporación a vacío y liofilización, obteniendo los extractos *C* y *D*, respectivamente. (Santos et al., 2010). En la Figura 3.1.2. se resume en esquema el procedimiento de extracción seguido.

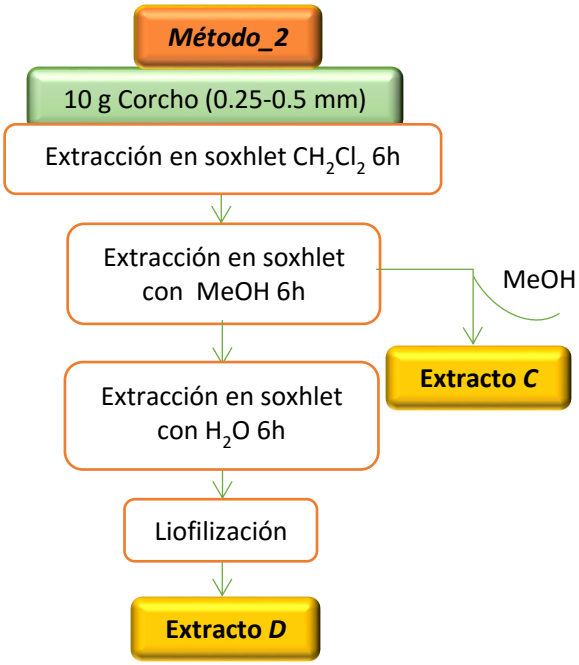


Figura 3.1.2. Esquema del método_2 de extracción.

Método_3

Al corcho, con un tamaño de grano entre 0.5 y 1.0 mm de diámetro, se le realiza una extracción, con una proporción de disolvente-corcho de 10 $mL \cdot g^{-1}$, en ausencia de luz y bajo atmósfera de nitrógeno utilizando una mezcla de acetona:agua (70:30, v:v), a temperatura ambiente y con agitación constante durante 24 h. La. Posteriormente, se filtra la suspensión y el disolvente orgánico se elimina por evaporación a bajas presiones. De la fase acuosa, se elimina el agua mediante liofilización, obteniendo un extracto estable, denominado extracto *E* (Zhentian et al., 1999).

Se ha procedido a realizar este mismo proceso lavando previamente el corcho con diclorometano durante 6 horas, obteniéndose el extracto *F*. En la Figura 3.1.3. se ha esquematizado el procedimiento de extracción seguido.

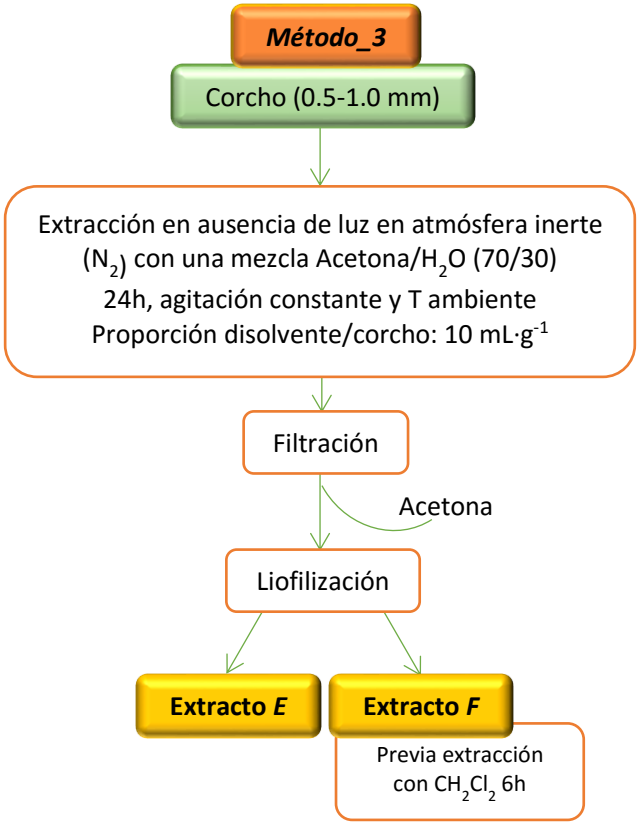


Figura 3.1.3. Esquema del método_3 de extracción.

Método_4

Las muestras de corcho, con un tamaño de grano entre 0.5 y 1.0 mm de diámetro, se le realiza una extracción en soxhlet con diclorometano durante 6 horas para eliminar los componentes lipofílicos. Una vez seco el corcho y seco (24h a 35˚C), 10 g de corcho seco se tratan con 250 mL de una mezcla metanol-agua, 80/20 (v/v), a temperatura ambiente durante 24 h con agitación constante. La suspensión se filtra y el metanol se elimina por evaporación a bajas presiones. La fase acuosa resultante se divide en dos fracciones (I y II).

La fracción I se somete a un proceso de liofilización, obteniéndose el extracto *G*. A la fracción II se le realiza una extracción con dietil éter, conservando la fase acuosa para proceder posteriormente al proceso de liofilización, obteniéndose el extracto *H*. (Conde et al., 1998).

Se ha procedido a realizar este mismo proceso sin lavar previamente el corcho con diclorometano durante 6 horas, obteniéndose los extractos *I* (fracción I) y *J* (fracción II), respectivamente.

En la Figura 3.1.4. se muestra un esquema del procedimiento de extracción llevado a cabo.

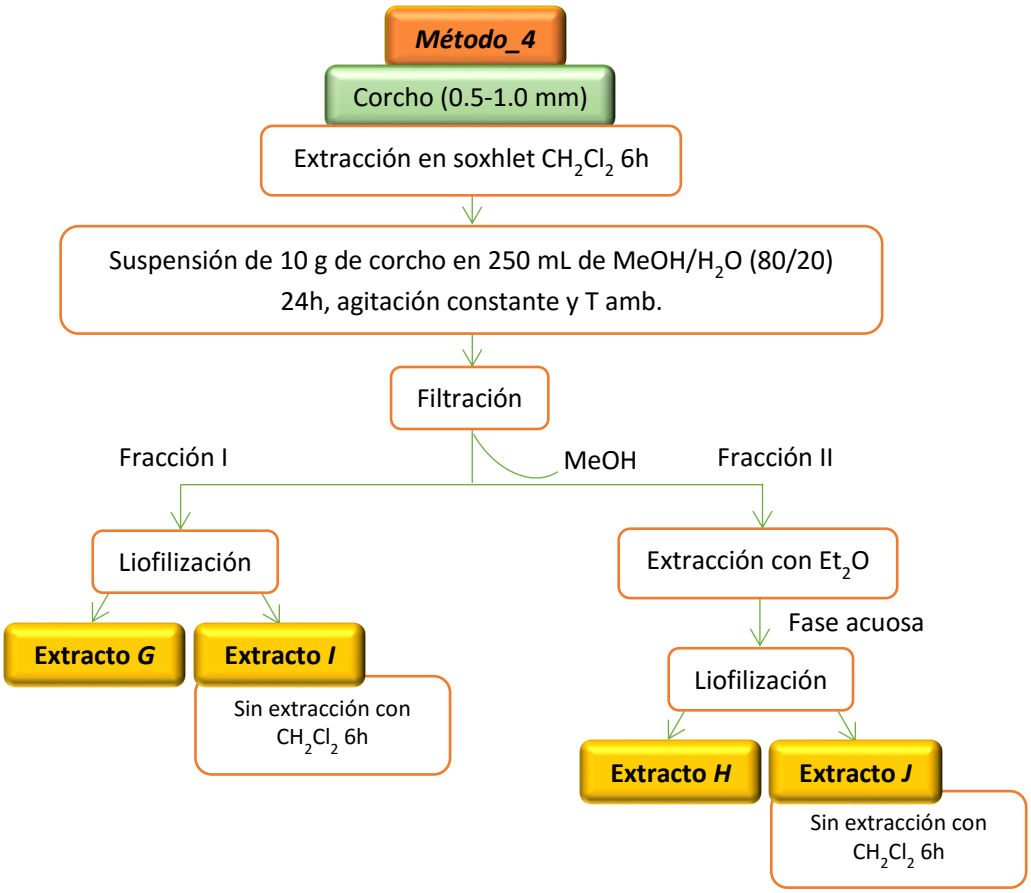


Figura 3.1.4. Esquema del método_4 de extracción.

3.1.2.2. Caracterización de compuestos bioactivos del corcho

Los extractos obtenidos aplicando los métodos indicados en el apartado anterior, se han caracterizado a través de la determinación de fenoles y taninos totales, actividad antioxidante, compuestos fenólicos de bajo peso molecular y elagitaninos presentes en los diferentes extractos. A continuación, se describe la metodología empleada para cada uno de ellos.

- Análisis de fenoles totales

La determinación de fenoles totales se ha basado siguiendo el procedimiento de Singleton y Rossi, (1965). Este método es el más utilizado y se basa en la capacidad de los fenoles para reaccionar con agentes oxidantes. El reactivo utilizado para este fin es el denominado Folin-Ciocalteau, éste contiene molibdato y tungstato sódico, que reaccionan con los fenoles u otros agentes oxidantes, formando complejos fosfomolíbdico-fosfotúngstico (Peterson, 1979). La absorbancia medida a 670 nm es directamente proporcional al contenido de fenoles totales de la muestra.

Con el objetivo de minimizar el efecto matriz de las muestras, se ha utilizado el método de adición patrón utilizando el ácido gálico (0.5-8.0 $\mu g \cdot mL^{-1}$) y expresando el contenido de fenoles totales como g de equivalente de ácido gálico $\cdot$ Kg^{-1} de corcho seco.

El procedimiento seguido fue el siguiente: se tomaron 10 mg del extracto de corcho (procedente de los diferentes extracciones del apartado 3.1.2.1.) y se suspendieron en 50 mL de metanol:agua (50:50). Las muestras preparadas se mantuvieron refrigeradas a 4˚C hasta el análisis de las mismas (1, 2, 8, 15 y 22 días), con el objetivo de estudiar la estabilidad de los extractos.

Para el análisis, las muestras se prepararon en matraces aforados de 25.0 mL conteniendo 0.5 mL de la disolución del extracto, 0.5 mL de reactivo de Folin-Ciocalteau, diferentes alícuotas de patrón de ácido gálico de 500 $mg \cdot L^{-1}$ y 10 mL de disolución de carbonato sódico (75 $g \cdot L^{-1}$), enrasando seguidamente con agua desionizada y agitando las muestras. Se dejaron durante una hora en oscuridad y reposo, para posteriormente medir la absorbancia de las muestras a una longitud de onda de 670 nm.

- Análisis de taninos totales

La determinación de taninos totales se ha realizado utilizando el reactivo Folin-Denis, compuesto por tugstato de sodio, ácido fosfomolibdico y ácido fosfórico. La reacción del reactivo Folin-Denis con la capacidad reductora de los taninos en solución alcalina produce una coloración azul, cuya absorbancia es proporcional a la cantidad de taninos presentes en la muestra medida a 760 nm (Schanderl, 1970).

Para la determinación de taninos se preparó una muestra que contenía 10 mg del extracto con 50 mL de metanol:agua (50:50).

La determinación de taninos totales se ha llevado a cabo utilizando el método de adición patrón. Para ello, en matraces aforados de 25 mL conteniendo unos 10 mL de agua desionizada (para evitar la precipitación del carbonato sódico), se añade 1.25 mL de la disolución del extracto del corcho procedente de los diferentes extractos indicados en el apartado 3.1.2.1., a los que se añade 0.5 mL de reactivo de Folin-Denis, diferentes volúmenes de patrón de ácido tánico de concentración de 100 $mg \cdot L^{-1}$ y 1.25 mL de disolución de carbonato sódico (200 $g \cdot L^{-1}$), enrasando con agua desionizada y agitando las muestras. Transcurrida media hora, en la oscuridad y en reposo, se mide la absorbancia de las muestras a una longitud de onda 760nm.

Se ha seleccionado como patrón el ácido tánico, de forma que el contenido de taninos totales de los extractos se ha expreseado en mg equivalentes de ácido tánico (EAT) $\cdot g^{-1}$ de corcho seco.

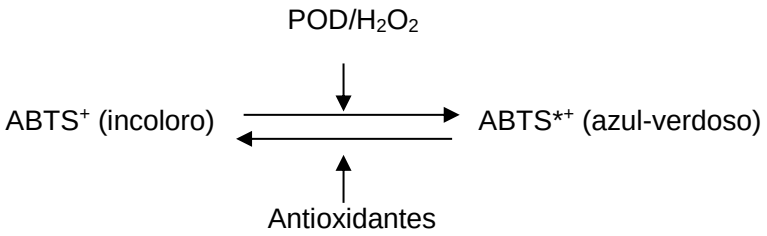
- Análisis de la actividad antioxidante

La actividad antioxidante es la capacidad que presenta un compuesto para inhibir la degradación oxidativa, es decir, el método se basa en la determinación de la reacción de un cromóforo de naturaleza radical metaestable con muestras de naturaleza antioxidante, comparando su eficacia antioxidante con un patrón: trolox (ácido 6-hidroxi-2,5,7,8-tetrametilcromano-2-carboxílico) un análogo estructural de la vitamina E, soluble tanto en medio acuoso como orgánico. La actividad antioxidante de las muestras se normaliza a concentración de trolox, expresando los resultados en $\mu mol \cdot L^{-1}$ de Trolox$\cdot g^{-1}$ de corcho seco.

Por otra parte, a través de un proceso de separación selectiva de los componentes solubles en agua y en disolventes orgánicos, se puede diferenciar en la misma muestra la actividad antioxidante hidrofílica (AAH, debida a componentes hidrosolubles) y la actividad antioxidante lipofílica (AAL, debida a componentes liposolubles). La suma de ambas se considera la actividad antioxidante total (AAT).

La siguiente reacción muestra el mecanismo de actuación:

El radical 2,2'-azino-bis-(3-etil-benzotiazolin-6-sulfónico) ($ABTS^{+}$), en presencia del sistema enzimático peroxidasa (POD)/peróxido de hidrógeno (H_2O_2), se oxida, formándose el radical $ABTS^{+*}$ (cromóforo, color azul-verdoso). Esta reacción se revierte sí en el medio existe alguna sustancia antioxidante. Por tanto, se utiliza como indicativo de la capacidad antioxidante de la muestra la disminución de la absorbancia del radical al añadir antioxidantes.



La determinación de la AAH y AAL conlleva una separación previa de los componentes hidrosolubles y liposolubles de las muestras en dos fases: acuosa y orgánica, mediante una extracción líquido/líquido (Cano et al.; 1998 y 2000).

El procedimiento de extracción es el siguiente:

1) Se realiza una primera extracción tomando de 0.5 g de extracto de corcho con 10.0 mL de tampón fosfato 50 $mmol \cdot L^{-1}$ a pH 7.5. Se homogeniza con agitador mecánico y se deja extraer unos 10 minutos. Se centrifuga durante 10 min a 3000 g y el líquido sobrenadante se lleva a un embudo de decantación de 25 mL.

2) Al residuo de extracto de corcho se añaden 2.0 mL de acetona al 80% y se procede como en el caso anterior. El sobrenadante se añade al mismo embudo junto con 1.0 mL de acetato de etilo. A continuación se adicionan 2.0 mL de acetato de etilo al residuo, se

homogeniza con agitador mecánico y se trasvasa el sobrenadante otra vez al mismo embudo. No suele necesitar centrifugación.

3) El embudo se agita enérgicamente y se dejan separar las dos fases (fracción acuosa y fracción orgánica).

La AAH se analiza y determina en un medio de reacción consistente en $ABTS^+$ radical acuoso 0.71 $mmol \cdot L^{-1}$, peróxido de hidrógeno 27.5 $\mu mol \cdot L^{-1}$ y POD 19.6 $nmol \cdot L^{-1}$, en un volumen final de 1.02 mL de tampón fosfato 50 $mmol \cdot L^{-1}$ (pH 7.5). La AAL se mide en un medio de reacción consistente en $ABTS^+$ radical orgánico 0.71 $mmol \cdot L^{-1}$, peróxido de hidrógeno 49.0 $\mu mol \cdot L^{-1}$ y POD 78.4 $nmol \cdot L^{-1}$, en un volumen final de 1.02 mL de etanol acidificado.

El procedimiento conlleva la utilización del espectrofotómetro UV-2401PC con el programa de cinética siguiente:

1) Se añade a la cubeta 1.0 mL de la disolución del radical $ABTS^+$ (orgánico o acuoso, según el caso).

2) Se deja estabilizar la medida durante 1 minuto y se toma como valor inicial de absorbancia a 730 nm en ese instante.

3) Se añade 20 µl, a la misma cubeta, de la disolución de la muestra en tampón (AAH) o en etanol acidificado (AAL), se agita y se sigue registrando la medida de absorbancia con el tiempo hasta el minuto 6. Se representa la diferencia de absorbancia obtenida del minuto 1 al minuto 6 frente a la concentración de trolox.

La actividad antioxidante se define como la concentración de patrón, expresada como mg trolox·100 g^{-1} de muestra.

- Análisis de compuestos fenólicos de bajo peso molecular

La determinación de compuestos fenólicos de bajo peso molecular en los extractos de corcho se ha llevado a cabo utilizando HPLC. La columna utilizada ha sido una Lichrospher RP-18 (250 mm x 4 mm x 5 µm) y la fase móvil ha sido agua (0.1% HCOOH, A) y metanol

(0.1% HCOOH, B). Las condiciones finales optimizadas para el análisis se muestran en la tabla 3.1.1.

Las muestras se preparan tomando 10 mg del extracto a caracterizar en 10 mL de metanol al 50%. Cada muestra ha sido refrigerada a 4˚C hasta el momento del análisis (1, 8 y 15 días) con el objetivo de estudiar la estabilidad del extracto.

Para cada análisis cromatográfico, se ha tomado 1.5 mL de la disolución anterior del extracto y se ha llevado a matraces de 3 mL con una mezcla 90:10 de H_2O (0.1% HCOOH): MeOH (0.1% HCOOH). Antes de inyectar las muestras se han filtrado mediante filtros de nylon de 0.22 µm.

Tabla 3.1.1. Condiciones cromatográficas para la determinación de compuestos fenólicos de bajo peso molecular en extractos de corcho.

Tiempo, min	A	B
0	100	0
10	100	0
30	80	20
65	50	50
80	0	100
90	0	100

Flujo: 0.5 $mL \cdot min^{-1}$

Volumen de inyección: 20 µL

Temperatura columna: 30˚C

Señales del detector: 255, 275, 305 y 345 nm

Como señal analítica se ha utilizado el área de pico. Las rectas de calibración se han obtenido utilizando los patrones de cada uno de los compuestos a analizar en el intervalo comprendido entre 0.5-16.0 $\mu g \cdot mL^{-1}$. Para aquellos compuestos de los que no se disponía de patrón, la cuantificación se ha realizado aplicando la recta de calibración de otro compuesto que pertenezca al mismo grupo fenólico.

La validación del método de HPLC propuesto para la determinación de compuestos fenólicos de bajo peso molecular en todas las muestras obtenidas de los extractos de corcho se ha llevado a cabo mediante un HPLC acoplado a un detector de espectrometría de masa de tiempo de vuelo (HPLC/Q-TOF-MS).

- Análisis de elagitaninos

Se han estudiado la vescalagina y la castalagina como elagitaninos presentes en los extractos de corcho. Se han determinado mediante HPLC utilizando una columna Lichrospher RP-18 y la fase móvil ha sido metanol (A) y agua (0.1% TFA, B). Se ha utilizado una elución en gradiente para el análisis y que se muestra en la tabla 3.1.2.

Tabla 3.1.2. Condiciones cromatográficas para la determinación de elagitaninos en extractos de corcho.

Tiempo, min	A	B
0	0	100
40	10	90
45	100	0
50	100	0
60	0	100
65	0	100

Flujo: 0.75 $mL \cdot min^{-1}$

Volumen de inyección: 20 µL

Temperatura columna: 30˚C

Señal del detector: 240 nm

Las muestras se preparan tomando 10 mg del extracto a caracterizar en 10 mL de metanol al 50%. Cada muestra ha sido refrigerada a 4˚C hasta el momento del análisis (1, 8 y 15 días) con el objetivo de estudiar la estabilidad del extracto.

Para cada análisis cromatográfico, se ha tomado 1.0 mL de la disolución anterior del extracto y se ha llevado a matraces de 5 mL con H_2O (0.1% TFA). Antes de inyectar las muestras se han filtrado mediante filtros de nylon de 0.22 µm.

3.1.2.3. Aislamiento de compuestos bioactivos del corcho mediante cromatografía preparativa

Los elagitaninos estudiados, como la vescalagina y castalagina, no son sintetizados industrialmente, por lo que se obtienen mediante aislamiento y purificación de los mismos de materiales vegetales, para poder ser utilizados como patrones.

El proceso de aislamiento de los elagitaninos se ha llevado a cabo utilizando la cromatografía de líquidos preparativa, consistente en la separación en una columna preparativa C18 de los extractos obtenidos anteriormente, utilizándose una elución en gradiente y posterior detección mediante UV a una longitud de onda de 240 nm. Una vez que se ha producido la detección, se recogen los compuestos en varias fracciones. Dichas fracciones se han sometido a vacío en rotavapor, a temperaturas inferiores a 40ºC, con el objetivo de evaporar el disolvente, y posteriormente eliminar el agua de los mismos mediante liofilización.

Los disolventes utilizados fueron metanol (A) y agua con 0,2% de ácido fórmico (B), con un flujo de 10 $mL \cdot min^{-1}$. La longitud de onda seleccionada para detectarlos fue 240 nm. Tras diferentes estudios previos de modificaciones del método, las condiciones del gradiente que mejores resultados proporcionaban para el colector de fracciones son las que se muestran en la tabla 3.1.3.

Tabla 3.1.3. Condiciones cromatográficas para el aislamiento de elagitaninos en extractos de corcho.

Tiempo (min)	A	B
0	0	100
5.0	5	95
15.0	10	90
17.5	100	0
22.0	100	0
27.0	0	100

Se ha partido del extracto denominado *B* para el aislamiento de elagitaninos debido a que este extracto contiene el mayor contenido de estos compuestos. También se ha utilizado los extractos denominados *C, D* y *G*, para la purificación de elagitaninos, dado que provienen de métodos de extracción más sencillos y rápidos. Sin embargo, dado que en estos extractos no se lleva a cabo una extracción posterior con dietil éter, los extractos contienen tanto compuestos fenólicos de bajo peso molecular como elagitaninos, y en las fracciones separadas se observan interferencias por la presencia de compuestos fenólicos, por lo que no se obtienen elagitaninos puros.

Se ha llevado a cabo un estudio previo acerca de la estabilidad del extracto seleccionado (extracto *B*) en disolución, estudiando diversas variables:

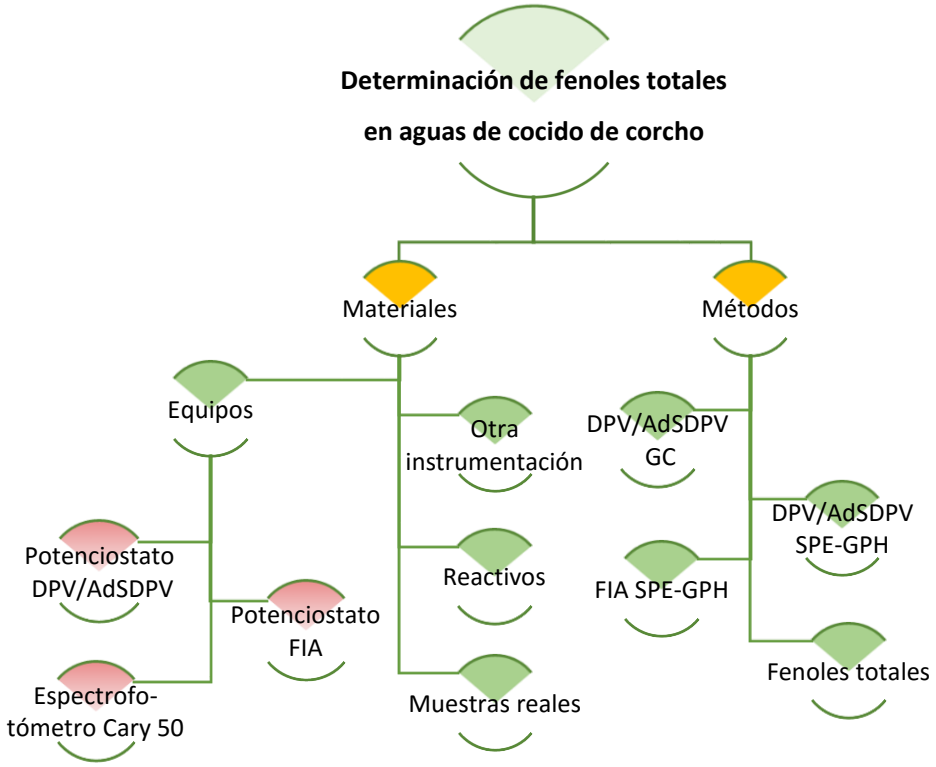
- Influencia de la temperatura. Para ello, la disolución del extracto *B* se mantiene, en estufa, durante una hora a diferentes temperaturas (25, 30, 35, 40, 50 y 60˚C).

- Estabilidad con el tiempo a temperatura constante. El extracto *B* se mantiene a temperatura constante (60˚C) y se analizan los elagitaninos a diferentes tiempos (1, 2, 3, 4, 5 y 24 horas).

- Estabilidad con el tiempo en presencia o ausencia de luz. Se ha estudiado la estabilidad de las disoluciones del extracto *B* en ausencia o presencia de la luz con el tiempo durante un período de 30 días a temperatura ambiente.

3.2. Análisis del contenido de fenoles totales en aguas de cocido de corcho.

Esta segunda parte del capítulo describe los materiales y métodos propuestos en esta Tesis para la determinación de fenoles totales en aguas de cocido de corcho mediante técnicas electroanalíticas. A continuación, se muestra un esquema global donde se resumen los principales apartados que se van a desarrollar.



3.2.1. Materiales

3.2.1.1. Equipos

- Instrumentación para voltamperometría de diferencial de pulso y de redisolución adsortiva

La instrumentación utilizada para los estudios electroanalíticos que se han desarrollado para la determinación de fenoles totales en muestras de agua de cocido de corcho mediante voltamperometría de diferencial de pulso (DPV) y de redisolución adsortiva (AdSDPV) han sido un potenciostato μAutolab PSTAT10 (ECO Chemie, Holanda) equipado con el Stand Metrohm 663 VA (Herisau, Suiza) y controlado mediante el software GPES 3.0 (General Purpose Electrochemical System). Se ha utilizado un sistema de tres electrodos: electrodo de trabajo (carbón vitrificado o impreso de carbono modificado con grafeno), electrodo de referencia Ag/AgCl y electrodo auxiliar de platino. Para la utilización de los electrodos impresos se ha necesitado un cable de conexión DRP-CAC, que actúa como interfase entre los electrodos impresos y el potenciostato (DropSens, España).

Como se ha mencionado anteriormente, se han utilizado dos tipos de electrodos de trabajo para el estudio, análisis y determinación de fenoles totales en agua de cocido de corcho:

- Electrodo de carbono vitrificado MF-2012 (Bioanalytical Systems, Inc., West Lafayette, Indiana, USA), con 3 mm de diámetro, lo que corresponde a una superficie activa de 0.07cm^2.

- Electrodos impresos comerciales fabricados sobre un soporte cerámico donde se encuentran serigrafiados los tres electrodos (trabajo, auxiliar y referencia). Concretamente, en esta Tesis Doctoral se han utilizado electrodos impresos de carbono modificados con grafeno (DRP-110GPH), suministrados por DropSens (Oviedo, España). Este electrodo consta de un electrodo de trabajo de carbono modificado con grafeno (WE), un electrodo auxiliar de carbono (CE) y electrodo de pseudo-referencia de plata (RE) (Figura 3.2.1.).

El electrodo de trabajo tiene 4 mm de diámetro, lo que corresponde a una superficie activa de 0.13 cm^2. Los contactos eléctricos también están fabricados en plata y se encuentran separados del área de trabajo por una capa de aislamiento.

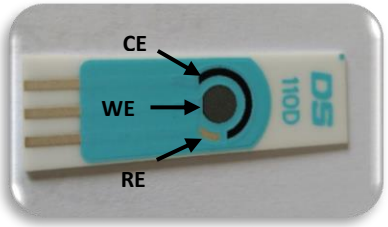


Figura 3.2.1. Electrodo impreso modificado con grafeno.

Las experiencias realizadas en el desarrollo de este trabajo, se llevaron a cabo en una célula electroquímica ámbar (6.1415.223, Metrohm) para preservar las muestras de la luz.

- Instrumentación para análisis por inyección en flujo con detección amperométrica

La instrumentación que se ha utilizado para el análisis por inyección de flujo (FIA, por sus siglas en inglés: Flow Injection Analysis), se compone de un bipotenciostato portátil µStat200 (DropSens, España) con técnicas tales como: amperometría y de pulsos; voltamperometría lineal y cíclica; diferencial de pulso y de onda cuadrada. El bipotenciostato dispone además de un adaptador serie-USB que se conecta a un ordenador portátil equipado con el software DropView versión 2.0; y un cable DRP-CAST que actúa como interfase entre los electrodos impresos y el bipotenciostato (DropSens, España). Se han utilizado los electrodos impresos de carbono modificados con grafeno, descritos anteriormente.

El equipo de FIA que se ha utilizado dispone de los siguientes componentes:

- Bomba peristáltica Rainin Dynamax RP-1 (Oakland, California), con una cabeza rotatoria para 4 canales de flujo ajustables entre 0.005 $mL \cdot min^{-1}$ a 37 $mL \cdot min^{-1}$.

- Válvula de inyección manual con loop de 0.2 mL.

- Celda de flujo para electrodos serigrafiados DRP-FLWCL (DropSens, España) de metacrilato.

3.2.1.2. Otra instrumentación

- Espectrofotómetro UV-Vis Cary 50 (descrito en el apartado 3.1.1.1.).
- Balanza analítica ATX84 con una precisión de 0.1 mg (Shimadzu, Japón).
- Baño de ultrasonidos (P-Selecta, España).
- pH-metro Basic 20+ (Crison, España).
- Equipo Milli-Q water system (Millipore S.A.S., Molsheim, Francia).

3.2.1.3. Reactivos

Se han utilizado patrones de los analitos que se indican a continuación y de una pureza superior al 99%:

- Ácido gálico, ácido protocatéquico, ácido vainíllico, ácido siríngico, ácido ferúlico, ácido elágico, siringaldehído, coniferaldehído, sinapaldehído y vainillina, suministrados por Sigma-Aldrich (Steinheim, Alemania).

Otros reactivos utilizados (calidad PA) para la preparación de diferentes disoluciones, disoluciones reguladoras, etc., necesarion, son los siguientes:

- Carbonato de sodio anhidro (Panreac, España).
- Reactivo de Folin-Ciocalteau (Panreac, España).
- Fosfato monosódico, 98% (Panreac, España).
- Fosfato disódico, 98% (Panreac, España).
- Ácido ortofosfórico, 85-88% (Panreac, España).
- Acetato de sodio, 99% (Sigma-Aldrich, Alemania).
- Ácido acético, 99% (Panreac, España).
- Ácido bórico, 99.8% (Panreac, España).
- Hidróxido sódico, 98% (Panreac, España).
- N-N-dimetilformamida, 99.9% (Scharlau, España).

- Lana de vidrio lavada, químicamente pura (Panreac, España).
- Agua destilada (Millipore, Francia).
- Agua ultrapura Milli-Q (Millipore, Francia).

3.2.1.4. Preparación de muestras de aguas de cocido de corcho

Se han preparado cinco muestras de agua de cocido de corcho de diferente composición a escala de laboratorio, siguiendo las mismas pautas del proceso a escala industrial. Para ello, se ha cocido en agua, durante una hora, diferentes cantidades de corcho y calidades mezcladas. Tras el proceso de cocción, se ha filtrado y separado el corcho. Cuando el agua alcanzaba la temperatura ambiente se ha medido el volumen final obtenido.

3.2.2. Métodos de análisis

3.2.2.1. Análisis del contenido fenólico total de compuestos de bajo potencial de oxidación en aguas de cocido de corcho mediante técnicas electroanalíticas, utilizando electrodo de carbono vitrificado (GCE)

La determinación de contenido fenólico total de compuestos de bajo potencial de oxidación en las muestras reales se ha llevado a cabo utilizando voltamperometría de diferencial de pulsos y voltamperometría de redisolución adsortiva. A continuación se indican las condiciones experimentales de ambos métodos:

✓ Voltamperometría de diferencial de pulso: Diferentes alícuotas (25, 50 y 75 µL) de aguas de cocido de corcho se han llevado a matraces de 25.0 mL que contenían 2.0 mL de tampón $H_3PO_4/H_2PO_4^-$ 0.5 mol·L^{-1} de pH 2.5 y se ha enrasado con agua ultrapura. Las muestras se han registrado en una celda electroquímica utilizando 50 mV de amplitud de pulso y 10 mV de paso de potencial.

✓ Voltamperometría de redisolución adsortiva de diferencial de pulso: Las muestras han sido preparadas como se ha comentado anteriormente, aplicando un potencial de acumulación de 0.0 V, tiempo de acumulaciónde 20 s y tiempo de equilibrio de 10 s. Las condiciones de registro han sido amplitud de pulso de 50 mV y paso de potencial de 10 mV.

Se ha utilizado como señal analítica la intensidad de pico integrada y el método de patrón externo utilizando ácido elágico, para expresar el contenido fenólico total en las aguas de cocido de corcho como equivalente de ácido elágico.

Tras cada registro, al electrodo de carbono vitrificado se le ha realizado una limpieza para obtener una buena reproducibilidad en las medidas electroquímicas. Dicha limpieza ha consistido en sumergir el electrodo en N-N-dimetilformamida y llevarlo a ultrasonido durante 2 min, seguidamente se ha enjuagado el electrodo y se ha repetido el proceso durante 40 s en agua ultrapura. Durante el proceso de limpieza se ha tenido especial cuidado en que el baño de ultrasonido estuviese siempre a temperatura ambiente para evitar el calentamiento del electrodo.

Para verificar que las muestras no se han quedado adsorbidas en el electrodo y que por tanto la limpieza del mismo ha sido efectiva, se procede a realizar un registro del tampón entre cada medida de las muestras.

3.2.2.2. Análisis del contenido fenólico total de compuestos de bajo potencial de oxidación en aguas de cocido de corcho utilizando electrodos impresos de carbono modificados con grafeno (SPE-GPH)

Los electrodos impresos de carbono modificados con grafeno precisan de un acondicionamiento electroquímico para obtener una buena línea base y una mayor reproducibilidad en la respuesta del analito. Este pretratamiento consiste en la aplicación de un potencial de 1.4 V durante 300 s en un blanco (tampón, electrolito soporte, etc.) (Moreno et al., 2010).

Del mismo modo que el anterior método, también la determinación de contenido fenólico total de compuestos de bajo potencial de oxidación en las muestras problema se ha realizado utilizando voltamperometría de diferencial de pulso y de redisolución adsorptiva:

- ✓ Voltamperometría de diferencial de pulso: Diferentes alícuotas (75-100 µL) de aguas de cocido de corcho se han llevado a matraces de 25.0 mL que contenían 1.0 mL de tampón HAcO/NaAcO 0.5 mol·L^{-1} de pH 4.5 y 80 µL de KCl 3 mol·L^{-1}, posteriormente se ha enrasado con agua ultrapura. Las muestras se han registrado en la celda

electroquímica utilizando los siguientes parámetros instrumentales: 50 mV de amplitud de pulso y 10 mV de paso de potencial.

- ✓ Voltamperometría de redisolución adsortiva por diferencial de pulso: En matraces de 25.0 mL, se ha añadido alícuotas (25-50 µL) de agua de cocido de corcho, 1.0 mL de tampón HAcO/NaAcO 0.5 mol·L^{-1} de pH 4.5, 80 µL de KCl 3 mol·L^{-1} y se ha enrasado con agua ultrapura. Las condiciones de registro han sido amplitud de pulso de 50 mV, paso de potencial de 10 mV, potencial de acumulaicón de +0.05 V durante 30 s y tiempo de equilibrio de 10 s.

Se ha utilizado como señal analítica la intensidad de pico y el método de patrón externo, utilizando ácido gálico y ácido elágico, para la determinación del contenido fenólico total en las aguas de cocido de corcho, expresándolo como equivalente de ácido gálico o elágico.

A diferencia de los GCE, los electrodos impresos de carbono modificados con grafeno no necesitan un proceso de limpieza exhaustivo, sólo es necesario lavarlos, introducirlos varias veces en agua ultrapura. No obstante, cuando se han llevado a cabo análisis de muestras reales de agua de cocido de corcho, sólo tenía un uso, es decir, hay que desechar el electrodo.

3.2.2.3. Análisis del contenido fenólico total en aguas de cocido de corcho mediante análisis de inyección de flujo utilizando como electrodos de trabajo los electrodos impresos de carbono modificados con grafeno (SPE-GPH-FIA)

Como ya se ha descrito en el apartado 3.3.2.2., a los SPE-GPH se les ha realizado un acondicionamiento electroquímico antes de su utilización por primera vez.

Se preparó la muestra que contenía una alícuota de la muestra de agua de cocido de corcho y se llevó a un matraz aforado de 25.0 mL, al que se le añadió 1.0 mL de tampón HAcO/NaAcO 0.5 mol·L^{-1} de pH 4.5, 80 µL de KCl 3 mol·L^{-1}, para posteriormente enrasar con agua ultrapura. Las condiciones optimizadas fueron las siguientes: flujo de la bomba de 2.0 mL·min^{-1}, potencial aplicado +0.65 V y número de puntos por segundo 0.12. La fase móvil se preparó con una concentración de 0.02 mol·L^{-1} de tampón HAcO/NaAcO a pH 4.5 y 9.6 mmol·L^{-1} de KCl en agua ultrapura.

Con objeto de cuantificar el contenido de fenoles totales en aguas de cocido de corcho mediante análisis por inyección de flujo se ha utilizado la altura del fiagrama como señal analítica y el método de patrón externo, utilizando ácido gálico para expresar el contenido fenólico total en aguas de cocido de corcho como equivalente de ácido gálico.

3.2.2.4. Análisis de fenoles totales en aguas de cocido de corcho mediante espectrofotometría UV-Vis

Con objeto de comparar resultados con los métodos propuestos en esta Tesis, se ha procedido a la cuantificación de fenoles totales en muestras de agua de cocido de corcho mediante el método de espectrofotometría propuesto por Singleton et al., en 1965. (descrito en el apartado 3.1.2.2.).

El procedimiento seguido ha sido el siguiente: en matraces aforados de 25.0 mL se introduce 0.5 mL de la muestra, 0.5 mL de reactivo Folin-Ciocalteau, 10.0 mL de carbonato sódico (75 $g \cdot L^{-1}$) y se enrasa con agua destilada. Seguidamente se agita y se deja reposar durante 60 minutos a temperatura ambiente y en la oscuridad, para posteriormente hacer la medición en el espectrofotómetro.

El contenido de fenoles totales se ha calculado como equivalentes de ácido gálico o elágico utilizando el método de patrón externo, y expresando los resultados de fenoles totales de las muestras en mg equivalentes de ácido gálico (GAE)$\cdot Kg^{-1}$ de corcho seco o bien en mg equivalentes de ácido elágico (EAE)$\cdot Kg^{-1}$ de corcho seco.

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4. Determination of low molecular weight phenolic compounds in cork of Quercus suber L. *by HPLC-DAD*

La determinación del contenido de compuestos fenólicos, compuestos bioactivos, es de gran interés debido a sus propiedades antioxidantes y anticancerígenas. Estos compuestos están presentes en diferentes materiales lignocelulósicos, entre el que se encuentra el corcho.

En este capítulo 4, se han estudiado diferentes métodos de extracción de dichos compuestos en corcho de *Quercus suber* L., utilizando diferentes disolventes tales como metanol, agua o acetona, así como mezcla de ellos. Una vez obtenidos los diferentes extractos de corcho, se ha procedido a caracterizarlos mediante la determinación de fenoles totales por el método espectrofotométrico de Folin-Ciocalteau, así como por la identificación y cuantificación de compuestos fenólicos de bajo peso molecular por HPLC. Se ha optimizado el método cromatográfico y se ha validado por HPLC/Q-TOF. Así mismo, se ha estudiado la estabilidad de los extractos con el tiempo de almacenamiento.

Este trabajo está en proceso de remisión, y se ha presentado como póster al *XVIIIth Scientific Meeting of the Euroanalysis*, celebrado en Burdeos (Francia) entre el 6 y 9 de septiembre del 2015.

Determination of low molecular weight phenolic compounds in cork of *Quercus suber* L. by HPLC-DAD

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Abstract

The determination of phenolic compounds in plants is of a great interest due to their antioxidant and anticancer properties. These compounds are present in different lignocellulosic materials, including cork from cork oak. The determination of phenolic compounds from cork has been carried out by HPLC-DAD following different extraction methods, using different solvents such as methanol, water or acetone, as well as mixtures of them. On the other hand, the study of the stability of these phenolic compounds in the different extractshas been carried out. Total phenolic compounds content has been determined by Folin-Ciocalteau method (1.0 to 11.8 g GAE·Kg^{-1} dry cork) while low molecular weight phenolic compounds have been determined by HPLC-DAD method. The HPLC-DAD method has been optimized and validated by HPLC-QTOF. More than twenty phenolic compounds have been identified and quantified in the different cork extracts, including phenolic acids and aldehydes, hidroxycinnamic acids, coumarins, flavonoids, ellagitannins, and ellagic acid and their derivatives. Quantification of the main compounds showed until 10 times higher content of total phenolic content between different extracts (1724.6 μg·g^{-1} vs 160.7 μg·g^{-1}). The main compounds found were ellagic acid (871.2 μg·g^{-1}), castalagin (223.6 μg·g^{-1}), vescalagin (199.7 μg·g^{-1}) and gallic acid (100.8 μg·g^{-1}).

Keywords: phenolic compounds, HPLC, cork, extraction

4.1. Introduction

Cork is the outer bark of an oak tree known botanically as *Quercus suber* L. The bark of the tree is removed periodically, generally every 9 years depending on the region. It consists of dead cells and hollow covered by a five-layer membrane: two external cellulosic layers, two suberized intermediate layers and a lignified core.

The cells that form the cork are rectangular prisms, pentagonal and hexagonal, mostly, as a honeycomb structure, with their axes along the radial direction of the trees, stacked into columns (Gibson et al., 1981, Pereira et al., 1987).

Due to its peculiar properties such as high elasticity and low permeability, the cork is used in a wide range of applications including the production of stoppers for wine and other alcoholic drinks is the most important, followed by their use in thermal and acoustic insulation materials (Pereira, 2007).

Is essentially composed of suberin, lignin and cellulose, but also contains a small amount of removable substances (Pereira et al., 1979, 1988).

Most important removable components are waxes and phenolic compounds. The waxes are composed of various aliphatic and aromatic compounds. Phenolic group includes family's flavonoid and tannin, in addition to phenolic acids, phenolic aldehydes and coumarins. Tannins can be monomeric or polymeric and may be divided into hydrolysable and condensed tannins, which in turn comprise a gallotannins and ellagitannins (Silva, 2005).

There are several studies about the content of polymeric phenols in cork (lignins and tannins), and low molecular weight phenolic compounds (Conde et al., 1997, 1998). Furthermore, recent studies also submitted that cork stoppers give in some of these phenols in wine (Varea et al., 2001).

The importance of these phenolic compounds lies in that are ascribed multitude of health benefits, such as antibacterial, antivirals, anticarcinogenic and anti-inflammatories.

Since these compounds can be extracted with solvents, cork samples have been submitted to different extraction procedures using methanol and acetone as solvents. Then, the content of phenolic compounds of different extracts obtained have been analysed. The total phenol content by UV-Vis spectrophotometry (Singleton and Rossi, 1965) has been determined, as well as low molecular weight phenolic compounds by HPLC. The structures of phenolic compounds studied on cork are shown in Fig 4.1.

Extraction of bioactive compounds in plant material can be easily performed with various solvents, although the extraction of these compounds in lignocellulosic material is not very abundant. There are precedents about the extraction solvent in fruits and vegetables using extractions with acetone: water (Vrhovsek et al., 2004), ethanol: phosphoric acid: water or formic acid: acetonitrile: water (Vagiri et al, 2012.), ethanol: sodium bisulfate: water (Romani et al, 1996), acetone: water: formic acid (León-González et al, 2013).

There are few antecedents for extraction of bioactive compounds in lignocellulosic material. For example, extraction of cork with methanol:water (Conde et al., 1998; Santos et al., 2010).

Regarding to chromatographic techniques, there are few papers about the determination of phenolic compounds in lignocellulosic materials. These papers use solvent isolation of bioactive compounds with mixtures of water and methanol with phosphoric acid (Cadahía et al., 2001), water and acetonitrile with formic acid (Santos et al., 2010), water and methanol with trifluoroacetic acid and water (Zhentian et al., 1999); acetic acid: isopropyl alcohol: methanol (Mazzoleni et al., 1998). All mentioned methods have low recoveries for phenolic compounds content and poor chromatographic resolution.

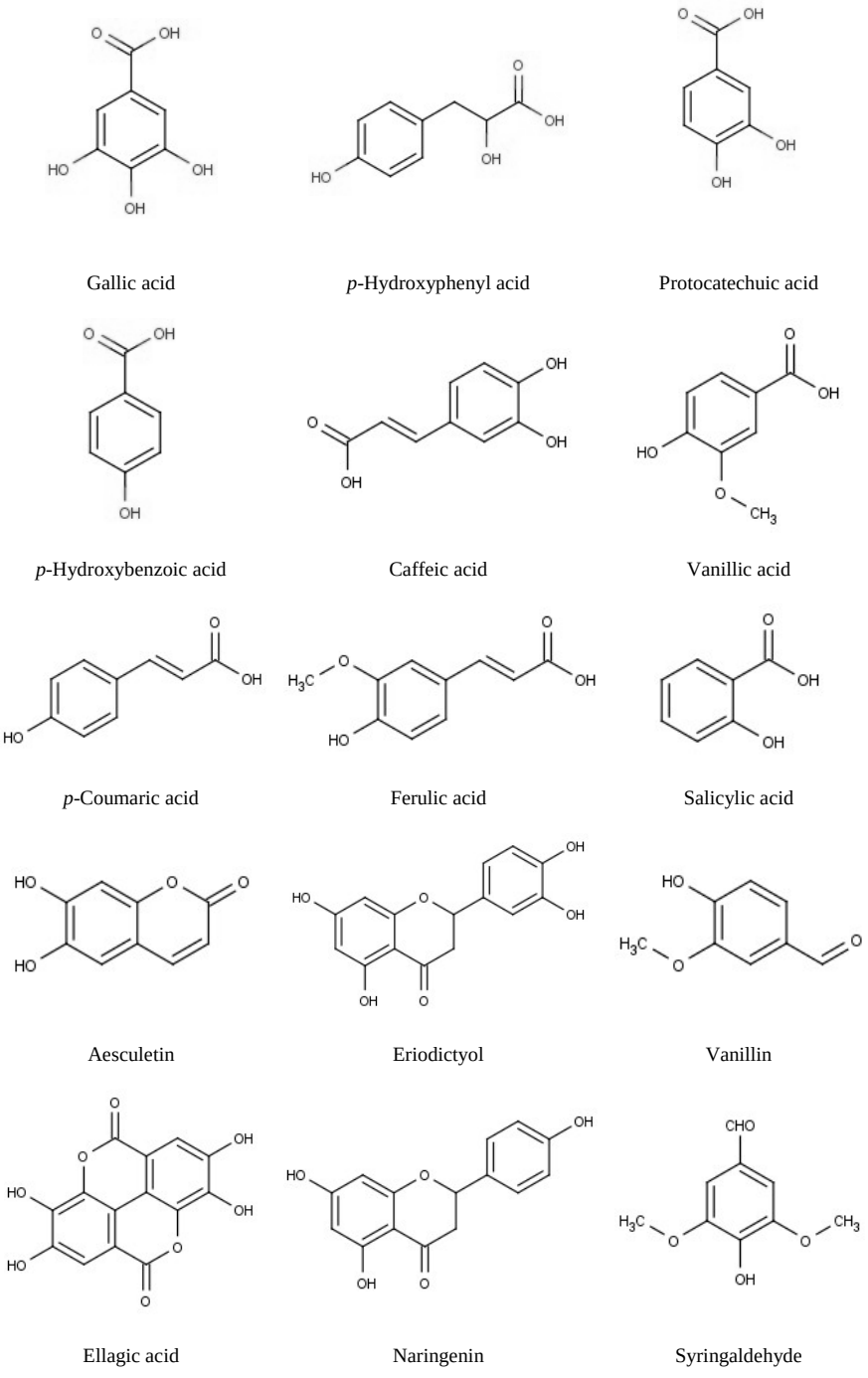


Figure 4.1. Structures of low molecular weight phenolic compounds found in cork.

4.2. Material and methods

4.2.1. Reagents

Standards of low molecular weight phenolic compounds; gallic, protocatechuic, *p*-hydroxyphenyl, *p*-hydroxybenzoic, vanillic, caffeic, syringic, coumaric, ferulic, salicylic and ellagic acids, syringaldehyde, coniferyl and sinapic aldehyde, aesculetin, vanillin, eriodictyol and naringenin, were supplied by Sigma-Aldrich (Madrid, Spain). Folin-Ciocalteau reagent, sodium carbonate, methanol (MeOH) HPLC-grade, acetone HPLC-grade, ethanol (EtOH) HPLC-grade, dichloromethane and formic acid (HCOOH) were provided by Panreac (Barcelona, Spain). Diethyl ether was supplied by Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water system (Millipore S.A.S., Molsheim, France).

Individual phenolic standard solutions of gallic, protocatechuic, *p*-hydroxyphenyl, *p*-hydroxybenzoic, vanillic, caffeic, syringic, coumaric, ferulic, and salicylic acids, syringaldehyde, coniferic and sinapic aldehyde, vanillin and naringenin were prepared in EtOH in concentration of 200μg·mL^{-1}; aesculetin, eriodictyol and ellagic acid stadandard solutions were prepared in MeOHwith the same concentration . All standards solutions were prepared separately and stored in darkness at 4ºC, except eriodictyol, which was stored at -20ºC, until analysis. Work solutions were prepared daily by dilution of appropriate amounts of individual standard solutions with H_2O (0.1% HCOOH):MeOH (0.1% HCOOH) mixture (90:10).

4.2.2. Raw material

Q. *suber* L. natural cork planks ("refuge" grade) were sampled from several local companies of San Vicente de Alcántara (Badajoz-Spain). An average sample composed of fragments of several planks from different trees was milled in a Retsch cross beater mill SK1 (Haan, Germany), and the granulometric fraction of 0.25-0.50 mm and 0.50-1.00 mm were used for analyses.

4.2.3. Phenolic compounds extraction

Several extraction methods have been carried out in this study:

- *Method_1*

About 10 g of cork, with a grain size between 0.25 and 0.5 mm, was submitted to a soxhlet extraction for 6 hours with dichloromethane to remove lipophilic components.

Cork, once washed and air dried, was suspended in a methanol-water mixture, 80/20 (v/v) for 24 h at room temperature under constant stirring. The suspension was then filtered and the methanol removed by low-pressure evaporation. The aqueous solution, free of methanol, was extracted tree times with diethyl ether. The organic phase was then removed in the rotary evaporator yielding extract *A*, and the aqueous phase proceeds to be lyophilized, giving the extract *B*.

- *Method_2*

About 10 g of cork sample, with a grain size between 0.25 and 0.5 mm, was submitted to a soxhlet extraction for 6 hours with dichloromethane to remove the lipophilic components. Then, the solid cork residue -once washed and air dried- was submitted to a methanol extraction for 6 h (extract *C*), followed by a reflux with water for 6 h. Then the solvents were removed from the liquid extracts by low-pressure evaporation and freeze drying yielding extracts *C* and *D*, respectively (Santos et al., 2010)

- *Method_3*

Cork sample with a grain size between 0.5 and 1.0 mm, was extracted in the dark under nitrogen atmosphere with an acetone-water mixture, 70/30 (v/v) at room temperature with constant stirring for 24 h. The solvent to cork ratio was kept al $10 mL \cdot g^{-1}$. The extract was filtered, the solvent removed by low-pressure evaporation and then freeze dried, yielding extract *E* (Zhentian et al., 1999).

Extract *F* is obtained with cork samples previous washed with dichloromethane for 6 hour.

- *Method_4*

Cork, with a grain size between 0.5 and 1.0 mm, was submitted to a soxhlet extraction for 6 hours with dichloromethane to remove lipophilic components. Once washed and air dried, 10 g of cork was suspended in 250 mL methanol-water mixture, 80/20 (v/v), at room temperature with constant stirring for 24 h. The suspension was then filtered and the methanol removed by low-pressure evaporation. The aqueous solution, free of methanol, was divided into two fractions (I and II).

Fraction I was subjected to a lyophilization process, obtaining the extract *G*. The fraction II was performed by extraction with diethyl ether, maintaining the aqueous phase to proceed subsequently to the lyophilization process, obtaining the extract *H.*

The same process has been carried out without prior washing of the cork with dichloromethane for 6 hours, obtaining extracts *I* (fraction I) and *J* (fraction II), respectively.

4.2.4. Low molecular weight phenolic compounds determination

Low molecular weight phenolic compounds of the extracts were analysed on an Agilent 1200 liquid chromatograph instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with an online degasser, quaternary pump, auto-sampler, thermostatted column compartment, UV-Vis diode-array detector, and the Chemstation software package to control the instrument, data acquisition and data analysis. Chromatographic studies and analytical separation were carried out in column purchased from Agilent. The column used was Lichrospher RP-18 (250 nm x 4 mm x 5 µm) and the mobile phases were Water (0.1% formic acid, A) and Methanol (0.1% formic acid, B). The gradient employed was the following: 0 min, 0% B; 10 min, 0% B; 30 min, 20% B; 65 min 50% B, 80 min, 100% B and was maintained for 10 min before returning to initial conditions. A flow rate of 0.5 $mL \cdot min^{-1}$ was used together with an injection volume of 20 µL and column temperature was fixed to 30ºC. Detection was performed with a diode-array detector at 255, 275, 305 and 345 nm, and peak areas were used as analytical response.

Method validation and qualitative identification of the low molecular phenolic compounds in all samples were performed by HPLC coupled to mass spectrometry time of

flight (HPLC-QTOF) on an Agilent 6530 Series Accurate Mass QTOF-MS (Agilent Technologies, Santa Clara, CA, USA).

The samples were prepared by dilution to 10 mg of each extract in 10 mL aqueous methanol (MeOH:H_2O, 50:50). Each sample was stored until the next time for analysis (day 1, day 8 and day 15) in order to study the stability of the extract.

For each extracted sample, 1.5 mL of previous sample were added in a 3 mL volumetric flask with H_2O (0.1% HCOOH):MeOH (0.1% HCOOH) mixture (90:10). Resulting solutions were filtered through a 0.22 µm nylon filter, and aliquots of 20 µL were injected in the chromatographic system.

The low molecular weight phenolic compounds were identified by comparing their retention time, UV-Vis and mass spectra with those obtained from standard solutions, when it was available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature and/or provided by HPLC-QTOF.

For quantitative analysis, a calibration curve (0.5–16.0 $\mu g \cdot mL^{-1}$) for each available phenolic standard was calculated based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in μg compound$\cdot g^{-1}$of dry weight, as mean ± standard deviation. At least, two replicates of each extract were made for quantification purposes.

4.2.5. Total Phenolic content

The total phenolic content of the extracts has been determined by the Folin-Ciocalteau method (Singleton and Rossi, 1965). 10 mg of extract were dissolved in 50 mL aqueous methanol (MeOH:H_2O, 50:50).Each sample was refrigerate stored until the next time for analysis (1, 2, 8, 15 and 22 days) in order to study the stability of the extract. The following spectrophotometric procedure was carried out: a 0.5 mL of Folin-Ciocalteau reagent and 10 mL of aqueous sodium carbonate (75 $g \cdot L^{-1}$) was added to an aliquot of 0.5 mL of each extract, to a final volume of 25 mL. Each mixture was kept for 60 min at room temperature in the dark. After homogenizing, the absorbance was measured at 670 nm, using a UV/Vis

Varian Cary-50 spectrophotometer (Palo Alto, California, USA). The total phenolic content was calculated as gallic acid equivalent using the standard addition method to avoid matrix effects caused in such extracts, for it has been used gallic acid standard solutions (0.5–8.0 $\mu g \cdot mL^{-1}$) and expressed in g gallic acid equivalent (GAE)$\cdot g^{-1}$ of extract and also as g GAE$\cdot Kg^{-1}$ of dry cork, as mean ± standard deviation of as a minimum of three extracts. The analyses were carried out at least three times for each extract and the average value was calculated in each case.

4.2.6. Statistical analysis

The results were expressed as mean ± standard deviation and the differences of both cases, total phenolic contents between the five sampling days (day 1, day 2, day 8, day 15 and day 22) and low molecular weight phenolic compounds between the three sampling days (day 1, day 8 and day 15), were tested using one-way ANOVA test. Differences were considered as statistically significant at a value of $p< 0.05$. The statistical analyses were carried out using Excel 2010 software (Microsoft, Redmond, Washington, USA).

4.3. Results and discussion

4.3.1. Extraction yield and total phenolic content

Yields of extracts of cork studied and their total phenolic content is shown in Table 4.1. Yield of the extracts displayed correspond to the values obtained after removal of lipophilic compounds using dichloromethane. The content of these lipophilic compounds has been 3.2% and 3.6% for the grain size of 0.25-0.50 mm and 0.5-1.0 mm, respectively, in agreement with previously published data (Sousa et al., 2006).

Table 4.1. Extraction yield and total phenolic contents of cork extracts from *Q. suber* L.

Extraction Method	Extract	Yield ± SD[a]	Total Polyphenol ± sd[a]					
		g extract·Kg^{-1} dry cork	g GAE·g^{-1} extract	g GAE·Kg^{-1} dry cork				
			Initial	Day 1	Day 2	Day 8	Day 15	Day 22
1	A	3.1 ± 0.8	0.32 ± 0.02	0.90 ± 0.18	0.74 ± 0.10	0.72 ± 0.09	0.71 ± 0.07	0.72 ± 0.03
	B	29.8 ± 2.9	0.40 ± 0.01	11.99 ± 0.32	11.96 ± 0.07	11.87 ± 0.03	11.65 ± 0.99	11.77 ± 1.29
2	C	32.6 ± 1.4	0.34 ± 0.02	11.23 ± 0.46	11.31 ± 0.31	11.19 ± 0.11	10.90 ± 0.28	10.70* ± 0.41
	D	13.9 ± 2.0	0.29 ± 0.01	4.37 ± 0.21	4.40 ± 0.24	4.42 ± 0.25	4.41 ± 0.28	4.45 ± 0.22
3	E	29.1 ± 1.8	0.29 ± 0.02	8.27 ± 0.55	8.39 ± 0.53	8.30 ± 0.53	8.58 ± 0.57	8.46 ± 0.35
	F	22.5 ± 2.1	0.37 ± 0.04	8.77 ± 0.46	8.75 ± 0.39	8.68 ± 0.57	8.84 ± 0.59	8.65 ± 0.37
4	G	22.2 ± 0.7	0.34 ± 0.02	7.55 ± 0.43	7.60 ± 0.43	7.58 ± 0.41	7.66 ± 0.54	7.68 ± 0.36
	H	19.8 ± 0.8	0.35 ± 0.01	7.00 ± 0.26	7.07 ± 0.32	7.18 ± 0.40	7.23 ± 0.32	6.95 ± 0.36
	I	27.3 ± 1.0	0.30 ± 0.01	8.07 ± 0.38	8.13 ± 0.53	8.33 ± 0.54	8.36 ± 0.46	8,00 ± 0.46
	J	19.4 ± 1.3	0.35 ± 0.01	6.86 ± 0.26	6.90 ± 0.32	6.97 ± 0.34	7.02 ± 0.25	6.89 ± 0.28

[a]sd: standard deviation.

* Significant differences ($p<0.05$) between day 1 and cited day.

The results obtained show that, a larger amount of extract was obtained by extraction with methanol (extract *C*) and methanol/water with later extraction with diethyl ether (extract *B*).

The total phenolic content, determined by the Folin-Ciocalteau method in ten extracts, varies from 0.29-0.40 g GAE·g^{-1} extract. However, when the total phenolic content is expressed in g GAE·Kg^{-1}dry cork shown greater differences in the results have been found, included in a range of 0.9-12.0 g GAE·Kg^{-1}dry cork, being extracts *B* and *C* those that present a higher content, and extracts *A* and *D*, those with the lower content. As can be observed in Table 4.1, regarding the evolution of the total phenolic content during the refrigerated storage, significant change ($p < 0.05$) was found in only one case, in day 22 of extract *C*, decreasing a 4.7% from 11.23 g·Kg^{-1} in the first day of storage to 10.7 g·Kg^{-1}after 22 days.

From the analysis of these results, it is observed that the best method to obtain the higher values of phenolic yields correspond to *method_2* (extracts *C* and *D*), as well as *method_4*, both with a previous wash cork (extracts *G* and *H*) as without washing (extracts *I* and *J*). Lower values in total phenolic content have been obtained with *method_3* (extract *E* and *F*).

4.3.2. HPLC analysis of phenolic compounds

Analysed compounds were preliminarily chosen according to the phenolic composition described in the literature (Santos et al., 2010, Conde et al., 1997, 1998, Mazzoleni et al., 1998). This study was performed in order to develop a simple, reliable and robust method for efficient separation and quantification of these compounds. The most important parameters that influence chromatographic separation, such as mobile phase composition and flow rate, were optimized.

The composition of the mobile phase has a large influence in complete resolution of the peaks of the compounds. Due to the analytes differing widely in polarity, isocratic elution is unviable and it was necessary to use a binary gradient. The two solvents employed in the gradient elution were mixtures of acid:H_2O, as solvent A, and acid:MeOH as solvent B, where phosphoric and formic acids were assayed. The behaviour of the low molecular

weight phenolic compounds was identical independent of the acid used. Finally, to avoid deposits of phosphate salts in the chromatographic system, the formic acid was chosen for the following experiments.

Different gradients with MeOH (0.1% HCOOH) as solvent B were assayed to achieve baseline separation of all compounds in a time as short as possible. On the other hand, the flow rate has been optimized between 0.5 and 1.0 $mL \cdot min^{-1}$.

Once elution conditions were established, chromatographic parameters (column resolution (*R*), capacity factor (*k'*), theoretical plate number (*N*) and selectivity factor (α)) were calculated to obtain information about efficiency of chromatographic separation of the low molecular weight phenolic compounds. These parameters have been calculated and the obtained values are summarized in Table 4.2.

Retention time is the most important parameter to know the length of analysis, it can be seen in Table 4.2. Peak resolution describes the degree of separation between two compounds. Usually an *R* value of 1 is accepted to consider that a separation is satisfactory, but *R* must be equal to or higher than 1.5 to achieve a baseline resolution. Most compounds were correctly separated with very good resolution. However, the separation of syringic acid and vanillin does not shown a good resolution, but when both compounds have different signal, this would not affect the quantification of them. In relation to *k'* values were comprised between 2.8 and 12.5, which are near the ideal values (1 and 10) (Murphy et al., 1978), only four of eighteen analyzed phenolic compounds (22%) of their *k'* values fell outside the optimum range. On other hand, *N* values, calculated based on the width of peak at half height, indicating a more efficient chromatographic separation.

Table 4.2. Chromatographic parameters (column resolution (*R*), capacity factor (*k'*), theoretical plate number (*N*) and selectivity factor (*α*)) calculated for each low molecular weight phenolic compounds.

Compound	k' ± sd[a]	N ± sd[a]	Rs ± sd[a]	α ± sd[a]
Gallic acid	2,82 ± 0,01	13605 ± 1746	-	-
Protocatequic acid	4,43 ± 0,01	51419 ± 1465	14,12 ± 0,54	1,57 ± 0,01
p-Hydroxyphenyl acid	5,23 ± 0,01	78578 ± 2505	8,63 ± 0,11	1,18 ± 0,01
p-Hydroxybenzoic acid	5,82 ± 0,01	74158 ± 601	6,25 ± 0,04	1,11 ± 0,01
Vanillic acid	6,96 ± 0,01	101123 ± 1326	11,29 ± 0,05	1,20 ± 0,01
Aesculetin	7,08 ± 0,01	118979 ± 985	1,25 ± 0,02	1,02 ± 0,01
Caffeic acid	7,27 ± 0,01	116273 ± 985	2,00 ± 0,01	1,03 ± 0,01
Syringic acid	7,72 ± 0,01	99560 ± 2612	4,29 ± 0,04	1,06 ± 0,01
Vanillin	7,80 ± 0,01	125228 ± 7457	0,76 ± 0,02	1,01 ± 0,01
Syringaldehyde	8,51 ± 0,01	136153 ± 939	6,22 ± 0,11	1,09 ± 0,01
p-Coumaric acid	8,68 ± 0,01	141613 ± 1107	1,58 ± 0,02	1,02 ± 0,01
Salicylic acid	9,06 ± 0,01	77334 ± 3782	3,09 ± 0,03	1,04 ± 0,01
Ferulic acid	9,28 ± 0,01	165499 ± 1651	1,78 ± 0,06	1,02 ± 0,01
Coniferyl aldehyde	9,71 ± 0,01	166757 ± 1252	4,10 ± 0,02	1,05 ± 0,01
Sinapic aldehyde	10,02 ± 0,01	165565 ± 1828	2,89 ± 0,01	1,03 ± 0,01
Eriodictyol	11,34 ± 0,01	240743 ± 2876	12,56 ± 0,06	1,13 ± 0,01
Ellagic acid	11,62 ± 0,01	119540 ± 4877	2,32 ± 0,07	1,03 ± 0,01
Naringenin	12,49 ± 0,01	416207 ± 6410	7,49 ± 0,07	1,07 ± 0,01

[a] sd: standard deviation.

4.3.3. Method validation: Analytical parameters

In order to achieve the method validation a calibration curve of each compound was established employing the peak area as analytical signal and using the optimized conditions. The linearity of the method was assessed by preparing calibration standards with concentrations interval from 0.5 to 16.0 $\mu g \cdot mL^{-1}$ of each low molecular weight phenolic compounds. Standard solutions containing all phenolic compounds were prepared by triplicate for each concentration level and the analytical figures of merit were calculated employing the peak areas as analytical signal. The linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability for each phenolic compounds of the HPLD-DAD method are shown in Table 4.3, together with their time retention (R_t), wavelength UV-vis maxima, selected wavelength for DAD determination and $[M-H]^-$ obtained. Evaluation of the precision of the optimized method was done by analysing standard solutions of the phenolic compounds in the same day (intra-day precision, n = 12) and in consecutive days (inter-day precision, n = 6). Intra-day and inter-day repeatability values, expressed as relative standard deviation (RSD), are lower than 3.3% and 4.0%, respectively, so they may be considered as a guarantee of the goodness of the proposed method. The limits of detection and quantification were calculated as concentrations corresponding to 3 and 10 times the standard deviation of the signal from linear calibration curve (Shrivastava et al., 2011). Low values are obtained that are indicative of the high sensitivity of the developed method.

Table 4.3. Phenolic compound standards and retention time (RT), linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability obtained with the HPLC-DAD method.

Peak	Name	Detection				LOD ($mg \cdot L^{-1}$)	LOQ ($mg \cdot L^{-1}$)	Analytical Sensitivity ($mg \cdot L^{-1}$)	R^2	Repeatability (RSD %)			
		RT (min)	UV-Vis maxima (nm)	UV-Vis DAD (nm)	$[M-H]^-$ (*m/z*)					Intra-day (*n* = 12)		Inter-day (*n* = 6)	
										RT	Area	RT	Area
1	Gallic acid	20.8	272	275	169	0.13	0.44	0.13	0.9995	0.4	1.2	0.7	2.5
2	Protocatequic acid	29.4	259	255	153	0.24	0.79	0.24	0.9982	0.2	1.5	0.3	3.5
3	*p*-Hydroxyphenyl acid	33.6	275	275	181 [a]	0.16	0.53	0.16	0.9992	0.1	0.9	0.1	1.5
4	*p*-Hydroxybenzoic acid	36.9	255	255	137 [a]	0.14	0.45	0.14	0.9994	0.1	0.6	0.2	2.0
5	Vanillic acid	43.0	261	255	167	0.18	0.60	0.18	0.9990	0.1	0.8	0.1	0.9
6	Aesculetin	43.6	350	345	177	0.20	0.66	0.20	0.9988	0.1	1.3	0.1	1.9
7	Caffeic acid	44.7	323	305	179	0.18	0.58	0.18	0.9990	0.1	1.5	0.1	2.3
8	Syringic acid	46.8	265	275	197	0.19	0.64	0.19	0.9989	0.1	1.1	0.1	0.9
9	Vanillin	47.4	279, 312	345	151	0.21	0.69	0.21	0.9987	0.1	1.2	0.1	1.4
10	Syringaldehyde	51.2	307	305	182[a]	0.13	0.45	0.14	0.9994	0.1	0.7	0.1	0.8

Cont. Table 4.3. Phenolic compound standards and retention time (RT), linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability obtained with the HPLC-DAD method.

Peak	Name	Detection				LOD ($mg \cdot L^{-1}$)	LOQ ($mg \cdot L^{-1}$)	Analytical Sensitivity ($mg \cdot L^{-1}$)	R^2	Repeatability (RSD %)			
		RT (min)	UV-Vis maxima (nm)	UV-Vis DAD (nm)	$[M-H]^-$ (*m/z*)					Intra-day (*n* = 12)		Inter-day (*n* = 6)	
										RT	Area	RT	Area
11	*p*-Coumaric acid	52.3	311	305	173	0.11	0.36	0.11	0.9996	0.1	1.0	0.2	0.8
12	Salicylic acid	54.2	302	305	137[a]	0.15	0.51	0.15	0.9993	0.2	1.6	0.1	2.4
13	Ferulic acid	55.5	321	305	193	0.10	0.32	0.10	0.9997	0.1	1.3	0.1	2.0
14	Coniferyl aldehyde	57.7	340	345	177	0.12	0.41	0.12	0.9995	0.1	0.9	0.1	1.3
15	Sinapic aldehyde	59.3	340	345	218	0.13	0.45	0.14	0.9994	0.1	1.1	0.1	2.2
16	Eriodictyol	66.5	288	275	287	0.20	0.68	0.21	0.9987	0.1	1.1	0.1	2.6
17	Ellagic acid	68.0	255	255	301	0.17	0.58	0.18	0.9991	0.1	3.3	0.1	4.0
18	Naringenin	72.7	288	275	271[a]	0.12	0.40	0.12	0.9996	0.1	0.7	0.1	0.6

[a], identified in the literature.

4.3.4. Quantitative analysis of low molecular weight phenolic compounds in cork.

The basis for identification of the phenolic compounds grouped as phenolic acids, aldehydes and derivatives, coumarins derivatives, hidroxycinnamic acid derivates, flavonoids, ellagic acid and derivatives, and ellagitannins is described below.

Phenolic acids, aldehydes and derivatives. The chemical structure of this group of phenolic compounds consists of an aromatic ring, a hydroxyl group and a carboxylic group. Plants produce an extremely diverse array of low molecular weight phenolic compounds, often called secondary metabolites or natural products that bestow metabolic plasticity essential for anticipating and responding to biotic and abiotic stress (Dixon, 2001). Phenolic acids, characterised by hydroxylated aromatic ring are ubiquitous secondary metabolites in plants and provide one of the most studied and widely exploited metabolic pathways in plant research (Boudet, 2007; Whiting, 2001).

It has been observed that plants possess defense mechanisms that they use in response to the attack of pathogens (Chester, 1993). Secondary metabolites play important roles either as local or systemic resistance factors in protecting the plants against various pathogens (Redman et al., 2003; Metraux, 2001).

The different extracts obtained of cork showed a great number of phenolic acid and aldehydes, among them gallic acid (t_R = 20.9 min, MS m/z = 169), protocatechuic acid (t_R = 29.8 min, MS m/z = 153), protocatechuic aldehyde (t_R = 34.9 min, MS m/z = 137), vanillic acid (t_R = 43.1 min, MS m/z = 167), vanillin (t_R = 47.8 min, MS m/z = 151), syringic acid (t_R = 46.6 min, MS m/z = 197), coniferyl aldehyde (t_R = 58.1 min, MS m/z = 177) and sinapaldehyde (t_R = 59.7 min, MS m/z = 218). For the quantification of protocatechuic aldehyde was made using the protocatechuic acid in the calibration curve.

Coumarins derivates. Coumarin and its derivates are widely distributed throughout nature and many exhibit useful and diverse biological activities (Egan et al. 1990; Borges et al., 2005).

The only coumarin found has been aesculetin (t_R = 43.9 min, MS m/z = 177), this compound is a derivate of coumarin. It is a nature lactone that derives from the intramolecular cyclization of a cinnamic acid derivate.

Hidroxycinnamic acid derivates. These compounds possess a simple chemical backbone consisting of a phenylpropanoid C_6-C_3 structure and care the major subgroup of phenolic acids with ubiquitous distribution in the plant kingdom. They are abundant found in tea leaves, coffee, red wine, various fruits, vegetables and whole grains(Kroon et al., 1999).In the extracts of cork have been identified caffeic acid (t_R = 44.9 min, MS m/z = 179), *p*-coumaric acid (t_R= 51.7 min, MS m/z = 163) and ferulic acid (t_R = 55.8 min, MS m/z = 193).

Hidroxycinnamic acids, such as *p*-coumaric, caffeic, ferulic and sinapic acids are known to play an important role in nature. In fact, their wide distribution and high concentration provide them with a key role in the biosynthesis of more complicated phenolic systems. Hidroxycinnamic acids are secondary metabolites that are found also in several conjugated forms, including amides, esters, mainly esters of hydroxyl acids, such as tartaric acid and sugar derivates, and glycosides (Teixeira et al., 2013).

Flavonoids. Flavonoids have the general structure of a 15 carbon skeleton, which consists of two phenyl rings and heterocyclic ring. Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, bark, roots, steams, flowers, tea, and wine (Middleton, 1998). As in the case of coumarins, eriodictyol (t_R = 66.6 min, MS m/z = 287) is the only flavonoid found.

Ellagic acid and derivates. Ellagic acid is a natural phenol antioxidant found in numerous fruits and vegetables. The antiproliferative and antioxidant properties of ellagic acid have prompted research into its potential health benefits. Ellagic acid is the dilactone of hexahydroxydiphenic acid. Plants produce ellagic acid from hydrolysis of tannins such as ellagitannin and gerannin (Seigler, 1998).

This compounds have been identified as dehydrated tergallic-C-glucoside (t_R = 40.7 min, MS m/z = 613), valoneic acid dilactone (t_R = 54.3 min, MS m/z = 469), ellagic acid-hexose (t_R = 59.2 min, MS m/z = 463), ellagic acid-pentose (t_R = 67.2 min, MS m/z = 433) and ellagic acid (t_R = 68.5 min, MS m/z = 301).

Ellagitannins. Ellagitannins are esters of hexahydroxydiphenic acid (HHDP: 6,6'-dicarbonyl-2,2',3,3',4,4'-hexahydroxybiphenyl moiety) and a polyol usually glucose or quinic acid (Quideau et al., 1996). A key feature of ellagitannins is their ability to release the bislactone, ellagic acid, which is formed from the hydrolytic release of HHDP esters group, which undergo rapid, facile and unavoidable lactonization. Ellagitannins are commonly detected and quantified in the form of ellagic acid (Amakura et al., 2000). Hence ellagic acid may be considered as a chemical marker compound for hydrolysable tannins (Seeram, 2006).

In cork extracts, the found ellagitannins correspond to vescalagin (t_R = 25.2 min, MS m/z = 933), castalagin (t_R = 28.1 min, MS m/z = 933), HHDP-glucose (t_R = 32.4 min, MS m/z = 408) and di-HHDP-glucose (t_R = 33.3 min, MS m/z = 783). These compounds were quantified using the ellagic acid calibration curve.

In table 4.4 and 4.5, shows the results obtained from the determination and quantification by HPLC of low molecular weight phenolic compounds of in different extracts obtained from cork. Results are expressed as μg compound·g^{-1} of cork dry, specifying the standard deviation.

Analyzing the results obtained as a function of the extraction method, it can be seen that in the *method_1*, after the final extraction with diethyl ether, low molecular weight phenolic compounds are concentrated in the organic phase (extract *A*), and ellagitannins are the main compounds in the aqueous phase (extract *B*. In Extract *A*, the main compounds are gallic acid (40.5 μg·g^{-1}), protocatechuic acid (34.6 μg·g^{-1}) and ellagic acid (12.6 μg·g^{-1}), being 25.2%, 21.5% and 7.8% respectively of the final concentration of phenolic compounds found. HHDP-glucose, *p*-coumaric acid and eriodictyol have the lowest content, representing 1.5%, 1.0% and 1.6% of the total phenolic compounds. Regarding the evolution of the identified compounds during storage, significant changes (p< 0.05) were found in almost all cases. In this extract, a significant evolution during the 8-day period has been found, decreasing to 50.0% in vescalagin, 47.1% in castalagin and 37.5% in *p*-coumaric acid. After 15 days of storage, decreasing 44.4% in vescalagin, 35.3% in castalagin, 37.5% in *p*-coumaric acid and 47.1% in valoneic acid dilactone. The opposite behavior was observed for ellagic acid increasing 37.3% from 12.6 μg·g^{-1} in the first day storage to 17.3 μg·g^{-1} after 15 days, this is because HHDP groups spontaneously are lactonization to ellagic acid in

aqueous solution (Hagerman, 2010).The major compounds in extract *B* have been castalagin (223.8 $\mu g \cdot g^{-1}$), vescalagin (199.7 $\mu g \cdot g^{-1}$) and ellagic acid (79.2 $\mu g \cdot g^{-1}$), also a non-identified ellagitannin has been found (78.3 $\mu g \cdot g^{-1}$), obtaining in proportion to the total compounds determined 28.0%, 25.0%, 9.9% and 9.8%, respectively. Di-HHDP-glucose, ellagic acid-hexose and ellagic acid-pentose do not obtain more than 3% of the total each. Caffeic and ferulic acids have been determined below the limit of detection, and the limit of quantification has been protocatechuic acid and aesculetin, but di-HHDP glucose after 8 and 15 days of storage. In contrast to extract *A*, numerous compounds such as vanillic acid, vanillin, *p*-coumaric acid, coniferyl aldehyde, sinapaldehyde and eriodictyol have not been detected. Extract *B* revealed a great stability of their phenolic profile during the 15 days storage. In fact, only ellagic acid were significantly modified ($p< 0.05$), increasing 109.2% from 79.2 $\mu g \cdot g^{-1}$ to 165.7 $\mu g \cdot g^{-1}$.

About *method_2*, ellagic acid has been the main component in extract *C* (871.2 $\mu g \cdot g^{-1}$), followed by castalagin (199.9 $\mu g \cdot g^{-1}$), vescalagin (139.6 $\mu g \cdot g^{-1}$) and gallic acid (100.8 $\mu g \cdot g^{-1}$), corresponding to 50.5%, 11.6%, 8.1% and 5.8%, of the total phenolic compounds determined. Di-HHDP-glucose, ferulic acid and ellagic acid-hexose no was obtaining more than 1.5% of the total compounds determined in each. Below the limit of detection has been determined p-coumaric acid; and the protocatechuic aldehyde, aesculetin, caffeic acid, coniferyl aldehyde and sinapaldehyde of below the limit of quantification has been determined. Syringic acid and eriodictyol could not be determined. Showed a significant evolution during the 15-day period ($p< 0.05$), decreasing 18.1% in valoneic acid dilactone and 30.62% in ellagic acid.

Table 4.4. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 1 and 2.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
Method_1					
Extract A					
1[a]	19.8	Gallic acid	40.5 ± 7.9	37.9 ± 5.7	35.2 ± 2.0
2[a]	25.9	Vescalagin	3.6 ± 0.1	1.8* ± 0.2	2.0* ± 0.1
3[a]	28.4	Castalagin	3.4 ± 0.1	1.8* ± 0.2	2.2* ± 0.1
4[a]	30.4	Protocatechuic acid	34.6 ± 5.5	33.1 ± 5.1	31.9 ± 1.9
5[a]	32.1	HHDP-Glucose	2.4 ± 0.1	2.2 ± 0.3	2.6 ± 0.1
6[a]	34.3	Protocatechuic aldehyde	5.0 ± 0.8	4.6 ± 0.5	4.2 ± 0.1
7[a]	40.5	Dehydrated tergallic-C-Glucoside	<LOD	<LOD	<LOD
8[a]	42.8	Vanillic acid	9.5 ± 1.5	8.7 ± 0.5	8.8 ± 0.1
9[a]	43.2	Aesculetin	4.1 ± 0.9	3.1 ± 0.2	3.3 ± 0.3
10[a]	44.3	Caffeic acid	6.4 ± 2.5	4.5 ± 1.0	4.3 ± 0.4
11[a]	46.6	Syringic acid	5.2 ± 1.4	3.9 ± 0.2	4.1 ± 0.1
12[a]	47.3	Vanillin	8.2 ± 2.3	7.4 ± 0.8	7.2 ± 1.0
13[a]	51.9	Coumaric acid	1.6 ± 0.3	1.0* ± 0.2	1.0* ± 0.2

Cont. Table 4.4. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 1 and 2.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
14[a]	54.2	Valoneic acid dilactone	5.1 ± 0.3	3.1 ± 1.2	2.7* ± 0.1
15[a]	55.5	Ferulic acid	6.3 ± 1.7	4.5 ± 1.2	3.9 ± 0.1
16[a]	57.9	Coniferyl aldehyde	4.4 ± 1.2	3.2 ± 0.4	3.2 ± 0.4
17[a]	59.0	Ellagic acid-hexose	<LOD	<LOD	<LOD
18[a]	59.6	Sinapaldehyde	2.7 ± 0.7	2.0 ± 0.1	2.0 ± 0.1
19[a]	66.5	Eriodictyol	2.6 ± 0.2	<LOQ	<LOQ
20[a]	67.3	Ellagic acid-pentose	2.7 ± 0.4	1.9 ± 0.1	2.3 ± 0.1
21[a]	68.5	Ellagic acid	12.6 ± 0.4	15.0 ± 3.4	17.3* ± 0.3
Extract B					
1[b]	21.0	Gallic acid	26.9 ± 9.2	26.4 ± 7.8	26.6 ± 6.3
2[b]	23.6	Non-identified ellagitannin	78.3 ± 4.5	87.0 ± 9.7	87.0* ± 2.8
3[b]	25.2	Vescalagin	199.7 ± 14.7	206.1 ± 26.8	208.5 ± 8.2
4[b]	28.0	Castalagin	223.6 ± 16.2	228.3 ± 30.2	231.8 ± 18.9
5[b]	29.8	Protocatechuic acid	<LOQ	<LOQ	<LOQ

Cont. Table 4.4. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 1 and 2.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
6[b]	32.4	HHDP-Glucose	24.8 ± 1.7	26.2 ± 3.4	28.3 ± 3.8
7[b]	33.2	Di-HHDP-Glucose	17.2 ± 0.6	<LOQ	<LOQ
8[b]	35.0	Protocatechuic aldehyde	29.8 ± 3.4	29.9 ± 1.8	29.1 ± 1.4
9[b]	40.7	Dehydrated tergallic-C-Glucoside	28.0 ± 6.5	30.3 ± 4.0	30.0 ± 3.1
10[b]	43.9	Aesculetin	<LOQ	<LOQ	<LOQ
11[b]	44.6	Caffeic acid	<LOD	<LOD	<LOD
12[b]	46.6	Syringic acid	26.2 ± 3.8	25.4 ± 3.9	25.4 ± 3.1
13[b]	54.4	Valoneic acid dilactone	26.3 ± 5.6	34.7 ± 11.1	35.3 ± 10.3
14[b]	55.9	Ferulic acid	<LOD	<LOD	<LOD
15[b]	59.2	Ellagic acid-hexose	18.2 ± 2.1	18.2 ± 2.4	18.3 ± 2.2
16[b]	67.2	Ellagic acid-pentose	21.3 ± 2.2	22.4 ± 3.2	22.7 ± 2.9
17[b]	68.6	Ellagic acid	79.2 ± 29.4	142.0 ± 59.3	165.7* ± 63.6

Cont. Table 4.4. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 1 and 2.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
Method_2					
Extract C					
1[c]	20.9	Gallic acid	100.8 ± 3.1	103.0 ± 3.7	101.2 ± 4.5
2[c]	23.4	Non-identified ellagitannin	35.5 ± 5.3	35.3 ± 5.0	39.6 ± 4.5
3[c]	25.0	Vescalagin	139.6 ± 9.0	141.4 ± 11.2	144.3 ± 12.7
4[c]	27.9	Castalagin	199.9 ± 4.1	177.4 ± 19.4	196.1 ± 14.0
5[c]	29.6	Protocatechuic acid	70.3 ± 9.8	67.8 ± 5.9	60.6 ± 5.3
6[c]	32.2	HHDP-Glucose	29.8 ± 4.6	29.0 ± 6.1	30.2 ± 5.0
7[c]	33.2	Di-HHDP-Glucose	19.3 ± 0.0	19.1 ± 0.2	19.1 ± 0.2
8[c]	34.8	Protocatechuic aldehyde	<LOQ	<LOQ*	<LOQ*
9[c]	40.4	Dehydrated tergallic-C-Glucoside	34.7 ± 0.9	34.5 ± 2.7	35.4 ± 2.4
10[c]	43.2	Vanillic acid	28.5 ± 0.4	28.3 ± 0.6	28.5 ± 0.4
11[c]	43.9	Aesculetin	<LOQ	<LOQ	<LOQ
12[c]	44.9	Caffeic acid	<LOQ	<LOQ	<LOQ
13[c]	47.7	Vanillin	57.8 ± 0.7	52.3 ± 2.8	55.3 ± 1.4

Cont. Table 4.4. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 1 and 2.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
14[c]	51.8	Coumaric acid	<LOD	<LOD	<LOD
15[c]	54.1	Valoneic acid dilactone	70.0 ± 4.2	60.2 ± 8.0	57.3* ± 7.8
16[c]	55.7	Ferulic acid	16.5 ± 1.4	16.3 ± 1.2	15.9 ± 0.8
17[c]	57.9	Coniferyl aldehyde	<LOQ	<LOQ	<LOQ
18[c]	59.0	Ellagic acid-hexose	22.4 ± 1.0	22.2 ± 1.0	21.8 ± 0.9
19[c]	59.6	Sinapaldehyde	<LOQ	<LOQ	<LOQ
20[c]	67.0	Ellagic acid-pentose	28.3 ± 1.1	26.7 ± 3.4	26.8 ± 1.7
21[c]	68.3	Ellagic acid	871.2 ± 36.3	786.9 ± 12.7	604.5* ± 51.3
Extract D					
1[d]	21.0	Gallic acid	20.3 ± 1.6	20.7 ± 1.3	20.5 ± 1.9
2[d]	23.6	Non-identified ellagitannin	11.1 ± 1.6	10.7 ± 1.4	10.5 ± 1.5
3[d]	25.2	Vescalagin	45.1 ± 4.5	34.5* ± 4.7	25.9* ± 6.3
4[d]	28.0	Castalagin	49.7 ± 6.0	48.3 ± 4.9	44.9 ± 5.2
5[d]	29.6	Protocatechuic acid	13.5 ± 1.4	13.9 ± 1.5	14.6 ± 1.7
6d	32.5	HHDP-Glucose	10.6 ± 0.6	18.7* ± 0.2	23.8* ± 1.5

Cont. Table 4.4. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 1 and 2.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
7[d]	33.4	Di-HHDP-Glucose	8.7 ± 1.2	8.1 ± 0.6	8.1 ± 0.6
8[d]	34.9	Protocatechuic aldehyde	<LOQ	<LOQ	<LOQ
9[d]	40.7	Dehydrated tergallic-C-Glucoside	12.0 ± 0.4	12.7 ± 0.8	13.1* ± 1.1
10[d]	43.2	Vanillic acid	9.2 ± 0.6	9.1 ± 0.6	8.9 ± 0.7
11[d]	43.8	Aesculetin	<LOQ	<LOQ	<LOQ
12[d]	45.0	Caffeic acid	<LOD	<LOD	<LOD
13[d]	46.6	Syringic acid	16.7 ± 1.6	16.8 ± 1.3	16.7 ± 1.8
14[d]	54.3	Valoneic acid dilactone	12.8 ± 1.4	12.4 ± 1.0	11.7 ± 1.0
15[d]	55.9	Ferulic acid	<LOQ	<LOQ	<LOQ
16[d]	58.0	Coniferyl aldehyde	<LOQ	<LOQ	<LOQ
17[d]	59.2	Ellagic acid-hexose	9.1 ± 0.7	9.1 ± 0.6	9.0 ± 0.7
18[d]	59.6	Sinapaldehyde	<LOQ	<LOQ	<LOQ
19[d]	67.2	Ellagic acid-pentose	10.8 ± 0.7	10.8 ± 0.6	10.8 ± 0.9
20[d]	68.3	Ellagic acid	176.4 ± 19.5	173.1 ± 10.5	167.0 ± 15.3

[a] sd: standard deviation.

* Significant differences ($p<0.05$) between day 1 and cited day.

Ellagic acid (176.4 $\mu g \cdot g^{-1}$), castalagin (49.7 $\mu g \cdot g^{-1}$) and vescalagin (45.1 $\mu g \cdot g^{-1}$) have been the main compounds of extract *D*, with 43.4%, 12.2% and 11.1%, respectively. Di-HHDP-glucose, vanillic acid and ellagic acid-hexose have had the lowest quantification that has been determined, representing 2.1%, 2.3% and 2.2% of the total phenolics quantified. The value obtained for caffeic acid has been below the limit of detection, the compounds below the limit of quantification have been protocatechuic aldehyde, aesculetin, ferulic acid, coniferyl aldehyde and sinapaldehyde. Vanillin, *p*-coumaric acid and eriodictyol have not been determined in this extract. The stability of the phenolic profile was different in this extract; vescalagin decreased a 23.5% in the first day of storage a after 8 days and a 42.6% after 15 days; while HHDP-glucose increasing 76.4% after 8 days and 124.5% after 15 days, also the dehydrated tergallic-C-glucoside increases after 15 days of storage a 9.2%.

In extracts obtained by extraction *method_3*, the compounds with the highest concentration in extract *E* have been ellagic acid (305.1 $\mu g \cdot g^{-1}$), castalagin (114.6 $\mu g \cdot g^{-1}$) and vescalagin (93.6 $\mu g \cdot g^{-1}$); and castalagin (128.1 $\mu g \cdot g^{-1}$), ellagic acid (108.4 $\mu g \cdot g^{-1}$), vescalagin (82.9 $\mu g \cdot g^{-1}$) and gallic acid (72.8 $\mu g \cdot g^{-1}$) in extract *F*. HHDP-glucose, coniferyl aldehyde and ellagic acid-hexose have been the compounds with lower concentration determined in extract *E*, while in extract *F* they have been ferulic acid, coniferyl aldehyde and sinapaldehyde. In extract *F,* only *p*-coumaric acid and eridyctiol below the limit of detection have been found. In the extract *E*, di-HHDP-glucose, protocatechuic aldehyde, aesculetin, caffeic acid, *p*-coumaric acid and sinapaldehyde have been below the limit of quantification, on the contrary, in extract *F* only caffeic acid. The only compound that has not been determined from the initially assumptions in this investigation has been eriodictyol in the extract *E*. The stability of the phenolic profile was different among these extracts, in extract *E*, vescalagin and vanillin was decreased 12.1% and 21.7% from first day of storage to after 15 days, respectively. On the other hand, in extract *F*, protocatechuic aldehyde decreased a 26.4% from 26.1 $\mu g \cdot g^{-1}$ in the first day to 19.2 $\mu g \cdot g^{-1}$ after 8 days of storage, remaining significantly constant until after 15 days; and ellagic acid has increased a 242.8% and 275.9%, after 8 and 15 days of storage, respectively.

Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
Method_3					
Extract E					
1[e]	21.1	Gallic acid	57.5 ± 4.6	55.4 ± 5.8	55.5 ± 6.0
2[e]	23.6	Non-identified ellagitannin	44.6 ± 2.8	41.5 ± 4.1	44.1 ± 6.1
3[e]	25.1	Vescalagin	93.6 ± 1.6	89.4 ± 2.6	82.3* ± 3.3
4[e]	28.0	Castalagin	114.6 ± 3.5	111.7 ± 1.8	115.1 ± 5.7
5[e]	29.8	Protocatechuic acid	41.2 ± 3.9	41.5 ± 4.5	42.1 ± 6.3
6[e]	32.4	HHDP-Glucose	17.8 ± 1.2	18.3 ± 2.4	19.0 ± 2.2
7[e]	33.1	Di-HHDP-Glucose	<LOQ	<LOQ	<LOQ
8[e]	35.0	Protocatechuic aldehyde	<LOQ	<LOQ*	<LOQ*
9[e]	40.7	Dehydrated tergallic-C-Glucoside	22.4 ± 1.6	22.0 ± 1.3	22.5 ± 1.2
10[e]	43.3	Vanillic acid	22.9 ± 0.9	22.5 ± 1.0	22.1 ± 1.2
11[e]	43.9	Aesculetin	<LOQ	<LOQ	<LOQ
12[e]	45.0	Caffeic acid	<LOQ	<LOQ	<LOQ
13[e]	46.6	Syringic acid	24.1 ± 2.5	23.5 ± 1.0	23.9 ± 1.2
14[e]	47.8	Vanillin	39.6 ± 3.7	32.9* ± 2.7	31.0* ± 1.7

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
15[e]	51.7	Coumaric acid	<LOQ	<LOQ	<LOQ
16[e]	54.4	Valoneic acid dilactone	33.5 ± 3.3	31.2 ± 4.0	25.8 ± 4.2
17[e]	55.8	Ferulic acid	22.8 ± 3.3	22.0 ± 3.2	22.0 ± 3.5
18[e]	58.0	Coniferyl aldehyde	12.3 ± 1.5	<LOQ	<LOQ
19[e]	59.2	Ellagic acid-hexose	16.9 ± 1.1	<LOQ	<LOQ
20[e]	59.7	Sinapaldehyde	<LOQ	<LOQ	<LOQ
21[e]	67.3	Ellagic acid-pentose	21.2 ± 1.0	20.8 ± 1.1	20.6 ± 1.2
22[e]	68.4	Ellagic acid	305.1 ± 65.1	313.3 ± 62.4	301.7 ± 67.5
Extract F					
1[f]	21.0	Gallic acid	72.8 ± 4.3	72.5 ± 5.2	71.8 ± 5.2
2[f]	23.6	Non-identified ellagitannin	35.5 ± 3.4	42.5 ± 0.9	44.1 ± 1.8
3[f]	25.2	Vescalagin	82.9 ± 9.4	94.4 ± 3.5	94.8 ± 5.0
4[f]	28.0	Castalagin	128.1 ± 16.2	133.3 ± 16.1	135.1 ± 17.3
5[f]	29.8	Protocatechuic acid	52.1 ± 2.5	53.5 ± 2.7	54.0 ± 3.1
6[f]	32.4	HHDP-Glucose	18.0 ± 1.6	19.3 ± 2.0	19.8 ± 3.1
7f	33.2	Di-HHDP-Glucose	14.5 ± 0.4	14.9 ± 0.1	14.9 ± 0.2

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
8[f]	34.9	Protocatechuic aldehyde	26.1 ± 2.0	19.2* ± 2.2	18.9* ± 0.5
9[f]	40.7	Dehydrated tergallic-C-Glucoside	23.3 ± 1.3	24.8 ± 0.6	24.7 ± 1.1
10[f]	43.5	Vanillic acid	19.8 ± 1.3	19.9 ± 1.0	19.7 ± 1.1
11[f]	44.1	Aesculetin	15.3 ± 0.5	15.4 ± 0.2	15.4 ± 0.3
12[f]	45.1	Caffeic acid	<LOQ	<LOQ	<LOQ
13[f]	46.7	Syringic acid	24.7 ± 2.3	24.6 ± 0.6	24.8 ± 0.5
14[f]	48.0	Vanillin	17.1 ± 2.6	<LOQ*	<LOQ*
15[f]	51.8	Coumaric acid	<LOQ	<LOQ	<LOQ
16[f]	54.4	Valoneic acid dilactone	44.5 ± 3.8	46.1 ± 5.3	47.8 ± 3.0
17[f]	55.9	Ferulic acid	13.3 ± 1.5	13.9 ± 1.2	13.8 ± 1.0
18[f]	58.2	Coniferyl aldehyde	10.4 ± 0.9	9.4 ± 0.6	9.5 ± 0.6
19[f]	59.2	Ellagic acid-hexose	16.2 ± 0.9	16.8 ± 0.6	16.5 ± 0.5
20[f]	59.9	Sinapaldehyde	10.1 ± 0.4	10.1 ± 0.2	9.9 ± 0.2
21[f]	66.7	Eriodictyol	<LOQ	<LOQ	<LOQ
22[f]	67.3	Ellagic acid-pentose	21.3 ± 1.5	21.3 ± 0.6	21.1 ± 0.6
23f	68.6	Ellagic acid	108.4 ± 10.2	371.6* ± 20.7	407.5* ± 33.1

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
Method_4					
Extract G					
1[g]	21.0	Gallic acid	66.9 ± 5.2	62.9 ± 8.0	61.2 ± 10.9
2[g]	23.5	Non-identified ellagitannin	37.1 ± 7.3	44.5 ± 1.3	44.5 ± 4.4
3[g]	25.1	Vescalagin	105.5 ± 10.5	98.5 ± 5.4	99.5 ± 4.7
4[g]	27.9	Castalagin	129.3 ± 5.1	124.6 ± 8.7	124.9 ± 2.0
5[g]	29.6	Protocatechuic acid	37.0 ± 1.2	34.5 ± 2.4	33.3 ± 3.8
6[g]	32.3	HHDP-Glucose	29.9 ± 2.8	28.2 ± 3.4	30.6 ± 5.4
7[g]	33.9	Di-HHDP-Glucose	14.2 ± 0.0	13.6 ± 0.6	13.3 ± 0.6
8[g]	35.1	Protocatechuic aldehyde	24.7 ± 2.5	21.6 ± 1.5	20.4 ± 1.9
9[g]	40.5	Dehydrated tergallic-C-Glucoside	23.5 ± 1.3	22.7 ± 1.9	21.8 ± 1.0
10[g]	43.2	Vanillic acid	18.7 ± 0.3	17.8 ± 1.2	17.2* ± 0.9
11[g]	43.8	Aesculetin	<LOQ	<LOQ	<LOQ
12[g]	44.9	Caffeic acid	<LOQ	<LOQ	<LOQ
13[g]	46.4	Syringic acid	22.2 ± 0.6	21.4 ± 0.9	21.5 ± 1.8
14[g]	47.8	Vanillin	37.7 ± 2.1	38.4 ± 2.4	34.9 ± 1.4
15[g]	51.6	Coumaric acid	<LOQ	<LOQ	<LOQ

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
16[g]	54.1	Valoneic acid dilactone	47.9 ± 2.8	46.8 ± 2.5	42.5* ± 0.9
17[g]	55.7	Ferulic acid	12.4 ± 0.3	11.9 ± 0.4	11.7 ± 0.7
18[g]	58.0	Coniferyl aldehyde	<LOQ	<LOQ	<LOQ
19[g]	59.0	Ellagic acid-hexose	14.7 ± 0.5	14.7 ± 0.5	14.4 ± 0.5
20[g]	59.6	Sinapaldehyde	<LOQ	<LOQ	<LOQ
21[g]	66.6	Eriodictyol	<LOD	<LOD	<LOD
22[g]	67.0	Ellagic acid-pentose	18.9 ± 1.1	18.7 ± 1.0	17.9 ± 0.9
23[g]	68.3	Ellagic acid	386.9 ± 37.0	401.8 ± 51.7	419.7 ± 49.3
Extract H					
1[h]	21.0	Gallic acid	20.0 ± 3.8	19.5 ± 3.7	19.9 ± 3.9
2[h]	23.6	Non-identified ellagitannin	42.2 ± 1.7	45.8* ± 1.3	48.5* ± 1.5
3[h]	25.2	Vescalagin	100.8 ± 1.5	102.1 ± 3.9	104.4 ± 2.2
4[h]	28.1	Castalagin	127.9 ± 1.3	125.7 ± 4.2	129.0 ± 1.9
5[h]	29.9	Protocatechuic acid	<LOQ	<LOQ	<LOQ
6[h]	32.4	HHDP-Glucose	15.0 ± 0.6	14.9 ± 1.1	14.8 ± 1.8
7[h]	33.2	Di-HHDP-Glucose	12.8 ± 0.3	12.5 ± 0.5	12.3 ± 0.6

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (μg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
8[h]	35.0	Protocatechuic aldehyde	25.5 ± 1.2	23.3 ± 2.2	22.5* ± 1.7
9[h]	40.7	Dehydrated tergallic-C-Glucoside	21.0 ± 0.7	20.7 ± 0.7	20.7 ± 0.7
10[h]	43.9	Aesculetin	<LOD	<LOD	<LOD
11[h]	44.7	Caffeic acid	<LOD	<LOD	<LOD
12[h]	46.7	Syringic acid	19.7 ± 0.8	19.0 ± 0.5	19.4 ± 0.4
13[h]	51.7	Coumaric acid	<LOD	<LOD	<LOD
14[h]	54.4	Valoneic acid dilactone	23.9 ± 1.3	23.2 ± 1.2	23.2 ± 1.0
15[h]	58.2	Coniferyl aldehyde	<LOD	<LOD	<LOD
16[h]	59.2	Ellagic acid-hexose	13.5 ± 0.3	13.3 ± 0.3	13.3 ± 0.1
17[h]	67.3	Ellagic acid-pentose	17.6 ± 0.5	17.2 ± 0.5	17.4 ± 0.4
18[h]	68.6	Ellagic acid	168.6 ± 21.6	180.9 ± 11.8	199.8* ± 13.1
Extract I					
1[i]	21.1	Gallic acid	55.2 ± 5.6	54.2 ± 6.1	52.9 ± 4.4
2[i]	23.7	Non-identified ellagitannin	45.3 ± 2.2	46.5 ± 2.5	51.2* ± 2.8
3[i]	25.3	Vescalagin	99.9 ± 0.3	101.1 ± 3.7	102.2 ± 1.8
4[i]	28.1	Castalagin	128.4 ± 6.4	128.3 ± 7.2	137.1 ± 3.1

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
5[i]	29.9	Protocatechuic acid	37.6 ± 4.5	36.2 ± 2.6	40.4 ± 7.5
6[i]	32.5	HHDP-Glucose	18.8 ± 1.0	19.7 ± 1.8	20.7 ± 3.3
7[i]	33.2	Di-HHDP-Glucose	17.2 ± 0.2	16.9 ± 0.4	16.1* ± 0.1
8[i]	35.0	Protocatechuic aldehyde	23.0 ± 1.4	<LOQ*	<LOQ*
9[i]	40.8	Dehydrated tergallic-C-Glucoside	24.3 ± 0.7	23.6 ± 1.1	23.8 ± 0.7
10[i]	43.5	Vanillic acid	23.8 ± 1.8	23.7 ± 1.6	24.2 ± 2.1
11[i]	44.1	Aesculetin	<LOQ	<LOQ	<LOQ
12[i]	45.2	Caffeic acid	<LOQ	<LOQ	<LOQ
13[i]	46.7	Syringic acid	24.6 ± 0.6	24.8 ± 3.9	25.0 ± 0.9
14[i]	48.0	Vanillin	34.6 ± 3.4	34.8 ± 3.6	33.7 ± 3.3
15[i]	54.4	Valoneic acid dilactone	33.0 ± 2.9	28.5 ± 1.5	25.9* ± 4.8
16[i]	55.9	Ferulic acid	26.1 ± 1.9	25.4 ± 3.1	25.8 ± 1.9
17[i]	58.2	Coniferyl aldehyde	<LOQ	<LOQ	<LOQ
18[i]	59.2	Ellagic acid-hexose	17.1 ± 0.5	16.9 ± 0.6	17.1 ± 0.5
19[i]	59.8	Sinapaldehyde	<LOQ	<LOQ	<LOQ
20[i]	67.2	Ellagic acid-pentose	21.1 ± 1.2	20.9 ± 1.2	20.9 ± 1.0
21[i]	68.6	Ellagic acid	265.9 ± 10.7	270.5 ± 22.6	289.4 ± 19.6

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (μg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
Extract J					
1[j]	21.3	Gallic acid	17.8 ± 0.5	17.4 ± 1.3	17.7 ± 0.7
2[j]	23.7	Non-identified ellagitannin	43.1 ± 4.6	46.2 ± 5.0	47.8 ± 4.4
3[j]	25.2	Vescalagin	108.8 ± 7.3	109.6 ± 2.9	111.9 ± 6.9
4[j]	28.1	Castalagin	137.1 ± 10.5	133.4 ± 8.4	135.4 ± 12.6
5[j]	29.9	Protocatechuic acid	<LOQ	<LOQ	<LOQ
6[j]	32.5	HHDP-Glucose	15.1 ± 1.6	16.0 ± 2.3	17.2 ± 3.6
7[j]	33.2	Di-HHDP-Glucose	12.9 ± 0.4	12.6 ± 0.4	12.1* ± 0.4
8[j]	34.9	Protocatechuic aldehyde	25.0 ± 1.9	22.3* ± 1.1	20.9* ± 1.4
9[j]	40.8	Dehydrated tergallic-C-Glucoside	21.7 ± 1.5	21.3 ± 1.8	21.1 ± 1.3
10[j]	44.1	Aesculetin	<LOQ	<LOQ	<LOQ
11[j]	45.1	Caffeic acid	<LOD	<LOD	<LOD
12[j]	46.7	Syringic acid	19.8 ± 1.0	19.1 ± 0.6	19.5 ± 1.1
13[j]	54.4	Valoneic acid dilactone	19.3 ± 0.9	19.2 ± 2.3	18.9 ± 0.9

Cont. Table 4.5. Retention time (R_t), tentative identification and concentration of low molecular weight phenolic compounds in the cork extract of extraction methods 3 and 4.

Peak	R_t, min	Tentative identification	Quantification (µg compound·g^{-1} dry cork± sd[a])		
			Day 1	Day 8	Day 15
14[j]	58.4	Coniferyl aldehyde	<LOD	<LOD	<LOD
15[j]	59.3	Ellagic acid-hexose	13.6 ± 0.1	13.4 ± 0.1	13.4 ± 0.2
16[j]	59.9	Sinapaldehyde	<LOQ	<LOQ	<LOQ
17[j]	67.3	Ellagic acid-pentose	17.4 ± 0.4	17.1 ± 0.5	17.2 ± 0.2
18[j]	68.7	Ellagic acid	95.1 ± 9.4	105.2 ± 10.9	119.1* ± 12.6

[a] sd: standard deviation.

* Significant differences ($p<0.05$) between day 1 and cited day.

In *method_4*, extracts *G* and *H* (previously washed with dichloromethane), have been as major components the ellagic acid with 386.9 and 168.6 $\mu g \cdot g^{-1}$, castalagin with 129.3 and 127.9 $\mu g \cdot g^{-1}$, and vescalagin with 100.8 and 100.8 $\mu g \cdot g^{-1}$, respectively; while minority compounds to extract *G* have been di-HHDP-glucose, ferulic acid and ellagic acid-hexose; and HHDP-glucose, di-HHDP-glucose and ellagic acid-hexose in the case of extract *H*.In extract *G*, only eriodictyol has been below the limit of detection, whereas aesculetin, caffeic acid, *p*-coumaric, coniferyl aldehyde and sinapaldehyde have not exceeded the limit of quantification. In the case of extract *H*, vanillic acid, vanillin, ferulic acid, sinapaldehyde and eriodictyol have not been identified; in addition, the aesculetin, caffeic acid, *p*-coumaric acid and coniferyl aldehyde have been below the limit of detection, and protocatechuic aldehyde of the limit of quantification. In the extracts *I* and *J* (previous washing with dichloromethane has not been performed), the results obtained have been similar to their previous homologues. Ellagic acid (265.9 $\mu g \cdot g^{-1}$), castalagin (128.4 $\mu g \cdot g^{-1}$) and vescalagin (99.9 $\mu g \cdot g^{-1}$) have been the major compounds in extract *I*, being castalagin (137.1 $\mu g \cdot g^{-1}$), vescalagin (108.8 $\mu g \cdot g^{-1}$) and ellagic acid (95.1 $\mu g \cdot g^{-1}$) in extract *J*. The three minority compounds for both extracts are HHDP-glucose, di-HHDP-glucose and ellagic acid-hexose. In extract *I* have been five compounds which were below the limit of detection, protocatechuic aldehyde, aesculetin, caffeic acid, coniferyl aldehyde and sinapaldehyde, and without being able to determine *p*-coumaric acid and eriodictyol. In extract *J*, below the limit of detection have been caffeic acid and coniferyl aldehyde, and protocatechuic acid, aesculetin and sinapaldehyde of the limit of quantification. Vanillic acid, vanillin, *p*-coumaric, ferulic acid and eriodictyol have not been determined in this extract.

As can be seen from the results obtained, the stability of these extracts differs greatly between them. The extract *G* was one of the most stable, with only vanillic acid and valoneic acid dilactone showing significant changes ($p<0.5$), decreasing by 8.0% and 11.24% after 15 days, respectively. On the contrary, in extract *H*, non-identified ellagitannin increases 8.5% from the first day to the day 8, and increasing by 6.4% more after 15 days of storage. Also after 15 days, protocatechuic aldehyde is reduced by 11.8%, while ellagic acid increased by 18.5% after the first day. In the case of extract *I*, the stability of its compounds has been affected only after 15 days of storage, resulting in an increase of 13.0% of the non-identified ellagitannin and a reduction of 6.4% and 21.5% of di-HHDP-glucose and valoneic acid dilactone, respectively. On the contrary, in extract *J* there has been a decrease after 15 days

of 6.2% in di-HHDP-glucose; and the protocatechuic aldehyde of 10.8% from 25 $\mu g \cdot g^{-1}$ on the first day of storage to 22.3 $\mu g \cdot g^{-1}$ after 8 days, further decreasing by 5.6% more after 15 days. On the other hand, ellagic acid has increased its concentration by 25.2% after 15 days of storage.

It can be concluded that extraction with methanol (*method_2*-extract *C*) is that which provides an extract in greater total concentration of low molecular weight phenolic compounds, obtaining ten times the happens to him in total concentration (extract *A*). The extracts with the highest number of quantified phenolic compounds are extract *A* (*method_1*) and extract *F* (*method_3*), on the other hand, extracts from the aqueous fraction extracted with diethyl ether (extracts *B*, *H*, and *J*) have been found to be those with a low quantitative determination of these compounds. From the point of view of identified phenolic compounds, extracts *F* (*method_3*) and extract *G* (*method_4*) are the most identified, whereas extract *B* (*method_1*) is found in contrast. Respect to the previous washing process with dichloromethane to eliminate lipophilic compounds, a significant difference between the extracts that have been made this process and its homologues has not been observed, although at the qualitative level, there have been observed more refined chromatograms in the extracts a those who have done this step. The results obtained for the different extracts confirm that the phenolic composition of each extract is differently affected during the same storage conditions. The total phenolic content of extract *F* (*method_3*) changed significantly during storage, and if the phenolic profile is taken into account, the extract that shows the greatest significant difference among its compounds is extract *A* (*method_1*), while in the other extracts only punctual changes in different compounds were noticed.

Ellagic acid has been the compound with the highest concentration found in extract *C* (*method_2*), followed by vescalagin and castalagin in extract *B* (*method_1*), and gallic acid in the extract *C*. The eriodictyol has only been identified in extracts *A* (*method_1*), *F* (*method_3*) and *G* (*method_4*). On the other hand, of the phenolic compounds of low molecular weight initially reported in cork, were not detected in any of the following compounds: *p*-hydroxyphenylacetic, *p*-hydroxybenzoic and salicylic acids, naringenin and syringaldehyde. Taking into account the stability of phenolic compounds, protocatechuic aldehyde in most of the extracts does not become stable even after 8 days, and ellagic acid after 15 days of storage.

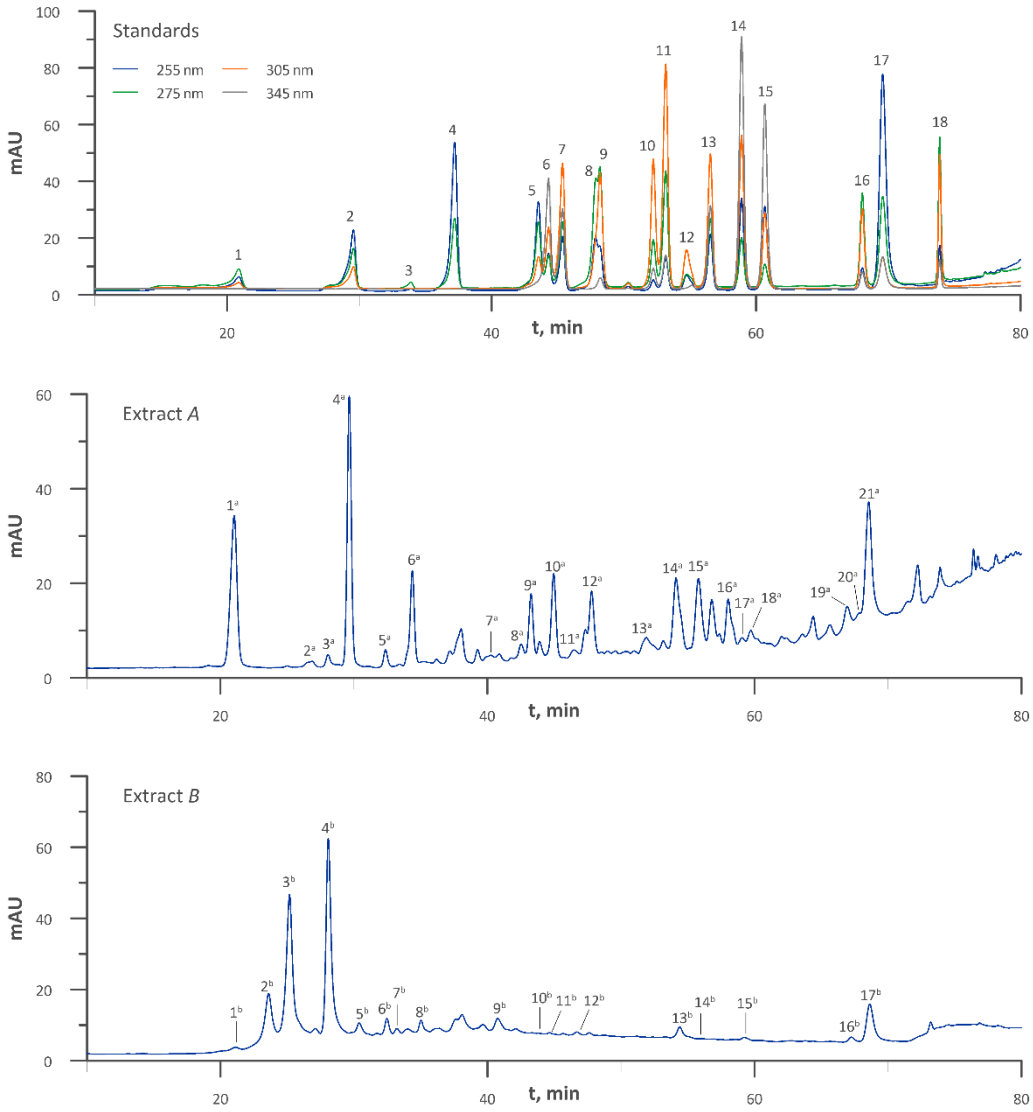
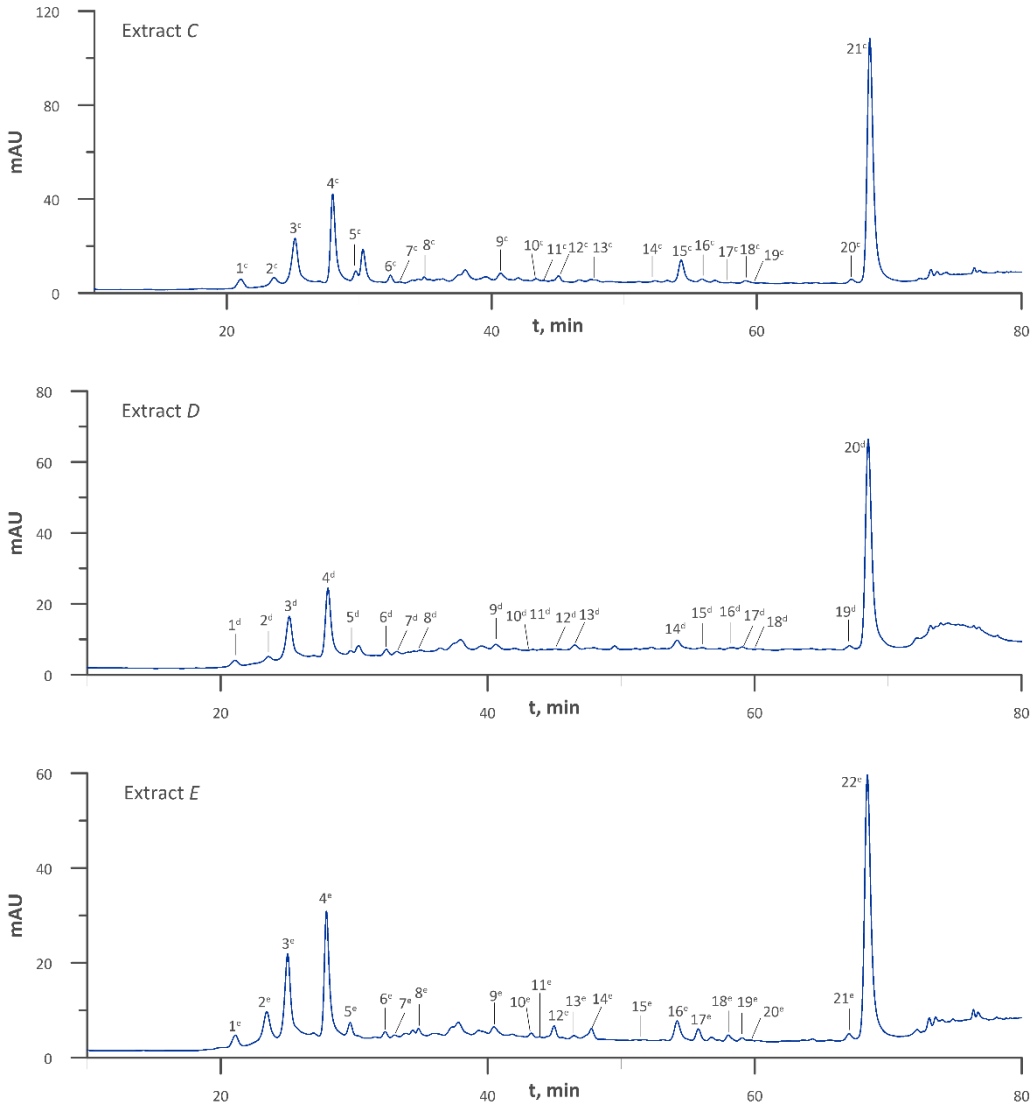
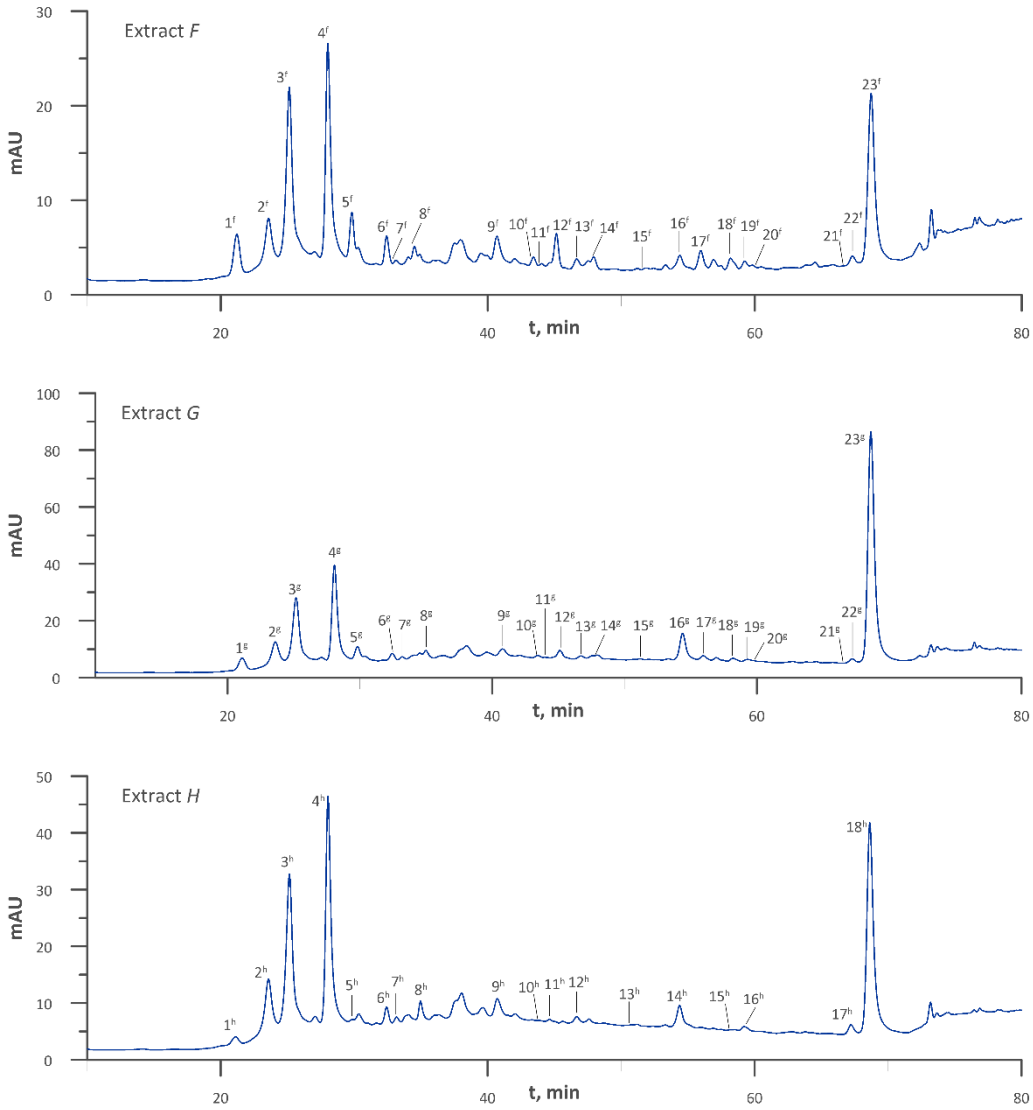


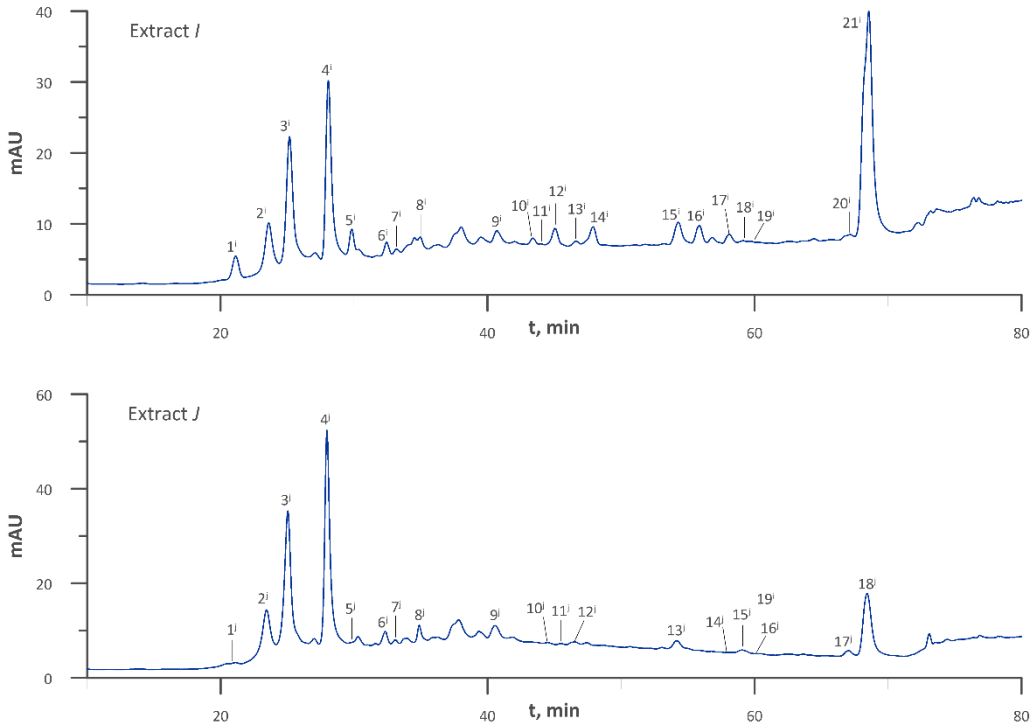
Figure 4.2. HPLC-DAD chromatograms of the phenolic standards and cork extract. A gradient solvent elution system was used over 80 min with water (0.1% formic acid) and methanol (0.1% formic acid) at a flow rate 0.5 mL·min^{-1}. Detection is shown at 255, 275, 305 and 345 nm. Peaks were identified by comparison with reference standards when available or by the HPLC-QTOF. Numbering of peaks refers to their identification as shown in Tables 4.4-4.5.



Cont. Figure 4.2. HPLC-DAD chromatograms of the phenolic standards and cork extract. A gradient solvent elution system was used over 80 min with water (0.1% formic acid) and methanol (0.1% formic acid) at a flow rate 0.5 mL·min^{-1}. Detection is shown at 255, 275, 305 and 345 nm. Peaks were identified by comparison with reference standards when available or by the HPLC-QTOF. Numbering of peaks refers to their identification as shown in Tables 4.4-4.5.



Cont. Figure 4.2. HPLC-DAD chromatograms of the phenolic standards and cork extract. A gradient solvent elution system was used over 80 min with water (0.1% formic acid) and methanol (0.1% formic acid) at a flow rate 0.5 mL·min^{-1}. Detection is shown at 255, 275, 305 and 345 nm. Peaks were identified by comparison with reference standards when available or by the HPLC-QTOF. Numbering of peaks refers to their identification as shown in Tables 4.4-4.5.



Cont. Figure 4.2. HPLC-DAD chromatograms of the phenolic standards and cork extract. A gradient solvent elution system was used over 80 min with water (0.1% formic acid) and methanol (0.1% formic acid) at a flow rate 0.5 mL·min^{-1}. Detection is shown at 255, 275, 305 and 345 nm. Peaks were identified by comparison with reference standards when available or by the HPLC-QTOF. Numbering of peaks refers to their identification as shown in Tables 4.4-4.5.

4.4. Conclusions

The extraction of phenolic compounds by different solvents has provided a high identification and quantification of these compounds according to the used method. Extraction with diethyl ether (extract *A*) provides the identification of a greater number of low molecular weight phenolic compounds, because these compounds is concentrated in organic phase, while ellagitannins are concentrated in aqueous phase (extract *B*). So much total phenolic content as in low molecular weight phenolic compounds gets a higher concentration of these compounds in the extract *C*, obtained with methanol as the solvent main.

A 15-day storage period revealed that the evolution of the phenolic compounds profile during refrigerated storage was different in every extract. In general, the differences between the overall phenolic compositions between the three sampling days were small, although statistically significant for extracts *B*, *C* and *F*. Nevertheless, some interesting relationships and variations among the different quantified components have been observed during the studied storage period.

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5. *Isolation and characterization of ellagitannis from cork of Quercus suber L.*

El corcho extraído del alcornoque (*Quercus suber* L.) es un recurso natural, que ha alcanzado un notable significado social y económico en España, especialmente en Extremadura. La mayor proporción de corcho se utiliza para elaborar tapones de vino, pero no todo el corcho extraído se puede utilizar para este fin. Este corcho puede ser una fuente explotable de antioxidantes fenólicos naturales, por lo que resulta de interés identificar, cuantificar y purificar los compuestos contenidos en el mismo.

Por todo ello, en este capítulo 5, se ha procedido primeramente a seguir caracterizando los extractos de corcho, descritos en el apartado 3.1.2.1. Se ha determinado en dichos extractos el contenido de taninos totales y actividad antioxidante. De forma análoga, se ha identificado y cuantificado los elagitaninos vescalagina y castalagina. Teniendo en cuenta los resultados obtenidos, se ha procedido a aislar los elagitaninos del extracto seleccionado mediante cromatografía preparativa. Para ello, se ha optimizado el método cromatográfico con el fin de obtener elagitaninos puros, validando el método mediante HPLC/Q-TOF.

Este trabajo está pendiente de remisión para su publicación.

Isolation and characterization of ellagitannins from cork of *Quercus suber* L.

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Abstract

The cork bark extracted from the cork oak (*Quercus suber* L.) is a renewable resource, which has achieved an outstanding social and economic meaning in Spain, particularly in Extremadura. The greater proportion of cork is used to make wine stoppers, but not all of the extracted cork can be used for this purpose. This cork may be an exploitable source of natural phenolic antioxidants, so it is of interest to identify, quantify and purify to compounds contained therein. Cork ellagitannins have been extracted by several methods using different solvents, obtaining a total of ten extracts. Castalagin and vescalagin are the main ellagitannins found in cork. The total tannin content, antioxidant activity and quantification of vescalagin and castalagin has been determinate in all extracts. The isolation of the ellagitannins has been carried out, obtaining yields of 846.5 μg vescalagin·g^{-1} dry cork and 912.4 μg castalagin·g^{-1} dry cork, corresponding to a 39.3 and 63.3% yield, respectively. HPLC and HPLC/Q-TOF have verified the purity of these isolated ellagitannins.

Keywords: elagitaninns, HPLC, cork, vescalagin, castalagin, isolation

5.1. Introduction

The ellagitannins are esters of glucose with hexahydroxydiphenic acid (HHDP), and belong together with the gallotannins to the group of hydrolysable tannins (Quideau et al., 1996; Hagerman, 2010). Ellagitannins are secondary metabolites biosynthesized in plants through the shikimic acid pathway. Over 500 ellagitannins have been identified in plants. Tannins are distributed differently in the plant kingdom, and rarely two species of plants share the same group of tannins (Juha-Pekka and Karonen, 2011). In many cases, the composition of tannins varies even within the same plant species, for example, plant cells do not simultaneously produce gallotannins and ellagitannins, and the production of ellagitannins causes a decrease in the biosynthesis of condensed tannins and flavonoids. For this reason, both ellagitannins and condensed tannins are not found in high concentrations in the same tissue of a plant (Ossipov et al., 2001; Salminen et al., 2001; Riipi et al., 2002). More than 150 molecules of ellagitannins have been isolated from Chinese medicinal plants by Okuda and Nishioka (Okuda et al., 1995; Nishioka, 1983).

Traditionally, the ellagitannins have been used as astringent drugs to cure gastric ulcers and burn injury (Okuda et al., 1981); tannins are able to decrease urea levels in the blood and inhibit Angiotensin-converting enzyme activity, they have antiviral and anti-HIV activity, and inhibit lipid peroxidation (Okuda et al., 1992). It has also been shown to have antitumor properties in animals (Yoshida et al., 1995). Recently, interest in the bioactive properties of ellagitannins in humans has grown. Different biological influences have been studied, including their antioxidant properties and their use in the treatment of cancer (Fridrich et al., 2008; Fernandes et al., 2009; Bialonska et al., 2009; Kasimsetty et al., 2010). In addition, several studies have concluded that tannins act as a defence mechanism against herbivores and pathogens, and as allelopathic agents (Kobayashi, 2004; Sosa et al., 2004; Juha-Pekka and Karonen, 2011).

Species of the family *Quercus* are some of the plants with higher content of tannins (Scalbert et al., 1990; Cadahía et al., 1998). Cork, the outer bark of *Quercus suber* L., is a natural product with exceptional qualities: low density, high compressibility, high water and gas impermeability, elasticity, high coefficient of friction, chemical stability and durability,

making it one of the most versatile natural raw materials (Rosa and Fortes, 1988; Vaz y Fortes, 1998; Silva et al., 2005; Gandini et al., 2006; Silvestre et al., 2008; Anjos et al., 2014).

Extremadura has one of the largest cork productions in Spain, almost 40% of Spanish production and more than 10% of world production (Martínez et al., 2012). More than 70% of cork produced all around the world is used to manufacture cork stoppers for wine (C.E. Liège), generating a lot of by-products, which are mainly used as biofuel or compost. Recent studies have sought new applications for cork and its by-products in order to promote their valorization (Pires et al., 2010; Pintor et al., 2012; Bejarano et al., 2015). Hence, cork may be an exploitable source of natural phenolic antioxidants.

The cork consists of suberine (~ 50%), lignin (20-25%), polysaccharides (~20%), extractable (14-18%) and inorganic compounds (1-2%) (Silva et al., 2005; Pereira, 2007). The proportions of this composition vary according to the methods used for its determination, as well as the quality of the cork analysed (Del Pozo et al., 1999).

Several studies have been carried out to study the phenolic composition of cork, with phenolic acids, aldehydes and flavonoids being identified predominantly (Conde et al., 1997, 1998; Santos et al., 2010; Fernandes et al., 2011). There are few studies on the composition of ellagitannins in cork (Cadahía et al., 1998), being most studies in oak wood (Zhentian et al., 1999; Cadahía et al., 2001), some fruits, nuts and seeds such as pomegranates, raspberries, strawberries, walnuts and almonds (Clifford and Scalbert, 2000).

Although there is a significant amount of studies focusing the composition of cork, we have been not find studies that carry out the isolation of ellagitannins in cork extracts. The majority of studies have carried out in oak wood (Quinn et al., 1985; Puech et al., 1988; Scalbert et al., 1990) or seeds (Karonen et al., 2010; Sudjaroen et al., 2012) through resins and semi-preparative HPLC.

The aim this work is the identification of the ellagitannins in cork extracts and the isolation of thereof in a simple and fast way, obtaining pure ellagitannins (vescalagin and castalagin).

5.2. Material and methods

5.2.1. Reagents

Standards of ellagitannins; vescalagin and castalagin were supplied by Doctor Stephane Quideau (European Institute of Chemistry and Biology, Pessac, Francia). Tannic acid, Folin-Denis reagent, sodium carbonate, methanol (MeOH) HPLC-grade, triflluoroacetic acid (TFA) and formic acid (HCOOH) were provided by Panreac (Barcelona, Spain). Peroxidase type VI (POD), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and H_2O_2 were purchased from Sigma-Aldrich (Madrid, Spain). 2.2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was obtained from Fluka (Munich, Germany). Ultrapure water was obtained from a Milli-Q water system (Millipore S.A.S., Molsheim, France).

Individual ellagitannins standard solutions were prepared in MeOH with the concentration to 100 $\mu g \cdot mL^{-1}$. All standards solutions were prepared separately and stored at -20 ºC, until analysis. During development of HPLC-DAD and HPLC-MS methods, a mixture of phenolic standards was prepared by dilution of the individual ellagitannins stock solutions with H_2O (0.1% TFA).

5.2.2. Raw material

Q. *suber* L. natural cork planks ("refuge" grade) were sampled from several local companies of San Vicente de Alcántara (Badajoz-Spain). An average sample composed of fragments of several planks from different trees was milled in a Retsch cross beatermill SK1 (Haan, Germany), and the granulometric fraction of 0.25-0.50 mm and 0.50-1.00 mm were used for analyses.

5.2.3. Ellagitannins extraction

The cork samples were extracted with different solvents (methanol/water, methanol, water and acetone/water) obtaining ten extracts of cork, according to the methods described by Martínez-Cañas et al. (2017).

5.2.4. Determination of ellagitannins from cork extracts

Ellagitannins from the obtained extracts were analysed on an Agilent 1200 liquid chromatograph instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with an online degasser, quaternary pump, auto-sampler, thermostatted column compartment, UV-Vis diode-array detector, and the Chemstation software package to control the instrument, data acquisition and data analysis. Chromatographic studies and analytical separation were carried out in a column Lichrospher RP-18 (250 nm x 4 mm x 5 µm) (Agilent Technologies) and the mobile phases were methanol (A) and water (0.1% TFA, B). The gradient employed was the following: 0 min, 100% B; 40 min, 90% B; 45 min, 0% B; 50 min 0% B, 60 min, 100% B and was maintained for 5 min (Zhentian et al., 1999). A flow rate of 0.75 $mL \cdot min^{-1}$ was used together with an injection volume of 20 µL, and 30°C column temperature. Detection was performed with a diode-array detector at 240 nm, and peak areas were used as analytical response.

The samples have been prepared by dilution to 10 mg of each extract in 10 mL aqueous methanol (MeOH:H_2O, 50:50). Each sample was stored at low temperature until the next time for analysis (day 1, day 8 and day 15) in order to study the stability of the extract.

For each extract samples, 1 mL of previous sample were added in a 5 mL volumetric flask with H_2O (0.1% TFA). Resulting solutions were filtered through a 0.22 µm nylon filter, and aliquots of 20 µL were injected in the chromatographic system.

The ellagitannins were identified by comparing their retention time, UV-Vis and mass spectra with those obtained from standard solution. For quantitative analysis, a calibration curve (0.5–14.0 $\mu g \cdot mL^{-1}$) for each available ellagitannin standard was constructed based on the UV signal. The results were expressed in µg $compound \cdot g^{-1}$ of dry weight, as mean ±

standard deviation of as a minimum three extracts. At least, two replicates of each extract were made for quantification purposes.

5.2.5. Isolation of ellagitannins

Isolation of ellagitannins of the cork extracts were carried out on an Agilent 1260 Infinity Preparative System (Agilent Technologies, Santa Clara, CA, USA) equipped with two binary pump, manual injector, UV-Vis diode-array detector, fraction collector and the Chemstation software package to control the instrument, data acquisition and data analysis. Chromatographic studies and analytical separation were carried out in a column PrepHT SB-C18 (21.2 x 150 mm, 7µm) from Agilent, and the mobile phases were methanol (A) and water (0.2% HCOOH, B). The gradient employed was the following: 0 min, 100% B; 5 min, 95% B; 15 min, 90% B; 17.5 min, 0% B, 22 min, 0% B and 27 min 100% B. A flow rate of 10 $mL \cdot min^{-1}$ was used together with an injection volume of 10 mL. Detection was performed with a diode-array detector at 240 nm, and the collector has collected the fractions as a function of time and selected peak parameters (slopes).

The cork extract samples were prepared by dilution of 200 mg of extract in 100 mL Milli-Q water. Resulting solutions were filtered through a 0.22 µm nylon filter, and aliquots of 10 mL were injected in the chromatographic system. Once the fractions were collected, the methanol was removed by low-pressure evaporation, and then was lyophilized. In this way, purified ellagitannins were obtained.

The validation of the proposed method to obtain purified ellagintannins were carried out by HPLC coupled to a detector mass spectrometry time of flight (HPLC/Q-TOF) on an Agilent 6530 Series Accurate Mass QTOF-MS (Agilent Technologies, Santa Clara, CA, USA).

5.2.6. Total Tannin content

The total tannin content of the extracts has been determined by the Folin-Denis method (Schanderl, 1970). 10 mg of extract obtained in preparative HPLC were dissolved in 50 mL aqueous methanol (MeOH:H_2O, 50:50). Folin-Denis reagent containing 0.5 mL, 10 mL deionized water and 1.25 mL of aqueous sodium carbonate (200 $g \cdot L^{-1}$) were added to an

aliquot of 0.5 mL of each extract, to a final volume of 25 mL. Resulting solutions were kept for 30 min at room temperature in the dark. After homogenizing, the absorbance was measured at 760 nm, using a UV/Vis Varian Cary-50 spectrophotometer (Palo Alto, California, USA). The total tannin content was calculated as tannic acid equivalent (mg TAE·g^{-1} of dry cork) using the standard addition method to avoid matrix effects caused in extracts. The linear range found was: 0.5–8.0 µg·mL^{-1}. Obtained results were expressed as a mean of mg TAE·g^{-1} of dry cork ± standard deviation of as a minimum three extracts. The analyses were carried out at least three times for each extract and the average value was calculated in each case.

5.2.7. Antioxidant activity

The antioxidant activity was measured using ABTS/POD decolouration method (Cano et al., 1998, 2000). This method is based on the capacity of different components to scavenge the ABTS radical cation ($ABTS^{\cdot+}$) compared to a standard antioxidant, Trolox, in a dose-response curve. For hydrophilic antioxidant activity (HAA), the reaction mixture contained 0.71 mmol·L^{-1} ABTS, 27.5 µmol·L^{-1} H_2O_2 and 19.6 nmol·L^{-1} POD in 50 mmol·L^{-1} phosphate buffer (pH 7.5) in a total volume of 1.02 mL. For lipophilic antioxidant activity (LAA) the reaction mixture contained 0.71 mmol·L^{-1} ABTS, 49.0 µmol·L^{-1} H_2O_2 and 78.4 nmol·L^{-1} POD in acidified ethanol, in a total volume of 1.02 mL. In both cases, 20 µL of the aqueous or organic phases was added to the reaction medium and the decrease in absorbance at 730 nm was determined after 5 min. The absorbance was measured using UV-2401PC Shimadzu spectrophotometer (Shimadzu Corporation, Tokyo, Japan). Antioxidant capacity was calculated as moles of $ABTS^{\cdot+}$ quenched by 1 mol of Trolox. In both cases, the antioxidant activity was expressed as µmol Trolox per g of dry cork.

5.2.8. Statistical analysis

The results were expressed as mean ± standard deviation and the differences of both cases, ellagitannins between the three sampling days (day 1, day 8 and day 15) and the stability of the extract, were tested using one-way ANOVA test. Differences were considered as statistically significant at a value of $p < 0.05$. The statistical analyses were carried out using Excel 2010 software (Microsoft, Redmond, Washington, USA).

5.3. Results and discussion

5.3.1. Total tannic content and antioxidant activity

The total tannic content, determined by the Folin-Denis method in the obtained extracts was in the range 1.5-23.3 mg TAE·g^{-1} dry cork, being extracts *B* and *C* are those that had got a higher content, and extract *A* the lowest content (see Table 5.1). Taking into account the extraction method, it is observed that both the extract with the highest quantification (extract *B*) and the lowest concentration (extract *A*) in *method_1* was obtained, due to the retention of low molecular weight phenolic compounds in the organic phase when diethyl ether is used, while ellagitannins are retained in the aqueous phase (Martínez-Cañas et al., 2017).

Table 5.1. Total tannin content and antioxidant activity of cork extracts from *Q. suber* L.

Extraction Method	Extract	Yield ± SD[a] (g extract·Kg^{-1} dry cork)	Total Tannin ± SD[a] (mg TAE·g^{-1} dry cork)	Antioxidant Activity (µmol Trolox·g^{-1} dry cork ± SD[a]) HAA	LAA
1	A	3.1 ± 0.8	1.5 ± 0.1	13.0 ± 0.1	6.9 ± 0.1
	B	29.8 ± 2.9	23.3 ± 1.2	152.6 ± 0.9	93.0 ± 2.1
2	C	32.6 ± 1.4	19.1 ± 1.9	156.5 ± 2.0	79.9 ± 1.6
	D	13.9 ± 2.0	6.8 ± 0.5	59.8 ± 0.6	31.3 ± 0.4
3	E	29.1 ± 1.8	14.5 ± 0.5	160.1 ± 2.3	85.0 ± 3.2
	F	22.5 ± 2.1	13.8 ± 0.6	128.3 ± 2.3	64.6 ± 2.0
4	G	22.2 ± 0.7	12.7 ± 1.0	97.9 ± 1.8	42.2 ± 2.0
	H	19.8 ± 0.8	11.1 ± 1.5	80.4 ± 1.0	36.8 ± 1.0
	I	27.3 ± 1.0	13.2 ± 1.3	113.3 ± 1.1	40.1 ± 1.9
	J	19.4 ± 1.3	12.3 ± 1.6	73.1 ± 2.1	26.0 ± 0.4

[a] SD: standard deviation.

Regarding the antioxidant activity, the hydrophilic antioxidant activity (HAA) in the ten extracts obtained comprises values from 13.0 to 152.6 μmol Trolox·g^{-1} dry cork, being in the extract *E* (*method_3)* the highest quantification was obtained; on the other hand, the lipophilic antioxidant activity (LAA) varies from 6.9 to 93.0 μmol Trolox·g^{-1} dry cork, in this case extract B (*method_1)* is the one with the highest concentration. From the point of view of total antioxidant activity (HAA plus LAA) is extract *B* this containing higher concentration, followed by extracts *E* and *C*.

5.3.2. Quantitative measurement of ellagitannins

The linearity of the method was assessed by preparing calibration standards with concentrations ranging from 0.5 to 14.0 μg·mL^{-1} of each ellagitannin. Standards were prepared by triplicate for each concentration level and the analytical figures of merit were calculated employing the peak areas as analytical response. The linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability for each ellagitannin compounds by using HPLD-DAD method are shown in Table 5.2. Evaluation of the precision of the method was done by analysing standard solutions of the phenolic compounds in the same day (intra-day precision, n = 10) and in consecutive days (inter-day precision, n = 5). The limits of detection and quantification were calculated as concentrations corresponding to 3 and 10 times the standard deviation of the signal from linear calibration curve (Shrivastava et al., 2011).

As can be seen in Table 5.3 and Figure 5.1, extracts with the highest concentration of vescalagin and castalagin are extract *B*, followed by extracts *C* and *F*; and the ones with the lowest concentration are extracts *A* and *D.*

About to the performed extraction methods, the extracts with the highest (extract *B*) and lowest (extract *A*) quantification were obtained by using *method_1*, moreover, in extract *A*, these ellagitannins were not detected, so the total tannin content and total antioxidant activity obtained are due to other compounds not analysed in this investigation.

Table 5.2. Analytical figures of merit of influence of concentration of ellagitannins.

Validation Parameters		Vescalagin	Castalagin
R_t, min		20.3	27.1
Slope, mAU·mL·µg^{-1}		50.060	80.136
Intercept, mAU		-29.592	-36.188
R^2		0.9982	0.9987
LOD, µg·mL^{-1}		0.43	0.32
LOQ, µg·mL^{-1}		1.45	1.07
Analytical sensitivity, µg·mL^{-1}		0.25	0.20
Repeatability, RSD%			
Intra-day	R_t	0.6	0.8
	Area	0.2	0.6
Inter-day	R_t	1.0	0.9
	Area	0.3	0.7

On the other hand, there is a difference in the quantification of both ellagitannins with respect to the two extracts obtained in *method_2*. The first extraction obtained in MeOH (extract *C*) provided extracts with the greater proportion of the ellagitannins while subsequent extraction with H_2O, provided the lower content of these compounds (extract *D*).

In *method_3*, the concentration of vescalagin and castalagin in extracts *E* and *F* is similar, although being slightly higher in extract *F*, suggesting that the previous extraction with dichloromethane to eliminate the lipophilic components benefits in the determination of these compounds.

Taking into account the obtained results by *method_4*, similar concentrations of ellagitannins have been found between the four different extracts and significant difference ($p < 0.05$) have been not found between those that have been previously extracted with dichloromethane (extracts *G* and *H*) and those that do not (extracts *I* and *J*).

Table 5.3. Quantification of ellagitannins in the cork extract.

Extraction Method	Extract	Vescalagin (μg·g^{-1} dry cork ± SD[a])			Castalagin (μg·g^{-1} dry cork ± SD[a])		
		Day 1	Day 8	Day 15	Day 1	Day 8	Day 15
1	A	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	B	2154.1 ± 170.1	1868.3 ± 106.4	1774.4 ± 85.3	1441.2 ± 153.7	1292.0 ± 119.2	1230.6 ± 114.9
2	C	1100.1 ± 178.0	949.4 ± 67.2	921.7 ± 91.2	1067.0 ± 29.6	1012.1 ± 43.1	1015.0 ± 84.6
	D	258.3 ± 51.1	203.6 ± 45.7	152.8* ± 24.3	210.6 ± 10.6	192.3 ± 0.9	198.6 ± 24.7
3	E	851.1 ± 72.1	845.0 ± 6.0	850.5 ± 47.0	559.1 ± 34.1	518.9 ± 29.2	504.1 ± 40.2
	F	1017.1 ± 111.2	1015.0 ± 98.4	881.3 ± 51.9	704.3 ± 51.6	672.8 ± 31.3	622.4 ± 30.9
4	G	873.1 ± 22.4	873.6 ± 21.9	885.8 ± 15.5	618.9 ± 17.9	619.2 ± 37.3	624.4 ± 24.4
	H	948.0 ± 41.8	754.3 ± 67.0	717.4* ± 66.8	665.6 ± 11.5	491.1 ± 61.0	476.2* ± 76.9
	I	899.9 ± 25.2	861.8 ± 36.6	862.3 ± 31.4	622.6 ± 50.5	632.9 ± 60.1	606.9 ± 11.7
	J	794.0 ± 51.2	785.5 ± 7.6	778.2 ± 30.5	614.7 ± 23.0	596.1 ± 22.7	592.1 ± 28.4

[a] sd: standard deviation.

* Significant differences ($p<0.05$) between day 1 and cited day.

Comparing extraction *methods 1* and *4*, focusing on the extracts *B* and *H*, the most significant difference between these methods is the particle size, it being possible to determine that at a smaller particle size, the extraction of these compounds is more effective.

About the study of the stability of these compounds in storage, a high stability of the compounds has been observed, determining in the vescalagin a significant difference ($p < 0.05$) in extracts *D* and *H* at 15 days of storage; and in the case of castalagin, only in extract *H* after 15 days of storage.

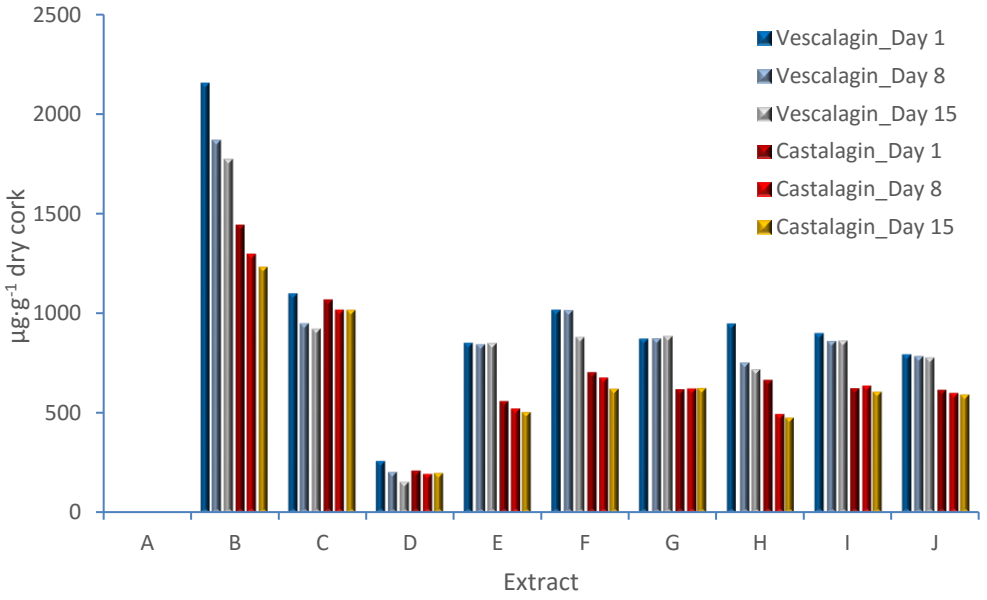

Figure 5.1. Variation of vescalagin and castalagin from cork extracts with storage time.

5.3.3. Isolation of ellagitannins

Taking into account the results obtained in the different cork extracts previously obtained, extract *B* has been selected for the isolation of the studied ellagitannins. Therefore, a stability study of this extract has been previously carried out. Selected variables have been the influence of temperature, time and exposure to light as a function of the concentration of ellagitannins, vescalagin and castalagin.

Influence of temperature: The Extract B solution has been kept, at different temperatures for one hour (25, 30, 35, 40, 50 and 60˚C). It is observed in Figure 5.2A that there were not variations in the concentration of the ellagitannins up to a temperature of 60˚C. However, significant differences ($p < 0.05$) were found comparing ellagitannins content at 60˚C and 25 ˚C.

Influence of time. Taking into account that ellagitannins from cork extract are not degraded at temperatures below 60˚C, the influence of the time on the ellagitannins content has been carried out. For this purpose, extract *B* has been kept at constant temperature (60°C) at different times, and the ellagitannins have been analysed (1, 2, 3, 4, 5 and 24 hours). It can be seen in Figure 5.2B, that concentration of castalagin does not change significantly ($p < 0.05$) when time is lower than 5 hours, reaching 30% reduction with respect to the initial concentration at 24 h. In the case of vescalagin, degradation is also observed, but although degradation of this compound begins at the first hour of exposure, reducing by 30% its initial concentration in the first 5 hours.

Influence of light exposure. The stability of extract *B* in solution in the absence (dark) or presence of light for time, until a period to 30 days at room temperature has been studied. As can be seen in Figure 5.2C, the concentration of the cork extract in the dark does not present significant variation until the sixth day, when the concentration begins to decrease to 25% after 30 days. If the extract is exposed to light, the degradation of vescalagin is observed at 48 hrs, decreasing by 60% at 30 days. On the other hand, as can be observed in the same figure, castalagin in the presence of light does not show significant difference until the eighth day, reducing after 30 days a 25%; however, in the dark, it remains stable up to 15 days, decreasing its concentration by only 13% at 30 days.

In view of the results obtained, it can be concluded that there is no degradation of the ellagitannins by raising the temperature for a given time, and yet a decrease of these compounds is observed with the passage of time at a given temperature. As regards the stability of these compounds in the absence or presence of light, greater degradation is noticed when the extract is exposed to light. Regarding the different compounds, it can be determined that castalagin is more stable than vescalagin.

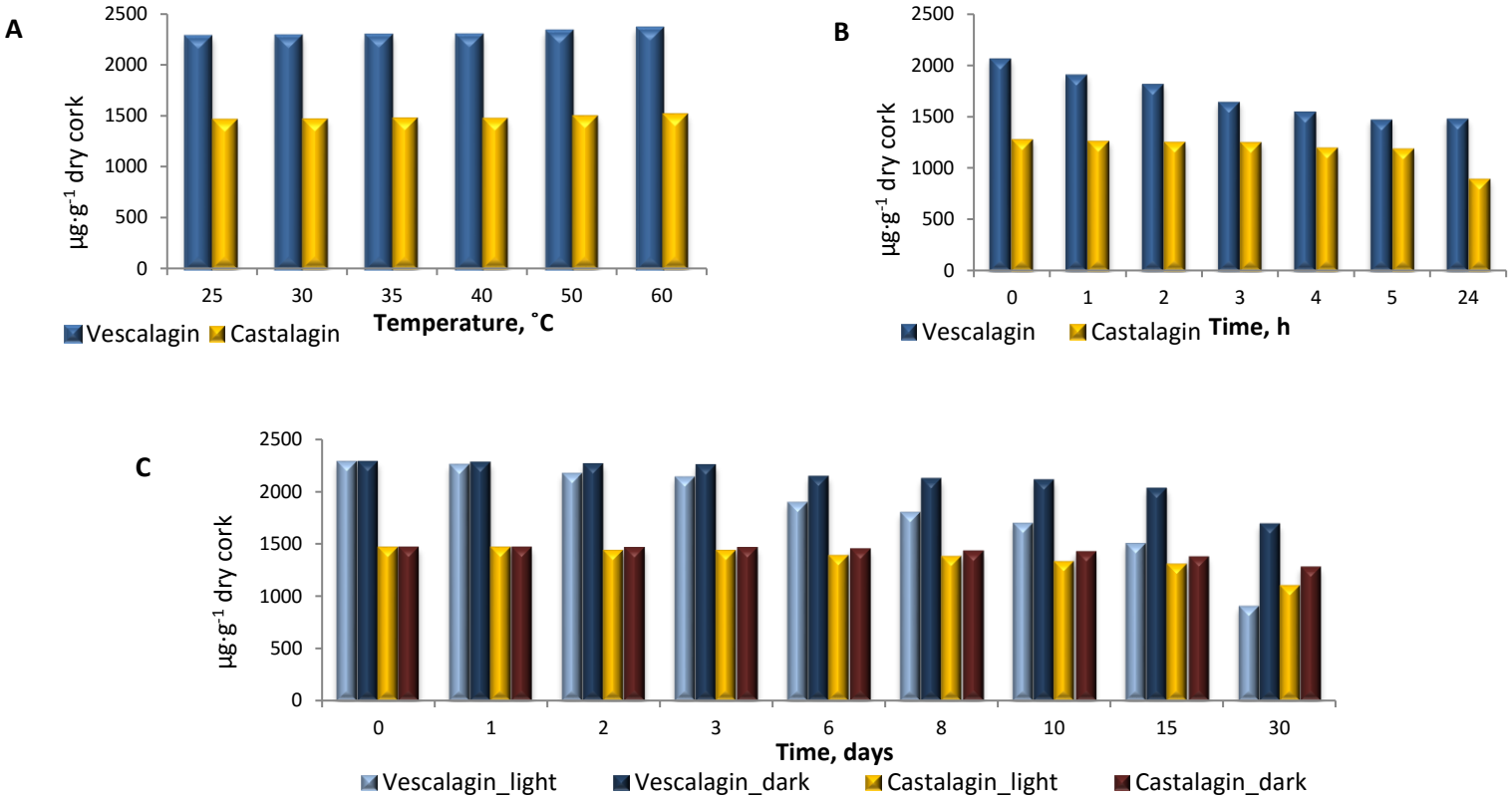

Figure 5.2. Stability extract *B*. A) Time (1h) vs temperature (25, 30, 35, 40, 50 and 60˚C). B) Temperature (60˚C) vs time (0, 1, 2, 3, 4, 5 and 24 h). C) Light/Dark vs time (0, 1, 2, 3, 6, 8, 10, 15 and 30 days).

The isolation of vescalagin and castalagin through extract *B* has been carried out. The average yield obtained has been 846.5 ± 28.9 and 912.4 ± 45.1 µg·g^{-1} dry cork of vescalagin and castalagin, respectively. Taking into account the concentrations of the elagitannins that have been determined in this extract B, 2154.1 µg vescalagin·g^{-1} dry cork and 1441.2 µg castalagin·g^{-1} dry cork, a 39.3 and 63.3% recovery of the compounds in the isolation has been obtained, respectively.

The isolated ellagitannins have been analysed by the HPLC method (described in section 5.2.4) and HPLC/Q-TOF to verify its purity. In Figure 5.3, is represented the obtained chromatograms of the ellagitannins isolated where only the peaks corresponding to the purified vescalagin and castalagin are observed. In Figure 5.4, it can be seen the 3D-plots of spectra of vescalagin (A) and castalagin (B), where it is observed that the spectra correspond only to the isolated ellagitannins. The peak purification factor within the threshold limit has been calculated, obtaining a 999.941 and a 999.876 for vescalagin and castalagin, respectively.

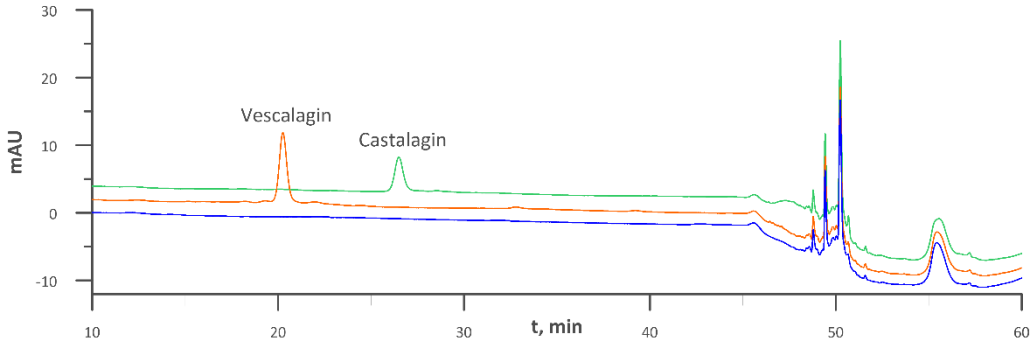

Figure 5.3. HPLC-DAD chromatograms of blank (blue line), purified vescalagin (orange line) and purified castalagin (green line).

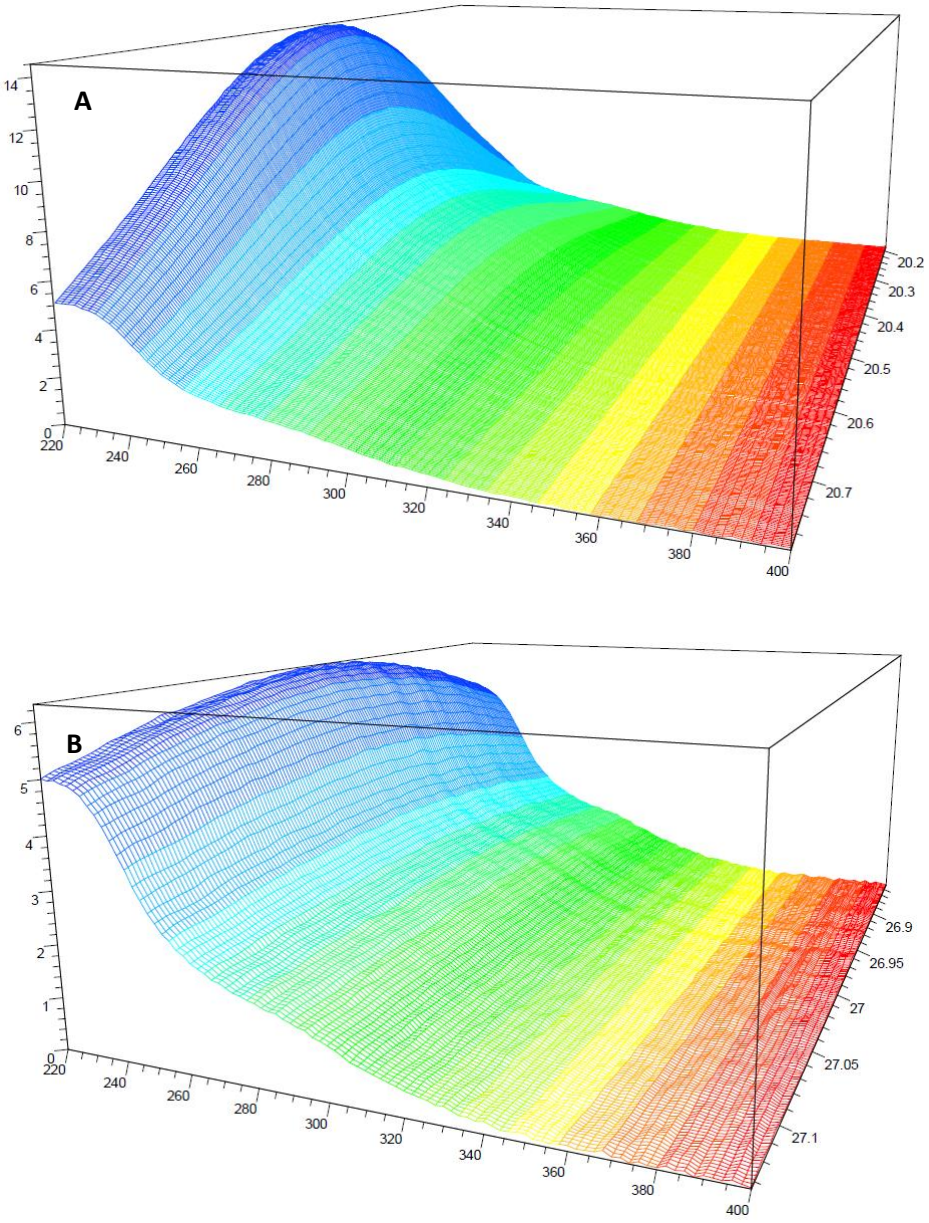

Figure 5.4. 3D-Plots of spectra of purified vescalagin (A) and purified castalagin (B) peaks.

On the other hand, the m/z ratio of ions (Table 5.4) of the purified ellagitannins has been determined by their mass spectra. In Figures 5.5A and 5.5B can be seen the mass spectra of castalagin and vescalagin. The m/z ratio of these compounds has been contrasted with literature references (Piwowarski et al., 2012). No contaminants have been detected in the isolated ellagitannins.

Table 5.4. m/z ratio of purified ellagitannins in the cork extract.

Ellagitannin	m/z (Abund. %)				
Vescalagin	466,04 (100)	933,08 (60,93)	466,54 (43,86)	934,08 (31,46)	933,58 (15,95)
Castalagin	466,04 (100)	933,08 (66,3)	466,54 (42,79)	934,08 (33,17)	933,58 (19,03)

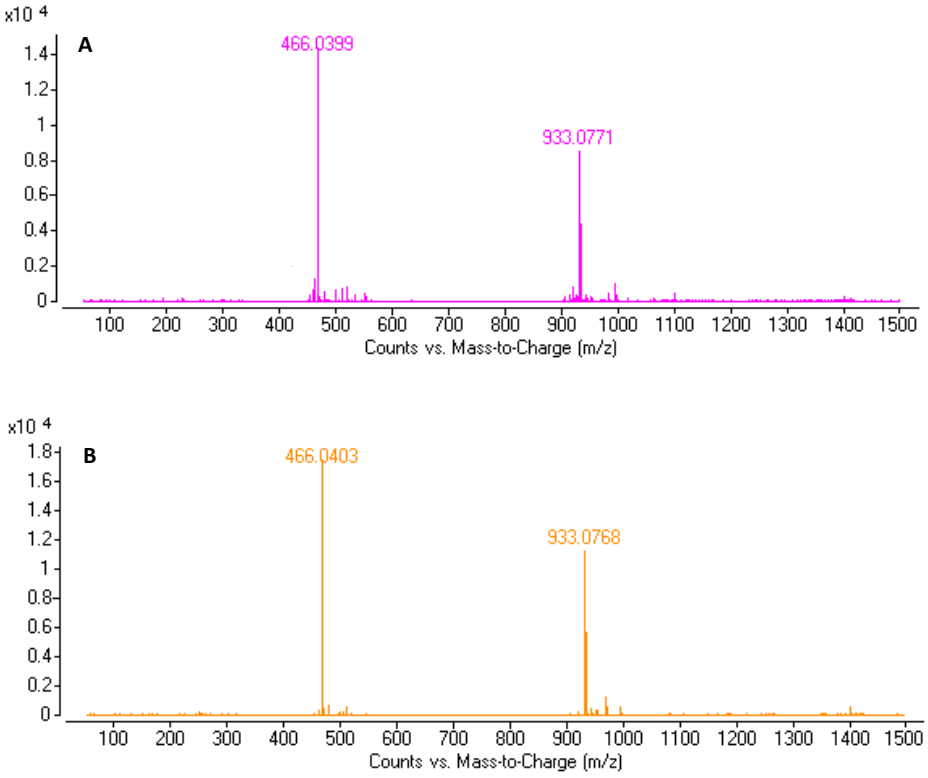

Figure 5.5. Mass spectra of ellagitannins isolated from cork extract. Vescalagin (A) and castalagin (B).

5.4. Conclusions

The extraction of ellagitannins through different solvents has provided a high quantification of these compounds according to the method used. . An extraction with diethyl ether (extract B) allows the high quantification so much of total tannins and antioxidant activity as in ellagitannins, because these compounds is concentrated in aqueous phase. The proposed method for the isolation of ellagitannins provides highly purified vescalagin and castalagin.

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6. *Quercus suber* L. *cork extracts induce apoptosis in human myeloid leukaemia HL-60 cells*

El corcho contiene una gran diversidad de compuestos fenólicos, como ya se ha indicado previamente. Varios autores apoyan que los fenoles provocan efectos pro-apoptóticos en células cancerosas; sin embargo, el estudio de la bioactividad de extractos de corcho para estos fines es escaso.

Con esta finalidad, se ha pretendido describir el potencial antitumoral de extractos de corcho en líneas celulares de leucemia promielocítica humana. Para ello se ha cuantificado la viabilidad de las células cancerosas, la exposición a fosfatidilserina, la actividad caspasa-3, el potencial de membrana mitocondrial y el ciclo celular. Con este estudio se ha pretendido abrir una nueva forma de utilizar el corcho mediante la explotación de sus propiedades anticancerígenas. Además, abre nuevas posibilidades de aplicación de subproductos de corcho.

En este capítulo se ha colaborado con el Departamento de Fisiología Animal de la Universidad de Extremadura, que ha estudiado el potencial antitumoral de los extractos de corcho, llevando a cabo los trabajos de aislamiento y purificación de extractos de compuestos fenólicos, descritos en el apartado 6.2.3 a 6.2.6 del trabajo que se recoge a continuación, el cual ha sido presentado como póster en *JOINT FEPS & SPANISH PHYSIOLOGICAL SOCIETY SCIENTIFIC CONGRESS*, en Santiago de Compostela del 8 al 11 de septiembre de 2012. También se ha publicado en la revista *Phytotherapy Research*, 29, 1180-1187 (Incluido en el Anexo).

Quercus suber L. cork extracts induce apoptosis in human myeloid leukaemia HL-60 cells

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Abstract

Quercus suber L. cork contains a diversity of phenolic compounds, mostly low molecular weight phenols. A rising number of reports support with convergent findings that polyphenols evoke pro-apoptotic events in cancerous cells. However, the literature related to the anti-cancer bioactivity of Q. suber L. cork extractives (QSE) is still limited. Herein, we aim to describe the antitumor potential displayed by cork extractives obtained by different extraction methods in the human promyelocytic leukaemia cells. In order to quantify the effects of QSE on cancer cells viability, phosphatidylserine exposure, caspase-3 activity, mitochondrial membrane potential and cell cycle were evaluated. The results indicated that the QSE present a time-dependent and dose-dependent cytotoxicity in the human promyelocytic leukaemia cells. Such a noxious effect leads these leukaemia cells to their death through apoptotic processes by altering the mitochondrial outer membrane potential, activating caspase-3 and externalizing phosphatidylserine. However, cells cycle progression was not affected by the treatments. This study contributes to open a new way to use this natural resource by exploiting its anti-cancer properties. Moreover, it opens new possibilities of application of cork by-products, being more efficient in the sector of cork-based agriculture.

Keywords: polyphenols, cork extracts, apoptosis, cancer cells

6.1 Introduction

It is broadly known that plants cannot rely on motion as animals do; however, they contain a wide variety of molecules that confer them a chemical compensation. Secondary metabolites play heterogeneous roles such as defence against predators, pigmentation, reproduction, growth and many other functions are given mainly by plant phenolic compounds, the most widespread family of molecules in plants. Phenolic compounds can be found in plants at their leaves, bark, roots, root exudates, flowers, fruits and so on (Giovannini and Masella, 2012). *Quercus suber* L., widely known as cork oak is natively spread across southwest Europe and northwest Africa. Spain is the second cork producer in the world, where Extremadura's production corresponds to the 50% of the Spanish cork; therefore, this sector reaches a huge meaning for the aforesaid region. Cork, the bark of *Q. suber*, has been used by different cultures along the history because of its peculiar characteristics such as elasticity, low permeability or heat-resistance giving it a large variety of applications. Production of wine stoppers is the main application of this material, apart from having the highest economical repercussion. Moreover, *Q. suber* cork contains a diversity of phenolic compounds, mostly low molecular weight phenols and tannins such as ellagitannins, which has polyphenolic composition. More than a few ellagitannins has been found in *Quercus sp*., for instance, roburin A and E, grandinin, vescalagin and castalagin, moreover others with related structures (Mayer *et al*., 1967; Mayer *et al*., 1971; Nonaka *et al*., 1990; Dos Remedios *et al*., 2003; Giovannini and Masella, 2012). Lipophilic and phenolic compounds are not chemically linked, and it is simply extracting them by polar and non-polar solvents (Fortes *et al*., 2004; Silva *et al*., 2005). Therefore, it was expected that raw extractives of *Q. suber* L. cork extractives (QSE) conserve a considerable amount of compounds contained in unprocessed bark (Santos *et a*l., 2010). The main composition QSE are based on phenolic, aliphatic and triterpenic components, both in cork and cork by-products throughout industrial processing (Sousa *et al*., 2006). In line with this, a great antioxidant potential of QSE, in the range of ascorbic acid, has been reported proposing nutraceutical applications (Santos *et al*., 2010). The nutraceutical perspectives of cork consist in its low molecular weight compounds. Phenolic components of cork, as ellagitannins, have been described to induce apoptosis in tumour cells (Fernandes *et al*., 2009; Quideau, 2009; Giovannini and Masella, 2012). In addition, other molecules

contained in cork have been described to have beneficial effects in health; likewise, castalagin and vescalagin have been shown to possesses a substantial inhibitory properties in colon cancer (Fridrich *et al*., 2008). Several phenolic compounds present in cork have been tested displaying an inhibitory effect in a dose-dependent manner (Fernandes *et al*., 2009). Previous works showed cytotoxic effects of casuarin and castalagin in promyelocytic leukaemia cells (Yang, 2000). More than few reports support with convergent findings that polyphenols evoke pro-apoptotic events in cancerous cells, such as activation of caspase-3 by ellagic acid and quercitin (Mertens-Talcott and Percival, 2005), promotion of reactive oxygen species (ROS) generation and elicitation of intrinsic by woodfordin I (Liu *et al*., 2004), among others (Giovannini and Masella, 2012).

Apoptosis is a process accurately controlled by a cell death signalling to keep the homeostasis of adult tissues. There are two canonical pathways by which apoptosis signal is transmitted, outer pathways or receptor dependent and inner pathway or mitochondrial dependent. During inner apoptotic pathways, mitochondria suffer a loss of their membrane potential, permeabilizing their membrane and allowing the free ion flow and releasing pro-apoptotic factors. Uncontrolled apoptosis plays an essential role in different pathologies, both by overactivation such as neurodegenerative diseases and by under-activation such as cancer. As mentioned earlier, a growing number of studies report the anti-cancer bioactivity of polyphenols in different cancer cell models (Giovannini and Masella, 2012; Cerella *et al*., 2013). Scarce are the studies about the anti-cancer bioactivity of QSE. Herein, we describe the anti-tumor potential displayed by cork extractives obtained by different methods in a leukaemia cell line. This study will contribute in the near future to design nutraceutics with antitumor and antioxidant properties from raw extractives of *Q. suber* L. cork.

6.2. Material and methods

6.2.1. Materials

Human promyelocytic leukaemia (HL-60) 15-12 cell lines (ECACC N° 88120805) were purchased from The European Collection of Cell Cultures (ECACC) (Dorset, UK). RPMI 1640

medium, fetal bovine serum, penicillin/streptomycin and N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin were obtained from Lonza (Porriño, Spain). Tetramethylrhodamine methyl ester (TMRM) was obtained from Molecular Probes (Eugene, Oregon, USA). Annexin binding buffer, propidium iodide (PI) and annexin V-fluorescein isotiocyanate (FITC) were obtained from Immunostep (Salamanca, Spain). All other reagents were of analytical grade.

6.2.2. Cell culture

Human promyelocytic leukaemia cells, a human promyelocytic leukaemia cell line, were cultured in RPMI 1640 complete medium (Lonza) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100U/mL penicillin and 100 µg/mL streptomycin. Cells were grown in a humidified air/CO_2 (5%) atmosphere at 37 °C and maintained in an exponential growth phase for all experiments. Cells were routinely plated at a density of 3 × 10^5 cells/mL into fresh flasks and the viability was >95% in all experiments as assayed by the trypan-blue exclusion method.

6.2.3. Cork samples

Reproduction cork planks (‘refuge’ grade) were provided by several local companies of San Vicente de Alcántara (Badajoz, Spain). Cork samples were reclassified according to industrial criteria. An average sample composed by fragments of several planks from different industries was made.

6.2.4. Phenolic extraction

Cork samples, free of outer bark, were ground and sieved (0.25–0.50 mm particle size). About 20 g of the cork powder sample was submitted to a soxhlet extraction with dichloromethane for 6 h to remove the lipophilic components. Then, the solid cork residue was divided into two fractions (I and II), which followed distinct extraction pathways. Extracts A, B and C were obtained according to the procedure of Santos *et al*. (Santos *et al*., 2010).

On the other hand, other reproduction cork planks ('refuge' grade), from the same local companies, were ground and sieved (0.5–1.0 mm particle size), free of outer bark too. About 20 g of the cork powder sample was extracted with 200 mL of aqueous acetone (Me_2CO: water, 7:3) in the dark and under nitrogen at room temperature with magnetic stirring for 24 h. The extracts were filtered, evaporated under reduced pressure (to remove Me_2CO) and then freeze dried, according to the procedure of Zhentian *et al*. (1999), yielding extract F.

6.2.5. Total phenolic content

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method (Singleton and Rossi, 1965). The 10 mg extracts of A, B, C and F were dissolved in 10 mL aqueous methanol (MeOH: water, 50:50). Folin–Ciocalteau reagent containing 0.5 mL and 10 mL of aqueous sodium carbonate (75 g/L) were added to an aliquot of 0.5 mL of each extract, to a final volume of 25 mL. Each mixture was kept for 60 min at room temperature in the dark. After homogenizing, the absorbance was measured at 670 nm, using a UV/Vis Varian Cary-50 spectrophotometer (Palo Alto, CA, USA). The total phenolic content was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (0.5–16.0 µg/mL) and expressed as microgram of gallic acid equivalent per gramme of dry extract. The analyses were carried out in triplicate and the average value was calculated in each case.

6.2.6. Antioxidant capacity

Total antioxidant capacity was evaluated by means of a colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions. This assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2-azino-bis (3-ethylbenzthiazoline sulfonate) (ABTS) to ABTS by metmyoglobin. The capacity of the antioxidants in the sample to prevent ABTS oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and quantified as millimolar Trolox equivalents.

6.2.7. Toxicity assay

Propidium iodide is fluorescence molecule, which passes across damaged plasma membranes and intercalates double-stranded DNA. PI was used as a dye to amplify the fluorescence of non- vital cells. After treatments with QSE and vehicle control (1% dimethyl sulfoxide (DMSO)), cells were stained with PI, and the later fluorescence monitoring allowed the evaluation of toxicity of the treatments regarding the time and dose. Each sample was tested 5–8 times in independent experiments.

6.2.8. Annexin-V/propidium iodide apoptosis assay

After treatments with QSE and vehicle control (1% DMSO), cells were washed twice with phosphate buffered saline (PBS) and centrifuged at 500 g for 5min; then the supernatant was discarded, and the pellet was resuspended in 95µL annexin V-binding buffer containing annexin V-FITC at a density of 106 cells/mL. Cells were analyzed by flow cytometry (Cytomycs FC-500; Beckman-Coulter, Hialeah, FL, USA) after addition of PI. Each sample was tested 3–5 times in independent experiments. Annexin V binds to those cells that express phosphatidylserine (PS) on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows for live cells (unstained with either fluorochrome) to be discriminated from early apoptotic cells (stained only with annexin V), late apoptotic or necrotic cells (stained with both annexin and PI) (Bejarano *et al*., 2011).

6.2.9. Measurement of caspase-3-like activity

The measurement of DEVD-AMC (Asp-Glu-Val-Asp 7-amido-4-methylcoumarin) (Sigma, Madrid, Spain) cleavage was performed using a modified version of a fluorometric assay. Treated cells with QSE and 1% DMSO (control) were pelleted and washed once with PBS. After centrifugation, cells were resuspended in PBS at a concentration of 2×10^6 cells/100µL; 25µL of the suspension were added to a microtiter plate and mixed with the appropriate peptide substrate dissolved in a standard reaction buffer (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 % sucrose, 5 mM dithiothreitol (DTT), 0.001% NP-40 (Nonidet P-40) and 0.1 % 3-[(3- Cholamidopropyl)dimethylammonio]-1-

propanesulfonate hydrate (CHAPS), pH7.25). Cleavage of the fluorogenic peptide substrate was monitored by aminomethyl coumarin (AMC) liberation in an automatic microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) using 355nm excitation and 460nm emission wave-lengths.

6.2.10. Mitochondrial membrane potential

The quantification of mitochondrial membrane depolarization was carried out by measuring the fluorescence intensity of the membrane-permeant cationic probe TMRM. The resulting signal of TMRM-stained cells thus represents only the probe accumulated in intact mitochondria. A collapse on mitochondrial membrane is indicated by a decrease in output fluorescence. Briefly, treated cells with QSE and 1% DMSO (control) (1×10^6 cells/mL) were washed once with PBS and loaded with 1 µM TMRM by incubation at 37 °C for 30 min. The fluorescence intensity of TMRM was measured in an automatic microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). Excitation wavelength was set at 443 nm and emission wavelength at 575 nm. Treatments were carried out in triplicate. The data are presented as fold increase over the pretreatment level (experimental/control).

6.2.11. Cell cycle

After treatment, treated cells with QSE and 1% DMSO (control) (approximately 1×10^6) were washed with PBS and fixed in 70 % ethanol for 30 min at 4 °C. The cells were again rinsed with PBS and resuspended in 500 µL of PBS containing 2.5 mg/mL PI and 50 mg/mL RNase. The sample were kept in the dark at 4 °C for 30 min and analyzed by flow cytometry with excitation at 488 nm and emission at measured at 560–640nm (FL2 mode) (Cytomycs FC-500; Beckman-Coulter). Cells undergoing apoptosis stain with PI and exhibit a reduced DNA content with a peak in the hypodiploid region (Bejarano *et al*., 2009). The percentage of every phase was represented, and the percentage of apoptosis was taken as the fraction containing hypodiploid DNA.

6.3. Results

After extraction and determination of total antioxidant capacity and phenolic content of the different QSE (Table 6.1), we firstly analyzed the toxicity of every QSE on HL-60 leukaemia cell line. It is noteworthy to mention that each QSE has been extracted by different methods. Therefore, it could be expected that different concentrations among the different kinds of phenolic molecules are present on QSE. The treatments were applied keeping the phenolic constant the phenolic content. Toxicity was studied through the loss of plasma membrane integrity and the consequent PI permeability. Despite not all the extractives have the same toxicity regarding to their phenolic content, PI fluorescence manifests a clear dose-dependence in all types assayed getting their maximal effect at the maximal concentration treated 30 µg phenol/mL during 24 h (Fig. 6.1A). Likewise, the evaluation of kinetic signature of toxicity exhibits a rising toxic behaviour along the analyzed times. Coherently, both curves of dependence indicate that the QSE type A possesses the most toxic effect on HL-60 cells at 30 µg phenol/mL (Fig. 6.1B). Unlike other types, the shortest time and the lowest phenolic concentration were enough for type A to carry out a significant increase of toxicity values (Fig. 6.1).

Table 6.1. Phenolic content and antioxidant capacity of cork extractives. The total phenolic content was calculated as gallic acid equivalent from the calibration curve of gallic acid and expressed as g gallic acid equivalent. The capacity of the antioxidants in the sample to prevent 2,2-azino-bis (3-ethylbenzthiazoline sulfonate) oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and quantified as millimolar Trolox equivalents.

Type of Extract	g GAE / g extract	µmol Trolox/mg extract
A	0.227 ± 0.010	6.43 ± 0.29
B	0.303 ± 0.015	7.25 ± 0.36
C	0.296 ± 0.006	6.55 ± 0.14
F	0.316 ± 0.013	8.42 ± 0.34

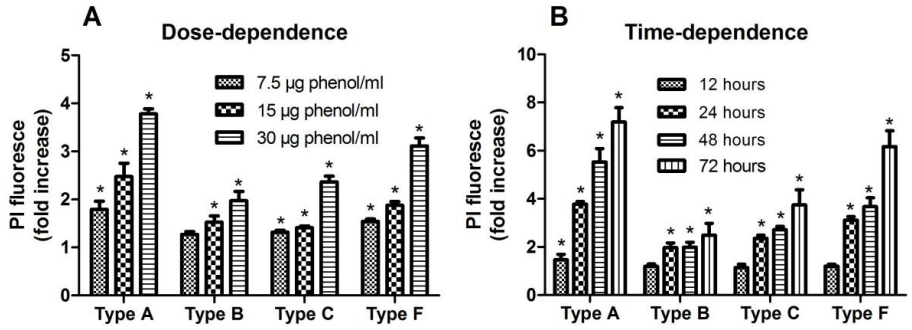

Figure 6.1. Cork extracts induce a dose-dependent and time-dependent decrease on viability of leukaemia cells. Human promyelocytic leukaemia cells were incubated for 24 h with increasing concentrations of phenols contained in cork extracts (A) or with cork extracts at a final concentration of 30 µg phenols/mL for several periods of time (B). Vehicle (1% dimethyl sulfoxide) was used as control. Viability was analyzed as described in the Material and Methods section. Values are presented as means ± SD of 5–8 separate experiments, which were carried out in triplicate and expressed as fold increase (experimental/control). *P<0.05, compared with control values. PI, propidium iodide.

To determine the apoptogenic capacity of the QSE in HL-60, we analyzed the fluorescence of annexin VFITC after treating cells with 30 µg phenol/mL for 24 h. The FITC fluorescence values evidenced an increase of the PS exposure at the outer leaflet of the plasma membrane after treatments. Such an apoptotic event indicates that the toxicity shown by QSE induces cell death mainly by apoptosis (Fig. 6.2).Moreover, no FITC negative cells were found during the analysis, fact which supports the absence of other types of cells death (Fig. 6.2).

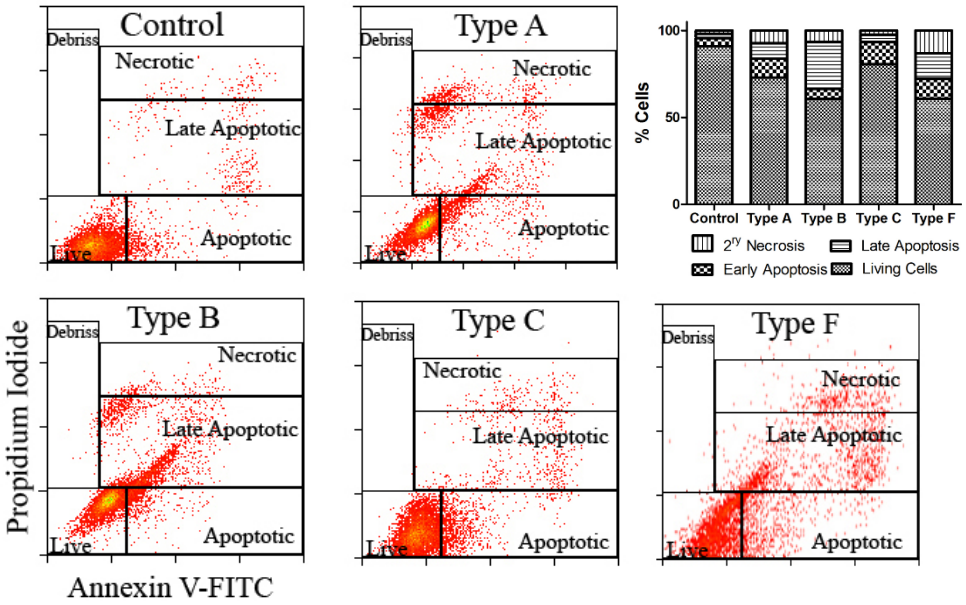

Figure 6.2. Cork extracts induce apoptosis in human promyelocytic leukaemia cells (HL-60). HL-60 were treated for 24 h with Quercus suber L. cork extractives containing 30 µg phenols/mL. Vehicle (1% dimethyl sulfoxide) was used as control. Apoptosis was analyzed as described in the Material and Methods section. Cytometer images shown were selected as the most representative. The results are represented as the distribution of the apoptosis phase's percentage, and they are representative of 3–5 independent experiments.

To make firm the results that indicate apoptogenic properties of the QSE on leukaemic cells, caspase-3 activity was analyzed. The signal of specific fluorogenic substrate revealed that the four QSE promote the caspase-3 activation significantly at 30 µg phenol/mL for 24 h (Fig. 6.3). These outcomes agree with PS exposure reinforcing that QSE trigger apoptotic cell death in HL-60 cells.

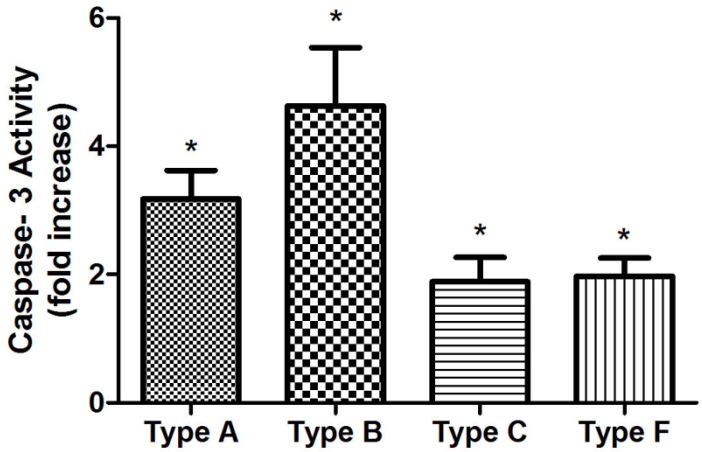

Figure 6.3. Cork extracts induce activation of caspase-3.Human promyelocytic leukaemia cells were treated for 24 h with Quercus suber L. cork extractives containing 30 µg phenols/mL. Vehicle (1%dimethyl sulfoxide) was used as control. Bars represent the means ± SD of 6–8 separate experiments and expressed as fold increase (experimental/control). *P<0.05, compared with control values.

In order to study the mitochondrial alterations under the stated QSE, TMRM assay was carried out. Interestingly, a high percentage of HL-60 cells suffered a loss of mitochondrial potential when were treated individually with the four QSE containing 30 µg phenol/mL for 24 h (Fig. 6.4). This indicates a mitochondrial damage, which could be involved in the apoptotic signal caspase-3 activation upstream.

Last but not the least, cell cycle was analyzed. The profile of DNA content shows that phases containing checkpoints, such as S, G1 and G2/M, did not increased their percentage. Therefore, cell cycle did not stop its progress because of the treatments. On the contrary, hypodiploid phase outcomes agree with previous values; extracts evoked an important increment of sub-G1 phase at the expense of the remainder phases, where accordingly, the type A also manifested the strongest effect on DNA fragmentation.

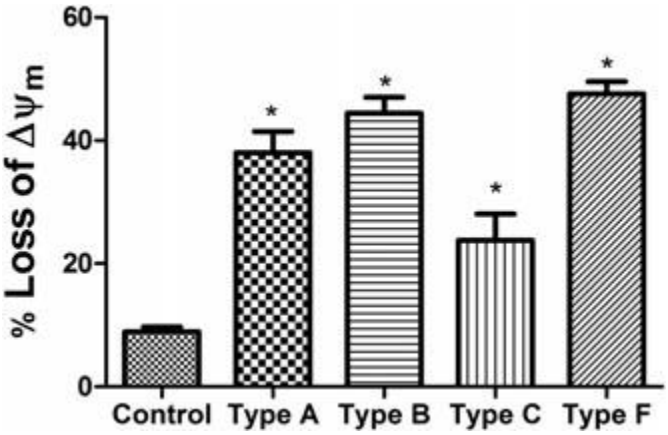

Figure 6.4. Cork extracts reduce the mitochondrialmembrane potential in leukemia human promyelocytic leukaemia cells (HL-60). HL-60 metramethylrhodamine methyl ester-loaded cells were treated for 24 h with Quercus suber L. cork extractives containing 30 μg phenols/mL. Vehicle (1% dimethyl sulfoxide) was used as control. Values are presented as percentage ± SD of 6–8 separate experiments and expressed as fold increase (experimental/control). *P<0.05, compared with control values.

6.4. Discussion

Plant polyphenols are being considered among the most promising anticancer chemopreventives (Giovannini and Masella, 2012). Actually, the risk of cancer has been shown to be relieved upon high dietary polyphenol intakes (Knekt *et al.*, 2002). Radical scavenging properties of polyphenols avoid lipid peroxidation and DNA damages, events which are closely linked with carcinogenesis. Malignant cells have accumulated mutations because of DNA damages becoming much more resistant to apoptosis than healthy cells. A growing number of works report that plant polyphenols reset apoptotic pathways in tumor cells (Orlikova and Diederich, 2012). The mechanisms through which these compounds act restarting apoptosis on tumor cells are heterogeneous. Certain phenolic compounds activate cell death acting as agonist of cell death receptors, such as luteolin (Horinaka *et al.*, 2005). Others perform their apoptotic action by triggering the intrinsic pathway, such as theaflavins and thearubigins, the major polyphenols of black tea, which has been reported to alter Bcl-2/Bax ratio, up-regulated by p53 and triggering mitochondrial apoptosis (Halder *et al.*, 2008). However, some phenols such as epigallocatechin-3-gallate, curcumin,

quercetin and resveratrol have been shown triggering simultaneously intrinsic and extrinsic pathways, where Bid links both apoptotic pathways (Orlikova *et al.,* 2012). In this study, we show the molecular events involved in the apoptotic effects induced by raw extracts obtained from *Q. suber* L. cork on human promyelocytic leukemia HL-60 cells. PI staining evidenced a dose-dependent and time-dependent mortality of HL-60 cells treated with the QSE, which was mediated by apoptotic cell death as phosphatidylserine externalization evidenced. Predictable outcomes taking into account that phenolic acids are outstandingly present in cork (reported by Conde *et al*., 1997; Cadahía *et al*., 1998; Conde *et al*., 1998; Sousa *et al*., 2006). In particular, in our current system HL-60 cells, cork phenolic molecules separately have been presented as potential therapeutic agents for the treatment of leukaemia or with preventive properties. Such is the case that, on HL-60 cells, cork phenolic molecules such as ellagic acid has been described as a potent inducer of apoptosis (Hagiwara *et al*., 2010); gallic acid was shown inducing apoptosis taking it place through both physiological and cell damage pathways (Yeh *et al*., 2011); results with caffeic acid suggest an apoptosis associated with mitochondrial dysfunction (Chen *et al*., 2001); HL-60 cells underwent apoptosis under protocatechuic acid treatment by unbalancing the Bcl-2/Bax ratio (Tseng *et al*., 2000); also, castalagin has been reported to exert cytotoxic effects by inducing DNA fragmentation and apoptotic cell death. Despite a rising number of studies showing anticancer properties of plant extracts, there is a great scarcity of literature describing the physiological events triggered by QSE on cancer cells. Accordingly to similar researches carried out with polyphenol-rich extracts (Benarba *et al.,* 2012; Li *et al*., 2013), QSE fostered caspase-3 activation, a key protease in most of apoptotic processes whose activation represents the point of no return in apoptosis signalling.

Preservation of mitochondrial potential is essential to keep at bay the release of mitochondrial pro-apoptotic factors. QSE-treated cells suffered disruption of mitochondrial functions as results revealed. This fact is in agreement with previous findings performed on cancer cells treated with both polyphenol-rich extracts (Benarba *et al*., 2012; Li *et al*., 2013) and cork phenolic molecules separately (Tseng *et al*., 2000; Chen *et al*., 2001; Alfredsson *et al*., 2014). Therefore, QSE could collaborate with the mitochondrial apoptotic pathway by altering the permeability of the mitochondria and releasing the apoptosis transducers. As evidenced by results, each QSE shows its particular behaviour for each assay performed. Such a fact could be expected given that each QSE likely contains different relative

composition among phenolic compounds. The relative concentration of phenolic compounds of QSE is not only determined by the extraction method but also it depends on the phenolic composition of the raw material. The location where cork is generated will affect the phenolic content of cork. The soil type, the weather conditions, the action phytofagous and so on are closely bound to the final composition of raw materials. Keeping in mind in Table 9.1 that neither the starting richness of phenolic compounds in QSE nor their antioxidant capacity does not show a correlation to their proapototic properties. Given that the phenolic composition of cork is not fixed, this entails that reproducing exactly the same results is unlikely. Nonetheless the main message of this study is that QSE keep proapoptotic effect on HL-60 cells to a greater or lesser extent.

On the other hand, some of the main compounds present in QSE have been reported to induce alterations in cell cycle kinetics (Agarwal *et al*., 2006; Kuriyama *et al*., 2013; Zhang, 2014). Surprisingly, in our study, cells did not suffer a block in the cell cycle, given that the ratio among phases did not undergo any change. In this regard, the literature about the effects of isolated QSE polyphenols on cycle of tumor cells remains still limited. The last but not least, the DNA quantitative analysis exhibited additional information. Figure 6.5 shows a substantial increase in the sub-G1 phase after a treatment with QSE at the expens of the rest of phases, what is indicative of DNA fragmentation, and is also coherent with the presence of apoptotic events described earlier. Dividing cells are able to interrupt the cycle progression in phase G1, S or G2 checkpoints to repair DNA damages before continuing. Depending on the gravity of the damage, the cell decides whether repairing or initiating intracellular apoptosis signaling in response to damage will remove potentially hazardous cells (Hanahan and Weinberg, 2000). Therefore, we hypothesize that the leukaemia cells sensitive to the treatment are directly prompted to apoptosis, as revealed in DNA content analysis (Fig. 9.5).

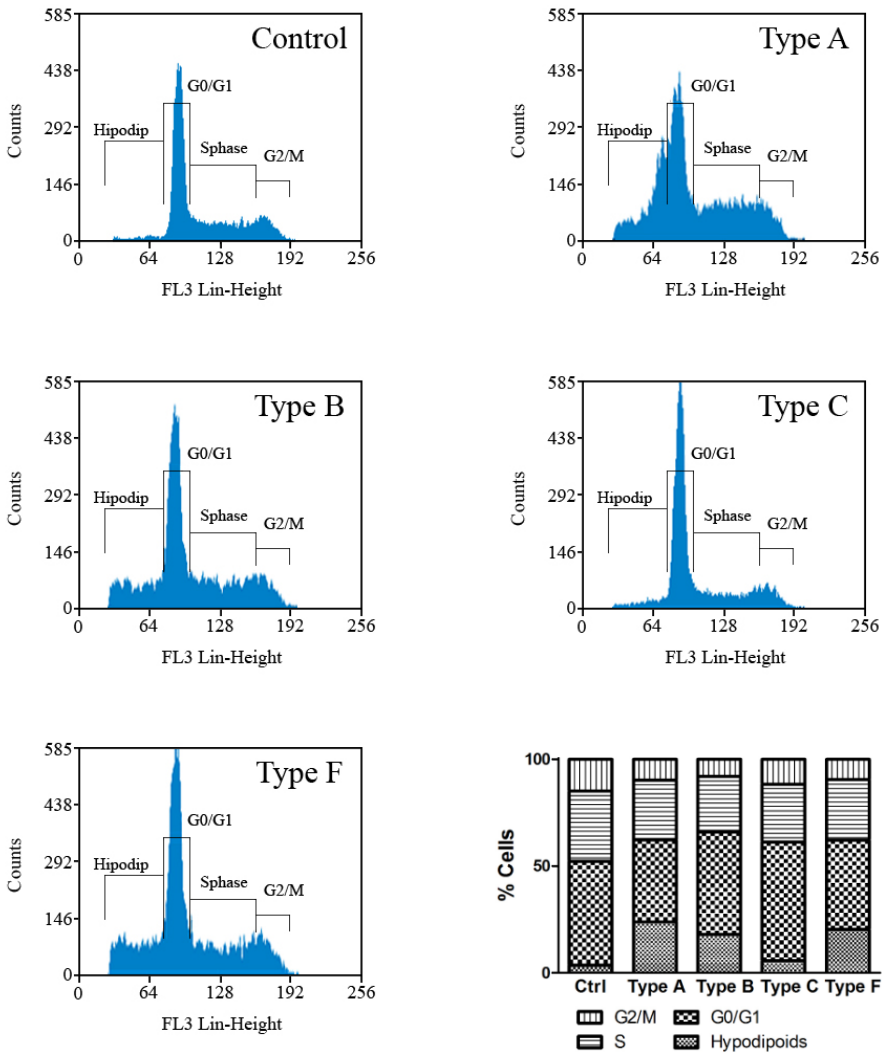

Figure 6.5. Cork extracts did not affect cell cycle progression. Human promyelocytic leukaemia cells were treated for 24 h with Quercus suber L. cork extractives containing 30 µg phenols/mL. Vehicle (1% dimethyl sulfoxide) was used as control. Cytometer images shown were selected as the most representative. The results are represented as the distribution of the cell-cycle phase's percentage and are representative of 6–8 independent experiments.

In summary, cork is a renewable resource, which has reached an outstanding social and economic meaning in Spain, particularly in Extremadura. More than 75 % of cork produced

all around the world is used to made cork stoppers for wine, generating a lot of by-products, which are mainly used as biofuel or compost. This study explores a door to new possibilities of application of cork by-products, being more efficient the sector of cork-based agriculture. The present study reports the QSE-induced apoptosis in HL-60 cells altering the mitochondrial outer membrane potential, activating of caspase-3 and externalizing phosphatidylserine. Nonetheless, additional studies regarding anti-tumor properties are needed, by analyzing isolated QSE compounds to be checked in a wide number of tumor cell lines. Anti-cancer and anti-tumor properties of cork extractives open a new way to exploit this natural resource.

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7. Voltammetric methods for analysis of total phenolic compounds content in cork waste water

Las propiedades anticancerígenas y antioxidantes de compuestos bioactivos, fenoles y polifenoles, es bien conocida, por lo tanto la propuesta de métodos para la determinación de estos compuestos en diferentes matrices es de interés.

El corcho, durante el proceso de fabricación del tapón para la industria vitivinícola, es sometido a un proceso de hervido, en agua a 100°C durante una hora. En esas aguas de cocido se encuentra fundamentalmente compuestos hidrofilicos extraídos del corcho, entre los que están, entre otros, los compuestos fenólicos.

El propósito que se aborda en este capítulo es desarrollar métodos electroanaliticos para la determinación del contenido total de fenoles en esas aguas de cocido, utilizando un electrodo de carbón vitrificado con y sin proceso de acumulación mediante DPV y AdSDPV. Se han optimizado las variables químicas e instrumentales y se han establecido las curvas de calibrado

El método ha sido aplicado a la determinación de fenoles totales de bajo potencial de oxidación en diferentes aguas de cocido de corcho. Los resultados obtenidos por los metidos electroanaliticos propuestos se comparan con los obtenidos mediante un método espectrofotométrico ampliamente utilizado, encontrándose una buena correlación entre ambos. Los métodos propuestos son rápidos y proporciona un valor más real que el espectrofotométrico que es menos selectivo.

Este trabajo está pendiente de remisión para su publicación y fue presentado como póster en el *19th Meeting of the Portuguese Electrochemical Society and XVI Iberian Meeting of Electrochemistry*, en Aveiro (Portugal) entre el 30 de junio y el 2 de julio de 2014.

Voltammetric methods for analysis of total phenolic compounds content in cork waste water

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Abstract

Determination of bioactive compound content, such as phenol, polyphenols or phenolic compounds, is of great interest because of their well-known antioxidant and anticancer properties. Due to physicochemical treatments in cork manufacturing for disinfection purposes essentially based on boiling of raw material, abundant cork wastewater, characterised by high content of mentioned compounds, is generated. The aim of this work was the development of electroanalytical methods for the determination of the total content of low oxidation potential phenolic compounds (LOPPCs) in cork wastewater samples using glassy carbon electrode. Voltammetric methods with and without accumulation steps were explored. Chemical and electrochemical parameters were studied and optimised. Calibration curves were established and Winefordner-Long and Clayton limits of detection, repeatability and reproducibility were calculated. The proposed electrochemical method was compared to Folin-Ciocalteau spectrophotometric method (non-selective in the reaction with any reducing species present in solution) reporting a good correlation between them. Subsequently, the electroanalytical method was successfully applied to the simple and fast determination of low oxidation potential phenolic compounds in real cork wastewater samples providing more realistic estimation of LOPPCs values.

Keywords: voltammetry, low oxidation potential, phenolic content, cork wastewater

7.1. Introduction

Cork oak (*Quercus suber L.*) is an evergreen tree native to the western and central Mediterranean region. The most important product deriving from the tree is cork, a vegetal tissue made of dead cells that protect oak's trunk and branches which is periodically extracted for many different commercial uses, mainly the production of wine stoppers (Demetzi et al., 2016). The largest cork production in the world is concentrated in the Mediterranean basin: Portugal, Spain, Algeria, Morocco, Tunisia, Italy and France (del Pozo et al., 2000). First step in cork manufacturing is the immersion of cork barks in boiling water for cleaning and softening the raw material (Ávila et al., 1998) and even eliminate soluble substances in water. Such cork boiling wastewater produced is characterised by high content of phenolic compounds (Bernardo et al., 2001; Minhalma and de Pinho, 2001; Guiberteau-Cabanillas et al., 2015). It is very well-known anticancer and antioxidant properties of those phenolic compounds (Gali-Muhtasib et al, 1999; Santos et al., 2013). On the other hand, pharmacological potentiality of cork lies in its low molecular weight components (Fernandez et al, 2009). For these reasons, the isolation and identification of different bioactive phenolic compounds from cork oak results in a very interesting field of research.

Traditionally, the term "total polyphenols" refers to the total phenolic compound content determined by spectrophotometric methods using Folin-Ciocalteau reagent (Singleton and Rossi, 1965). However, the disadvantage of this procedure is an overestimation of the "total polyphenolic" content. For example, sulphur dioxide acts as interferent in the analysis of polyphenols in wine samples, resulting in a synergetic effects (Blasco et al., 2005). Thus, most of phenolic compounds are active electrochemically and the estimation of "total polyphenolic" content applying electrochemical methods are preferable because of their high sensitivity and selectivity (avoiding interferences). These methods are also characterised by their simplicity, portability and low cost instrumentation, and good stability (Magarelli et al., 2013). Blasco et al (2005) proposed also an electrochemical protocol as analytical alternative to spectrophotometric method to obtain a new "total antioxidant/phenolics" index from electrochemistry. It is described in the literature the determination of total phenolic compounds using electroanalytical

techniques, such as cyclic voltammetry (CV) or square wave anodic stripping voltammetry (SWASV) using different working electrodes such as glassy carbon electrode (GCE) for the analysis of these compounds in wine (Blasco et al., 2005; Kilmartin et al., 2001; Kilmartin et al., 2002) and tea (Piljac-Žegarac et al., 2011); carbon nanotubes-modified glassy carbon electrodes (GC/CNT/PEI) (Sánchez et al., 2013) or enzymatic biosensors for the determination of polyphenols in wine (Carralero et al., 2005). In this way Guiberteau-Cabanillas et al (2015) have described the voltammetric determination of phenolic compounds in cork boiling water using graphene-modified screen-printed electrodes. However, due to the high cost of the implementation of screen-printed technology for the monitorisation of phenolic compounds in complex matrices such as cork boiling waters, voltammetric methods using common glassy carbon electrode (GCE) for the assessment of the total low potential polyphenol compounds (LOPPCs) in wastewater from cork manufacturing have been carried out in the present work. Ellagic acid has been selected as representative of LOPPCs because of the high content of this compound in this kind of waste water. The optimisation of chemical and instrumental variables was performed and standard spectrophotometric method was simultaneously carried out for comparative purposes. It is interesting to note that the advantage of using GC electrode rather than screen printed electrodes (SPEs) is due to the lower real cost of the whole analysis. In the particular case of the analysis of real water samples from cork industry, which are characterised by a highly complex composition, the concept of one-shot sensor associated to SPEs it is not useful whatsoever. The electrochemical cleaning of SPEs is not effective in this application what makes necessary the use of a large amount of SPEs increasing the price of the analysis

7.2. Experimental

7.2.1. Apparatus

All voltammetric measurements have been performed using a computer controlled potentiostatAutolab (ECO Chemie, Holland) and Metrohm 663 VA stand (Herisau, Switzerland). The working electrode consist in a glassy carbon electrode (GCE) (3.0 mm disk diameter) model MF-2012 (Bioanalytical Systems, Inc., West Lafayette, Indiana, USA); an

Ag/AgCl saturated reference electrode and a platinum wire auxiliary electrode. The spectrophotometric equipment was a UV-Vis Varian Cary-50 spectrophotometer (Palo Alto, California, USA).

7.2.2. Reagents

All chemicals were of analytical grade and Milli-Q water was used throughout the study. Gallic, protocatechuic, vanillic, syringic, ferulic and ellagic acids, syring, coniferic and sinapic aldehydes, and vanillin were supplied by Sigma-Aldrich (St. Louis, MO, USA) as well as acetic acid (PA grade). Sodium acetate, phosphoric acid, anhydrous monosodium phosphate, anhydrous disodium phosphate, sodium hydroxide, anhydrous sodium carbonate, methanol, ethanol, Folin-Ciocalteau reagent and boric acid were supplied by Panreac (Barcelona, Spain).

Britton- Robinson buffer solutions of different pH values were prepared daily from a stock solution 0.04 M. Other buffers solutions of different pH values were used: phosphoric acid/monosodium phosphate 0.5 M, monosodium phosphate/disodium phosphate 0.5 M, Acetic acid/Sodium acetate 0.5M.

7.2.3. Samples

A standard stock solution of ellagic acid (EA) (200 $\mu g \cdot mL^{-1}$) was prepared in a volumetric flask, by weighing the appropriate amount and dissolving it in methanol. A more diluted solution (25 mL) were prepared by taking an adequate amount of EA stock solution and diluting with Milli-Q water and adjusting pH using pH buffer solutions described above The pH was fixed by using different buffers solution indicated above.

Cork boiling water samples were obtained by immersing different amounts of cork samples in boiling water for about an hour. After that, the cork was immediately separated of the solution by filtration. Then, when the filtrate water reached room temperature, the final volume was determined.

7.2.4. Cleaning of the working electrode

In order to obtain a good reproducibility of the electrochemical measurements, the GCE was cleaned after each measurement, because the compound is adsorbed on the surface electrode. In this way, the GCE was systematically immersed in DMF in an ultrasonic bath for 2 min followed by 40 seconds in Milli-Q water. The ultrasonic bath must be cooled to prevent heating of the electrode.

7.2.5. Voltammetric determination of low oxidation potential phenolic compounds (LOPPCs) content in cork boiling water

The analysis of the total low oxidation potential phenolic compounds content in cork boiling waters, expressed as mg ellagic acid equivalent (EAE) / kg dry cork, was carried out according to the following two different proposed methods:

Differential pulse voltammetry (DPV): In the voltammetric cell containing 25.0 mL of 0.04 mol·L^{-1} $H_3PO_4/H_2PO_4^-$ pH 2.5 buffer solution, appropriate aliquots (25, 50 and 75 µL) of cork boiling water were added. The differential pulse voltammograms were recorded with pulse amplitude of 50 mV and a step potential of 10 mV.

Adsorptive differential pulse stripping voltammetry (AdDPSV): The samples were prepared as indicated above and voltammograms were recorded by AdDPSV (50 mV amplitude pulse and 10 mV), by using 0.00V as accumulation potential, 20 s as accumulation time, and 10 s rest time.

All measurements were performed at room temperature and analyses were carried out by triplicate, thus the average value of Ip measured was calculated in each case.

In both methods (DPV and AdDPSV), GCE was cleaned after each scan following the procedure described in section 7.2.4. In order to verify that neither analyte nor sample were not still absorbed onto GCE after cleaning process, a scan with only the buffer solution was recorded.

7.2.6. Spectrophotometric determination of total phenolic compounds content

The total phenolic content of the cork boiling water was determined by the standard spectrophotometric method (Singleton et al., 1965). Folin-Ciocalteau reagent (0.5 mL) and aqueous sodium carbonate (10 mL, 75g·L^{-1}) were added to a 0.5 mL aliquot of each cork boiling water up to final volume of 25 mL with Milli-Q water. Each mixture was kept in the dark for 60 min at room temperature. After homogenising, the absorbance was measured at 670 nm using a typical UV-Vis spectrophotometer. The total phenolic content was calculated as ellagic acid equivalents from the calibration curve of ellagic acid standard solutions (0.5–16.0 µg·mL^{-1}) and expressed as mg ellagic acid equivalent (EAE)/kg of dry cork. The analyses were carried out by triplicate and the average values of EA were calculated in each case.

7.3. Results and discussion

7.3.1. Electrochemical studies

An initial study on the electrochemical behaviour of the phenolic compound potentially present in cork boiling water and also the analysis of real cork boiling water samples were carried out. The phenolic compounds selected for the present study were: ellagic acid (EA), ferulic acid (FER), gallic acid (GA), protocatechuic acid (PA), vanillin (V), vanillic acid (VA), syringic acid (SA), syringic aldehyde (SIR), sinapaldehyde (SIN) and coniferaldehyde (CON).

Figure 7.1 shows voltammograms of phenolic compounds selected in this work (1 µg·mL^{-1}) together with the voltammograms of the cork boiling water samples (25 and 75 µL) in 0.02 mol·L^{-1} AcOH/NaAcO buffers solution of pH 4. Also, the results obtained from the electrochemical measurements are summarised in Table 7.1. Note that some of the compounds analysed shows two different oxidation peaks. It is also observed that in the real cork boiling water samples two peaks are revealed at +0.315V and +0.461V, respectively, resulting the current (Ip) of the first peak at lower oxidation potentials lower than the other one in front of when the SPE_GPH were used in which the first signal shows a higher intensity peak value than the second one (Guiberteau-Cabanillas et al., 2015).

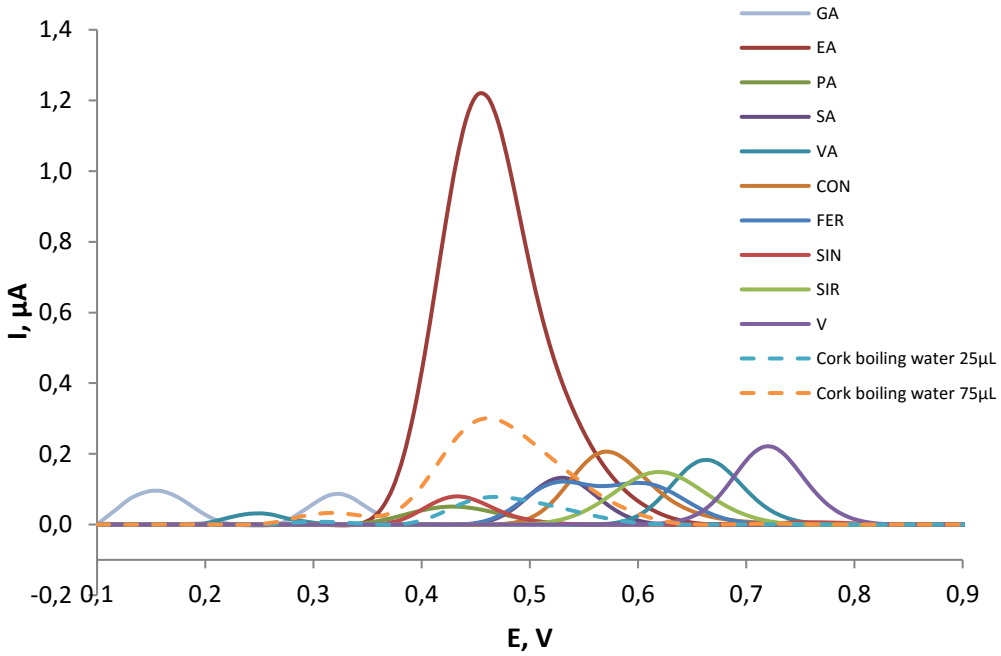

Figure 7.1. Voltammograms of DPV (baseline correction) of phenolic compounds assayed at a concentration of 1 µg·mL^{-1}, and different samples of cork boiling water. Conditions: acetic acid/ sodium acetate buffer solution pH 4.5, Pulse amplitude: 50 mV, Step potential: 10 mV

Table 7.1. Oxidation peaks of phenolic compound (1 mg·L^{-1}). GA: gallic acid; EA: ellagic acid; PA: protocatechuic acid; SA: syringic acid: VA: vanillic acid; CON: coniferaldehyde; FER: ferulic acid; SIN: sinapaldehyde; SIR: syringic aldehyde and V: vanillin. (pH of 4.5; 50 mV of pulse amplitude and 10 mV of step potential).

Compound	C, µmol·L^{-1}	E_{p1} (V)	I_{p1} (nA)	E_{p2} (V)	I_{p2} (nA)
GA	5.88	0.159	95	0.325	87
EA	3.31	0.452	1220	-	-
PA	6.49	0.432	51	-	-
SA	7.24	0.53	132	-	-
VA	5.95	0.247	31	0.667	182
CON	5.61	0.569	206	-	-
FER	5.15	0.53	121	-	-
SIN	4.80	0.432	80	-	-
SIR	5.05	0.618	148	-	-
V	6.57	0.715	219	-	-
Cork boilingwater 25 µL		0.305	8	0.471	78
Cork boilingwater 75 µL		0.315	33	0.461	301

In order to have an estimation of the content of low molecular weight phenolic compounds present in real cork boiling water, several samples were previously analysed by HPLC (figure not shown), which revealed the following results: 12.3 µg·mL^{-1} GA (34.4%), 7.1 µg·mL^{-1} PA (20.0%), 3.2 µg·mL^{-1} VA (9.1%), 2.8 µg·mL^{-1} SA (7.8%), 2.2 µg·mL^{-1} V (6.3%), 1.1 µg·mL^{-1} SIR (3.1%), 0.8 µg·mL^{-1} FER (2.3%), 0.8 µg·mL^{-1} CON (2.3%), 1.0 µg·mL^{-1} SIN (2.7%) and 4.3 µg·mL^{-1} EA (12.0%). It is demonstrated that ellagic, gallic and protocatechuic acids are the major phenolic compounds found in cork boiling water samples from the cork industry, in agreement with the results found in the bibliography (Santos et al., 2013; Minhalma et al., 2001).

Taking into account the high content of ellagic acid found in the cork boiling water analysed by HPLC, it is assumed that the voltammetric peaks exhibited by those samples,

which are close to the ellagic acid and protocatechuic acid oxidation potentials, corresponds to EA and PA, respectively. Therefore, ellagic acid was selected as analyte to express the content of total phenols in boiling cork water as mg of ellagic acid equivalents (EAE)/ kg dry cork, although protocatechuic acid could also be chosen as alternative due to the signal provided by ellagic acid is higher for the same concentration value.

For that, previous studies of voltammetric behaviour (DPV) of ellagic acid have been carried out, as well as cork boiling waters. In preliminary experiments it was observed that the EA accumulates in the GCE as well as the solution of cork boiling water (Figure 7.2). For this reason, the study of the electrochemical behaviour by adsorptive differential pulse stripping voltammetry (AdDPSV) has been also included in order to develop two methods for the analysis of total phenolic compounds in cork boiling water samples.

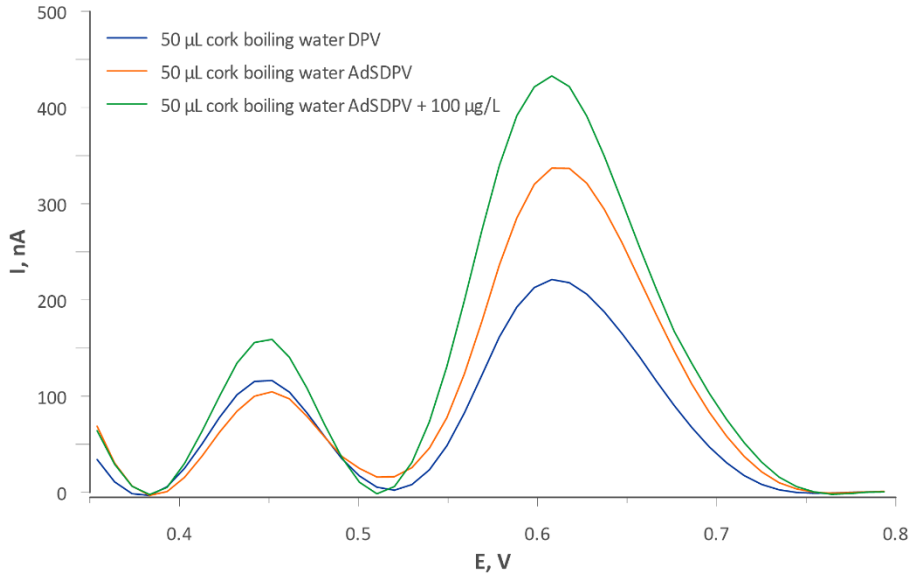

Figure 7.2**.** Voltammograms DPV (baseline correction) of 50µL cork boiling water by DPV (blue line), AdDPSV (orange line) and AdDPSV plus 100 $\mu g \cdot L^{-1}$ of EA (green line). Conditions: pH 4.5, Pulse amplitude: 50 mV, Step potential: 10 mV.

7.3.1.1. Influence of pH

The influence of pH on Ep (peak potential) and Ip (peak intensity) of EA was examined in the range of 2.0–6.0 using Britton-Robinson buffer solution. The obtained

voltammograms are shown in Figure 3A. The results show that the potential peak (Ep) shifts to more positive values linearly with the decreasing of pH [Ep (V)=-0.0662 pH+0.7471; r=0.9982] (data not shown). The peak current (Ip) also varies with the pH, reaching a constant value at the pH range of 2.5–3.5 and decreasing at more acidic pH (Figure 7.3B). For further study, a pH of 2.5 obtained with $H_3PO_4/H_2PO_4^-$ buffers solution was selected. As also illustrated in Figure 7.3B, similar behaviour was obtained applying the AdDPSV method with a significantly higher Ip values obtained compared to DPV.

Cuarteto et al., 2011 studied the influence of pH on Ip for EA , and a peak in the range 3.7-9.0 was found by using SWV about , but at ph> 6,1 a little peak of more higher potential values are show. The maximum peak intensity of the first peak has been obtained at pH= 5.5. In DPV only one peak are shown the Ep change with the pH.

However, Ghoreishi et al., 2011, studied the electrochemical behaviour of EA by DPV using a carbon paste electrode modified with carbon nanotubes. Only one peak was observed at pH 4, with a shoulder which could be seen. The intensity decrease with the pH, value of pH 2.5 was selected because of the higher intensity.

It has been observed that EA presents two partially overlapped peaks, being the first one higher than the second one. In the other hand, Ip remains at pH 2.5-3.5, but it decreases to pH when Ip is zero. However, in this case there are two peaks. In the influence of pH in Ep, similar behavior has been observed that those observed in literature (Cuarteto et al., 2011; Goreshi et al, 2011).

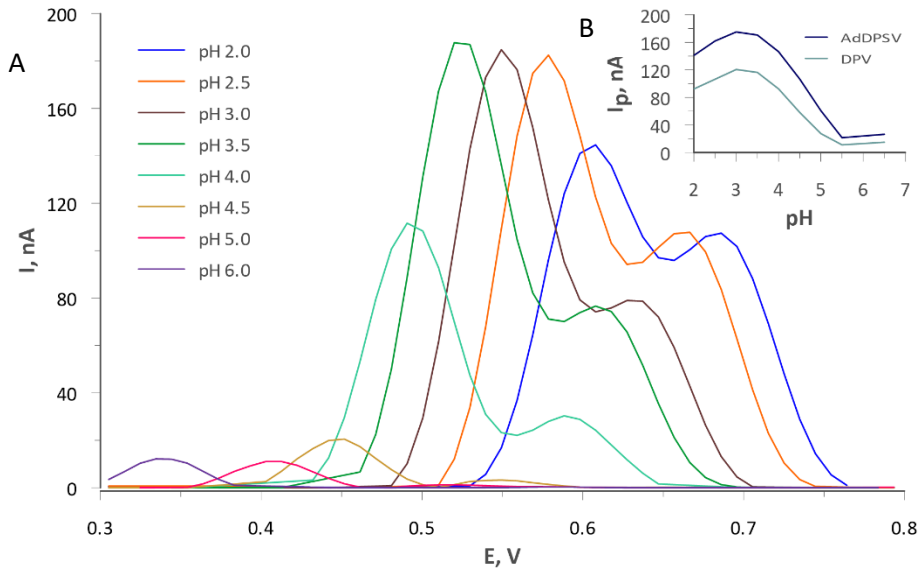

Figure 7.3. A) Differential pulse voltammograms (baseline correction) of 100 µg·L^{-1} EA at different pH values. B) Influence of pH on Ip by DPV (light blue line) and AdSDPV (dark blue line). Conditions: 0.016 mol·L^{-1} Britton-Robinson Buffer, Pulse amplitude 50 mV, step potential 10 mV and additionally for AdSDPV: Deposition potential +0.00 V, deposition time 20 s and equilibrium time 10 s.

7.3.1.2. Influence of instrumental parameters

The influence of instrumental parameters such as pulse amplitude and step potential on the EA signal has been studied by DPV and AdDPV. A solution of 100 µg·L^{-1} EA in 0.04 mol·L^{-1} buffer $H_3PO_4/H_2PO_4^-$ pH 2.5 was prepared. The optimal values selected of pulse amplitude and step potential were 50 mV and 10 mV, respectively.

On the other hand, others instrumental variables such as the influence of accumulation time and accumulation potential in waste water have been optimized, only for AdDPV.

A) Influence of accumulation time on Ip. Two solutions of EA (100 and 400 µg·L^{-1}) in 0.04 mol·L^{-1} $H_3PO_4/H_2PO_4^-$ pH 2.5 buffer solutions were prepared, and an E_{acc} of 0.00 V and a rest time of 10 s was applied. Voltammograms and the variation of Ip with accumulation time (t_{acc}) is shown in Figure 7.4. A linear relationship between the accumulation time and peak intensity up to 60 seconds is shown for the 100 µg·L^{-1} EA solution (Figure 7.4A), and

up to 30 s for the 400 µg·L^{-1} EA solution (Figure 7.4B). For further studies, an accumulation time of 20 s was selected.

B) The influence of the accumulation potential, using a solution of a 100 µg·L^{-1} EA in 0.04 mol·L^{-1} $H_3PO_4/H_2PO_4^-$ buffer, pH 2.5, in the range of 0.00 V to +0.25 V was performed. A potential value of 0.00V was chosen as the accumulation potential.

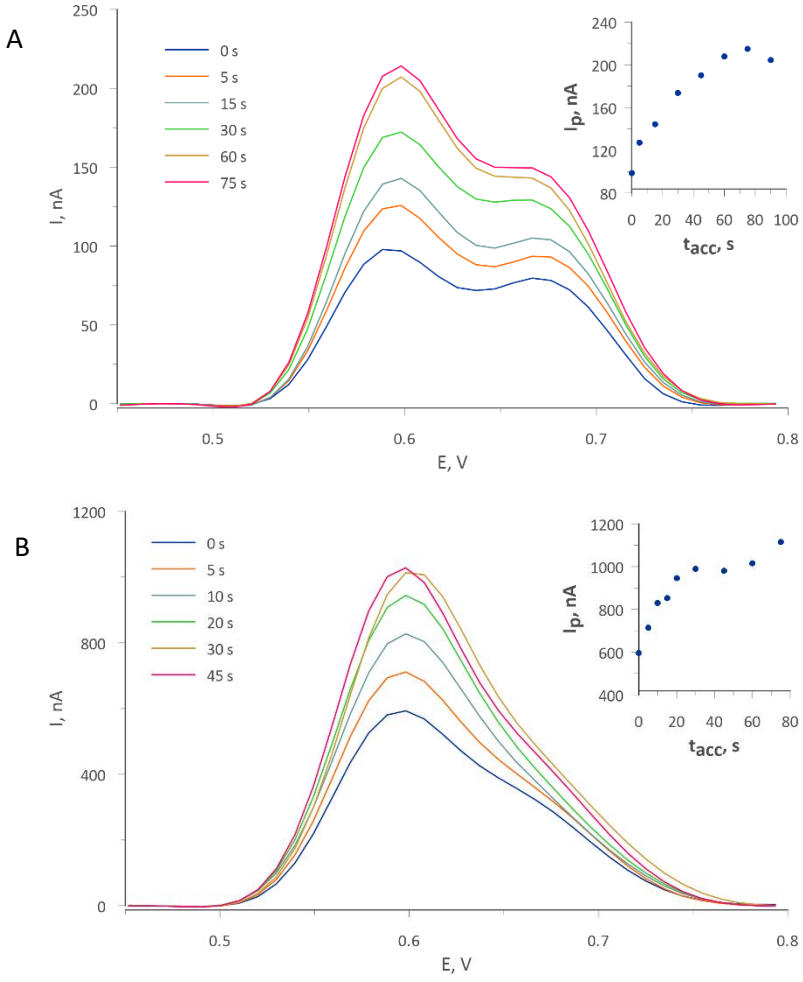

Figure 7.4. Voltammograms (baseline correction) of AdSDPV (pH 2.5, Pulse amplitude: 50 mV, Step potential: 10 mV), to different t_{acc}; also the influence on I_p of t_{acc}: A) 100 µg·L^{-1} of EA. B) 400 µg·L^{-1} of EA.

7.3.1.3. Influence of concentration of EA

The influence of concentration of EA on Ip was carried out by DPV and AdDPV.

A) Influence of EA concentration, by DPV: The study of concentration of EA (40–600 $\mu g \cdot L^{-1}$) on Ip in 0.04 $mol \cdot L^{-1}$ $H_3PO_4/H_2PO_4^-$ buffer, pH 2.5, by the above experimental conditions mentioned was carried out. From the calibration graph (DPV), in Figure 7.5 it can be seen that there is not a linear relationship between Ip and the EA concentration in all the studied range. This behaviour is probably due to the presence of one or two peaks which appear depend on the concentration of EA present in the samples. In this way, two peaks are observed at low EA concentrations, whereas at high concentrations, the two peaks are overlapped at high concentrations, causing the addition of their respective intensities (Figure 7.6 for cork boiling water samples).Thus, the integration of the registered voltammograms (Figure 7.7A) was carried out. Now, the intensity value at the plateau (where the signals for the two peaks are summed) was measured. In Figure 7.7B, the integrated values of the signal versus the EA concentration is shown. In this condition, a good linear relationship between the signal and EA concentration over the studied range was obtained, with a slope of 0.1924 $nA/\mu g \cdot L^{-1}$ and a correlation coefficient of 0.9969.

B) The influence of the EA concentration (40–400 $\mu g \cdot L^{-1}$), by using AdDPSV, on Ip, was carried out (E_{acc} of 0.00 V, rest time of 10 s, t_{acc} of 20 s and pulse amplitude of 50 mV). A relationship with two different slopes (Figure 7.5) was observed. This behaviour was also shown in the study of ellagic acid by DPV (without the accumulation process) as mentionated above. For this reason, an integration of the obtained voltammograms from the influence of the concentration was carried out like DPV (Figure 7.7B). Now, a linear relationship in over the range of studied EA concentrations was obtained, with a slope of $0.3165 nA/\mu g \cdot L^{-1}$ and with a correlation coefficient of 0.9968.

The figures of merit were performed by means of the ACOC program (Espinosa et al., 2005), in Matlab code version R2008a. The detection limits calculated for EA by the Winefordner-Long method (Long et al., 1983), which the following results: 18.4 $\mu g \cdot L^{-1}$ (DPV) and 48.1 $\mu g \cdot L^{-1}$(AdDPSV) respectively, and by the Clayton's method (Clayton et al., 1987): 14.3 $\mu g \cdot L^{-1}$ (DPV) and 34.1 $\mu g \cdot L^{-1}$(AdDPSV) respectively. Therefore, the developed methods have good sensitivity.

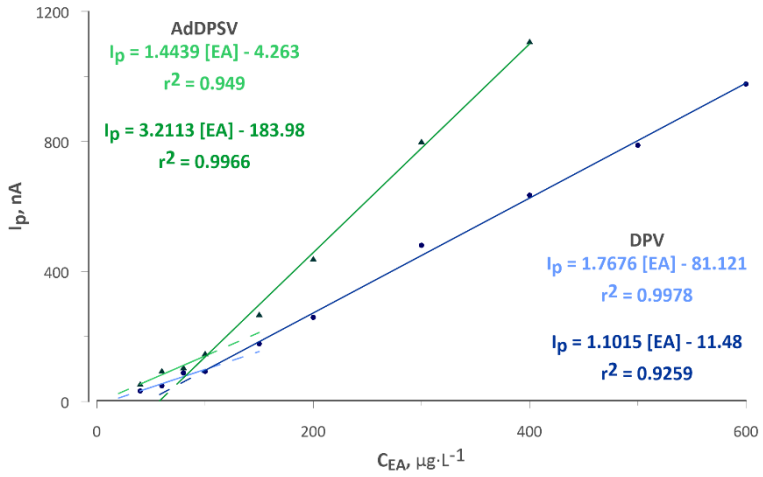

Figure 7.5. Influence of EA concentration on the peak current (Ip) by DPV (blue line) and AdDPSV (green line) methods (pH 2.5, Pulse amplitude: 50 mV, Step potential: 10 mV and E_{acc}+0.00 V, t_{acc}20 s, t_{eq} 10 s.)

The reproducibility was evaluated in terms of repeatability and between-day reproducibility. The coefficient of variation (CV) for nine consecutive measurements of the peak current, corresponding to an EA concentration of 100 µg·L^{-1}, was calculated as 2.3% and 2.6 % in DPV and AdDPSV methods respectively, which demonstrates good repeatability of the voltammetric methods. The reproducibility between-daywas studied through measurement of the peak current, corresponding to an EA concentration of 100 µg·L^{-1}, over a period of two weeks. A CV of 3.0 % and 4.6% (DPV and AdDPSV methods respectively) was obtained, showing satisfactory reproducibility.

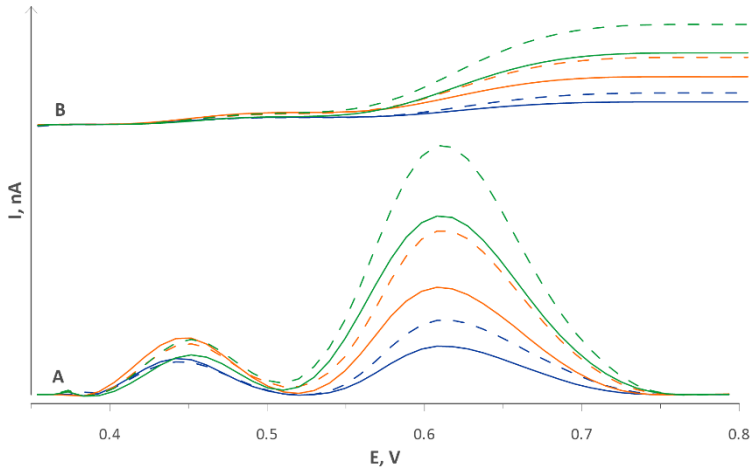

Figure 7.6. Voltammograms of samples containing different aliquots of cork boiling water (25 µL-blue, 50 µL-orange and 75 µL-green), DPV (solid line) and AdDPSV (dashed line). Conditions: pH 2.5, Pulse amplitude: 50 mV, Step potential: 10 mV, E_{acc}: +0.00 V, t_{acc}: 20 s, t_{eq}: 10. A) Baseline correction Voltammograms. B) Integration signal of voltammograms.

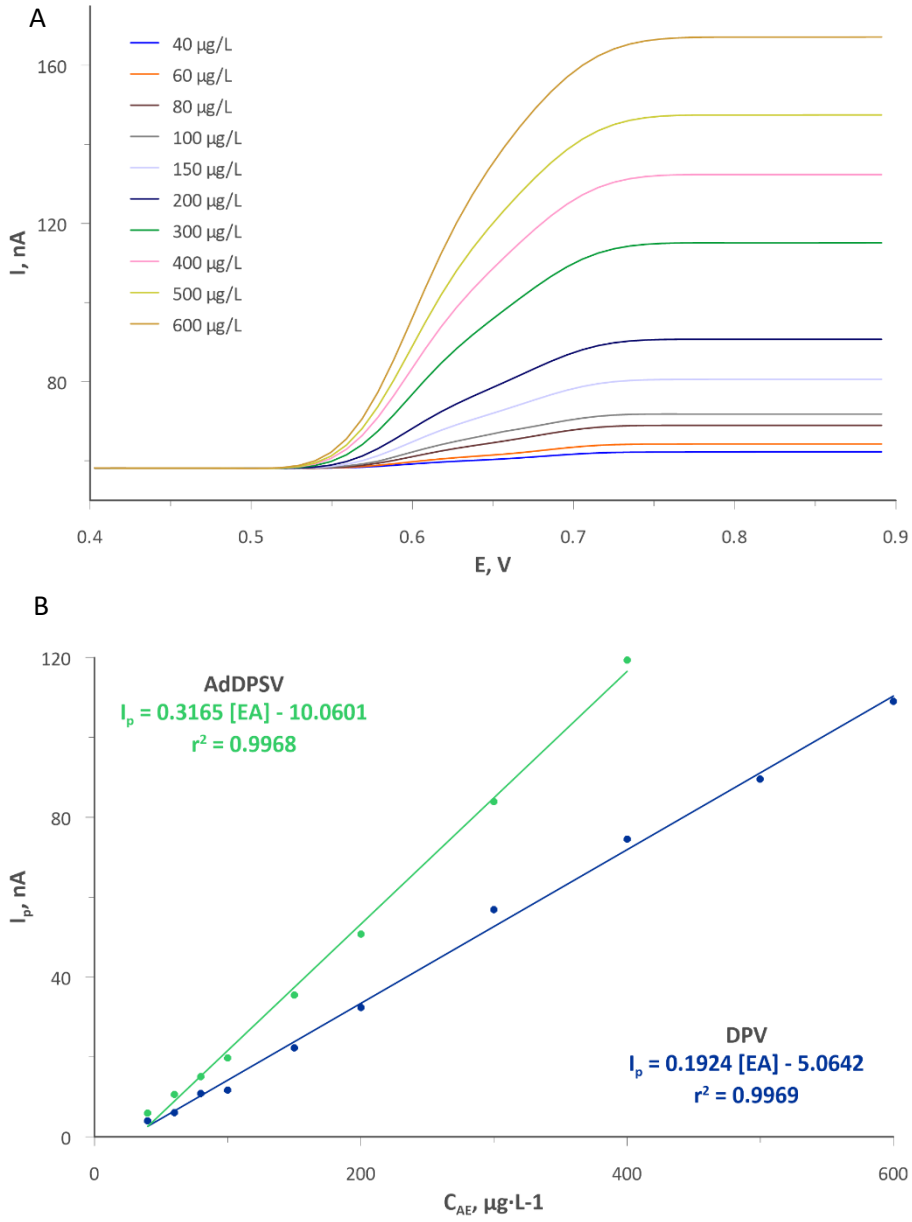

Figure 7.7. A) Voltammograms signal integrated (DPV) for different concentration of EA. B) Representation of the variation in peak intensity (integrated signal) with the concentration of EA. Conditions: pH 2.5, Pulse amplitude: 50 mV, Step potential: 10 mV.

7.3.2. Electrochemical application of DPV and AdDPSV for the determination of LOPPC's in cork boiling water. Correlation with spectrophotometric method

The analysis of total content of LOPPCs in cork boiling water was carried out following the previously optimized procedure, thoroughly described in the experimental section. For this study, four different cork boiling water samples were obtained by boiling different amounts of cork. For each four different samples of cork boiling water, three aliquots of each sample (25, 50 and 75 µL in a final volume of 25.0 mL), with and without accumulation process, have been analysed in triplicate.

In order to validate the proposed voltammetric methods, the results obtained were compared with those determined using the spectrophotometric method (Folin-Ciocalteau reagent). The results obtained are shown in Table 7.2. It is observed that the phenolic contents determined by voltammetric methods are lower than those obtained by the spectrophotometric method. The reason is that the spectrophotometric method is less selective. However, a good correlation between the results obtained by electroanalytical methods (DPV and AdDPSV) and the spectrophometric method are obtained, as depicted in Figure 7.8.

Table 7.2. Analysis of total phenolic content in cork boiling water by Electroanalytical (DPV and AdSDPV) and Spectrophotometric (FC reagent) methods.

Cork boiling water samples	Composition[a] (g dry cork/L)	FC[b] (mg EAE/Kg dry cork)		Volume, cork boiling water samples, (µL)	DPV[c] (mg EAE/Kg dry cork)		AdDPSV[d] (mg EAE/Kg dry cork)	
		Average	% RSD		Average	% RSD	Average	% RSD
A	860.8	1501	0,41	25	119	1,36	115	6,88
				50	109	4,09	99	7,6
				75	103	3,73	91	3,8
					110	**3,06**	**102**	**6,09**
B	1062.1	3131	0,37	25	205	4,57	171	7,49
				50	234	5,10	156	4,91
				75	225	8,49	157	7,48
					221	**6,05**	**161**	**6,63**
C	909.1	1760	0,74	25	114	7,98	109	7,50
				50	108	2,40	89	3,0
				75	97	4,90	78	4,9
					106	**5,09**	**92**	**5,1**

Cont. Table 7.2. Analysis of total phenolic content in cork boiling water by Electroanalytical (DPV and AdSDPV) and Spectrophotometric (FC reagent) methods.

Cork boiling water samples	Composition[a] (g dry cork/L)	FC[b] (mg EAE/Kg dry cork)		Volume, cork boiling water samples, (µL)	DPV[c] (mg EAE/Kg dry cork)		AdDPSV[d] (mg EAE/Kg dry cork)	
		Average	% RSD		Average	% RSD	Average	% RSD
D	1900.0	4961	0,46	25	365	4,66	263	1,76
				50	351	3,12	244	3,00
				75	289	8,42	198	3,85
					335	**5,40**	**235**	**2,87**

[a] Concentration of cork boiling water expressed as g cork/L.

[b]Folin-Ciocalteau Method, total phenolic content expressed as mg EAE/Kg dry cork.

[c] Differential Pulse Voltammetry Method, total content of low oxidation potential phenolic compounds, expressed as mg EAE/Kg dry cork.

[d] Adsorptive Differential Pulse Stripping Voltammetry Method, total phenolic content of low oxidation potential compound, expressed as mg EAE/Kg dry cork.

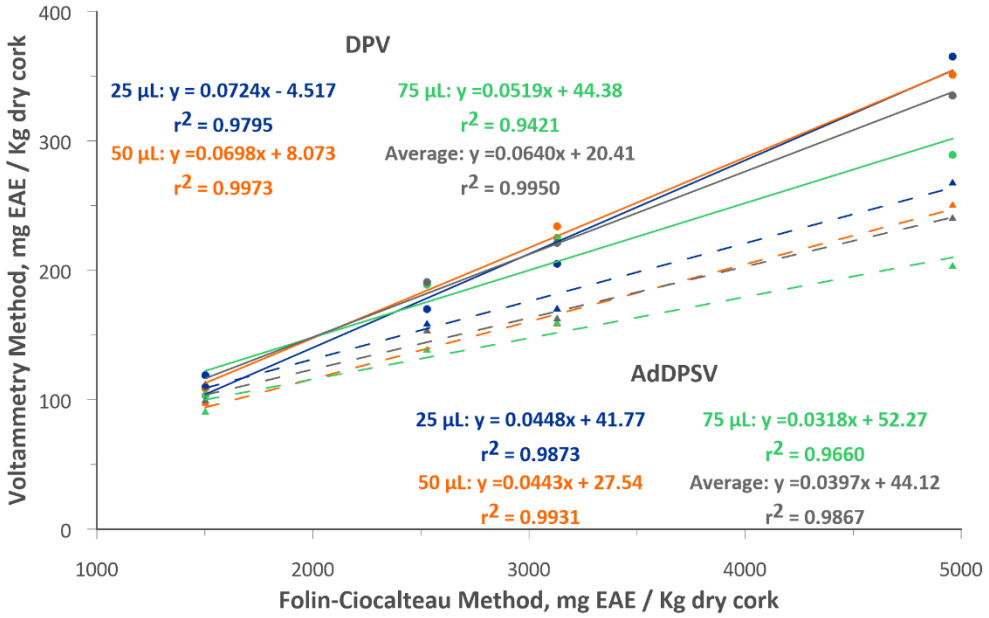

Figure 7.8. Correlation between the results obtained between by the electroanalytical method proposed DPV (solid line) and AdDPSV (dashed line) to by spectrophotometric method using the Folin-Ciocalteau reagent. Different aliquots (25 µL–blue; 50 µL–orange; 75 µL–green; and average values in gray) of cork boiling water. Conditions DPV and AdSDPV: pH 2.5, Pulse amplitude: 50 mV, Step potential: 10 mV and also by AdSDPV E_{acc}: +0.00 V, t_{acc}: 20 s, t_{eq}: 10 s.

7.4. Conclusions

The developed electroanalytical methods (DPV and AdSDPV), allows the determination of total LOPPCs in cork boiling water by using GCE.

A good correlation was obtained between the electrotroanalytical and spectrophotometric methods, even though the first one achieved lower results because of the higher selectivity and the quantification involves only the phenols with low oxidation potential, and while spectrophotometric methods is less selective.

The electroanalytical methods proposed using GCE are able to estimate the antioxidant capability of low oxidation potential phenolic compounds present in the samples, and are more selective, quick, low cost and do not require any sample pretreatment.

On the other hand, the possibility to profit the cork boiling water by obtaining the phenolic compounds (potential anticancer properties) is of interest and their analysis of such residues.

Finally, the assumed advantage of the utilisation of SPEs due to their fast kinetic, miniaturisation and simplicity is questionable in terms of the cost effectiveness of their implementation in the analysis of phenolic compounds in real cork water samples regarding the necessity to use one new sensor each time to avoid lack of reproducibility in the determination. In real situations one SPE cannot be used more than once, as we observed in this study, so its implementation in routine analysis is more expensive. However, a single GC electrode can be used for many years and it demonstrated it suitability for the analysis of those compounds in real complex samples. The performance of an appropriate cleaning of the surface of GCE can ensure the reproducibility and repeatability of the electrochemical signals, making the determination more reliable, reducing time consumption and general costs.

7.5. References

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8. Electrochemical behavior of gallic and ellagic acid using graphene modified screen-printed electrodes. Method for the determination of total low oxidation potential phenolic compounds content in cork boiling waters

Se propone un nuevo método electroanalitico, mediante la utilización de electrodos impresos modificados con grafeno, para la determinación del contenido total de compuestos fenólicos de bajo potencial de oxidación en aguas de cocido de corcho. Se ha calculado la influencia de la concentración y los valores relacionados con los límites de detección y cuantificación. Los resultados obtenidos se comparan con los obtenidos aplicando el método espectrofotometrico (utilizando el reactivo de Folin Ciacalteau), encontrándose una buena correlación entre ambos.

El método propuesto es rápido, sensible simple y de bajo coste.

Este trabajo se ha presentado como póster en el *15th International Conference on Electroanalysis*, en Malmö (Suecia) entre el 11 y 15 de junio de 2014, y se ha publicado en *Electroanalysis*, 27, 177-184. (Incluido en el Anexo)

Electrochemical behavior of gallic and ellagic acid using graphene modified screen-printed electrodes. Method for the determination of total low oxidation potential phenolic compounds content in cork boiling waters

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Abstract

New electroanalytical methods have been proposed for the estimation of the total content of phenolic compounds of low oxidation potential in cork boiling water, expressed as gallic acid equivalent/g dry cork, by using screen-printed electrode modified with graphene. The results obtained have been compared with spectro-photometric method (Folin-Ciocalteau) and a good correlation has been found between them. The detection limits obtained by Winefordner-Long and Clayton methods were 0.04 and 0.06 ppm for ellagic acid (EA), and 0.05 and 0.09 ppm for gallic acid (GA), respectively. The sensitivity, simplicity, selectivity, fast and low cost of the developed methodologies is also demonstrated.

Keywords: Gallic acid. Ellagic acid. Screen-printed electrodes. Phenolic compounds. Cork boiling water

8.1. Introduction

The analysis of bioactive compounds (phenols and polyphenols) is of great interest for its antioxidant antimutagenic, antithrombotic, anti-inflammatory and anti-carcinogenic properties [1, 2]. Some of these phenols or polyphenols, in particular the acids ellagic, vanillic, gallic, protocatechuic, salicylic, ferulic and syringic, or vanillin, coniferaldehyde and synapaldehyde are present in different amounts in several lignocellulosic materials, such as cork, and thereby in waste by-products of the associated industries, which has been object of a several studies in the last few years [3].

Cork comes from the outer bark of *Quercus suber* L, commonly known as oak. This bark is removed from the tree periodically, every 9 years approximately depending on the region. Cork forests extend over an area of nearly 2.7 million hectares (Ha) in seven countries: Portugal, Spain, Algeria, Morocco, Tunisia, Italy and France [4].

Cork is a vegetal tissue made of dead cells that protect the living parts of the oaks trunk and branches.

It is a versatile material that has been used for different purposes since ancient times, among which are thermal insulation, decoration, and its main use actually for the manufacturing of caps on wine. For these applications, cork has to be previously pretreated. One of the first steps after the extraction of the cork from the tree is the immersion in boiling water for one hour. The aim is to clean the material, remove any water-soluble substances or microorganisms, and improve its texture and plastic properties [5]

Further, this cork boiling waters are characterized by high levels of phenolic compounds [6], as tannic, gallic, protocatechuic, syringic, ellagic acid, vanillic and protocatechuic aldehyde [7]; and also vanillic and ferulic acids [8]. In fact, the isolation and characterization of phenolic compounds in cork is of interest to the pharmaceutical, food industry, etc. In the literature, several analytical methods have been reported for the determination of the "total phenols compounds" in different kind of samples. The spectrophotometric method [9], based on the reaction with the Folin-Ciocalteau reagent is the most used. This method

is characterized by low selectivity because the reagent does not only measure phenols, and will react with any reducing substances.

Nevertheless, since Blasco et al. [10] introduce the "electrochemical index" concept, which could be defined as the total phenolics concentration obtained by means of electrochemical techniques for predominant and representative phenolic classes. The electroanalytical methodologies began to play an important role and gain future prospects in the determination of the total phenolic content, considering always the inherent selectivity and sensitivity of electrochemical techniques versus spectrophotometric protocols.

As described in the literature over the past decade, these methods are particularly well suited for the analysis of polyphenols, because the antioxidant properties of these compounds are related to their ability to donate electrons. Thus, the electroanalytical analysis of the "total polyphenol" content has been carried out by using different electrodes i.e. carbon, bare or modified glassy carbon, biosensors, etc. [1, 11–13]. For example, several electroanalytical methods have been developed by using a glassy carbon working electrode and they have been applied to determine the content of phenolic compounds in wine [10, 14–17], tea [18] and juices [18, 19]. Also, other types of electrodes, such as glassy carbon modified with carbon nanotubes [1] or biosensors (tyrosinase biosensor based on glassy carbon electrode modified with gold nanoparticle [20]) have been also used for these purposes.

Recently, the advantage and succeed of screen printing technology has opened new exciting opportunity to apply electrochemical techniques for the analysis of this compounds. On the other hand, the screen-printed electrode without modification or modified with graphene, carbon nanotubes are opening new possibilities for the analysis of phenols/polyphenols because the screen-printed electrodes present suitable reproducibility, more versatility and improvement of the kinetic of the electrode process.

In the present work, the electroanalytical behavior of the gallic and ellagic acids is performed by using, for the first time, screen-printed electrode modified with graphene. The optimization of the instrumental and chemical variables is carried out, and the figures

of merits of the methods are also established. The proposed voltammetric methods are applied to cork boiling water samples, which gives oxidation peaks at potential values next to the mentioned polyphenols, in order to determine the total low oxidation potential phenolic compounds content.

8.2. Experimental

8.2.1. Apparatus

All voltammetric measurements were performed using a computer controlled potentiostat μAutolab (ECOChemie, Holland) and Metrohm (Herisau, Switzerland) 663 VA stand. Screen-printed carbon electrode modified with graphene (DRP-110GPH) were used with cable connector for SPEs (DropSens S.L., Oviedo, Spain)

The spectrophotometric equipment was a UV/Vis Varian Cary-50 spectrophotometer (Palo Alto, California, USA).

8.2.2. Reagents

All chemicals used were of analytical grade. Gallic, ellagic, protocatechuic, vanillic, syringic and ferulic acids, syringic, coniferyl and sinapyl aldehyde, and vanillin were supplied by Sigma-Aldrich. Acetic acid was provided by Sigma-Aldrich, while sodium acetate, phosphoric acid, monosodium phosphate anhydrous, disodium phosphate anhydrous, sodium hydroxide, sodium carbonate anhydrous, methanol, ethanol, Folin-Ciocalteau reagent and boric acid were supplied by Panreac.

8.2.3. Samples

A standard of ellagic acid (EA) and gallic acid (GA) solutions (200 mg/L) was prepared, in volumetric flask, by weighing the appropriate amount and dissolving into methanol or ethanol: water (20:80), respectively.

More diluted solution was prepared in 25 mL volumetric flask by taking the adequate amount and diluting with Milli-Q water to the mark, in presence of 80 µL of 3.0 M KCl electrolyte solution necessary to the electroanalytical determination by using silver pseudo reference screen-printed electrode.

Cork boiling water samples were obtained after immersing of different cork samples, with varying content, in boiling water for one hour. After that, the cork was immediately filtered. When the filtrate water achieved the room temperature, the final volume was determined.

8.2.4. Pretreatment of the Working Electrode

In order to obtain better reproducibility of the electrochemical measurements, an electrochemical pretreatment of screen-printed electrode modified with graphene (SPE-GPH) was performed by applying a constant potential of + 1.4 V during 300 s [21] in 0.02 M HAc/NaAcO (pH 4.5), which is the buffer solutions used for further electrochemical studies.

8.2.5. General Procedure. Differential Pulse Voltammetry (DPV)

Samples of the appropriate concentration of GA and EA were prepared in 25 mL volumetric flasks and the appropriate volume of the buffer solutions of 0.5 M HAc/NaAcO, $H_3PO_4/H_2PO_4^-$, $H_2PO_4^-/HPO_4^{2-}$ or 0.04 M Britton-Robinson buffer were added to obtain the desired pH. Furthermore, 80 µL of 3.0 M KCl were also added and finally Milli-Q water to the mark. The samples were introduced into the voltammetric cell and were analyzed by Differential Pulse (DPV). All measurements were performed at room temperature, and the DPV voltammograms at 50 mV of pulse amplitude and 10 mV of step potential. After each scan, the cleaning process of SPE-GPH was carried out, as following: firstly, the SPE-GPH electrode was immersed in Milli Q water, and secondly in buffer solutions to check the effectiveness of the process, ensuring that no signals appear in the voltammograms.

For the analysis of the cork boiling water, different diluted subsamples were prepared taking different volumes of cork boiling waters from 25 to 200 µL and Milli-Q water was

added to the mark in 25 mL volumetric flask. Buffers solution and 80 µL of 3.0 M KCl were also added.

8.2.6. DPV Determination of Total Phenolics Compounds of Low Oxidation Potential in Cork Boiling Waters

The determination of total phenolic content of low oxidation potential, present in the cork boiling water, expressed as gallic acid equivalents (GAE), was carried out by two different procedures:

- Without accumulation process: In a 25 mL volumetric flask, 75–100 µL of cork boiling water, 1 mL of 0.5 M buffer HAc/NaAcO (pH 4.5), 80 µL of 3.0 M KCl and Milli-Q water to the mark were added. Then, the sample was introduced into the electrochemical cell and the DPV voltammogram was recorder.

- With accumulation process: In a 25 mL volumetric flask, 25–50 µL of cork boiling water, 1 mL of 0.5 M buffer HAc/NaAcO (pH 4.5), 80 µL of 3.0 M KCl and Milli-Q water to the mark were added. Then, the prepared sample was introduced into the electrochemical cell recording its DPV voltammograms, 30s of accumulation time, and + 0.05 V of accumulation potential and 10 s equilibrium time.

The total phenolic content of low oxidation potential values was calculated as GAE from the calibration curve of GA standard solutions (0.2–1.0 ppm) and it was expressed as mg GAE/g of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case.

8.2.7. Total Phenolic Content by Spectrophotometric Method

The total phenolic content of the cork boiling water was determined by spectrophotometric method using the Folin-Ciocalteau reagent [9]. In a 25 mL volumetric flask, 0.5 mL of Folin-Ciocalteau reagent, 10 mL of aqueous sodium carbonate (75 g/L), aliquots of 0.5 mL of each cork boiling waters and Milli Q water were added. Each mixture was kept in the dark and at room temperature for 60 min. After homogenization, the

absorbance was measured at 670 nm using a UV/Vis spectrophotometer. The total phenolic content was calculated as GAE from the calibration curve of gallic acid standard solutions (0.5–16.0 ppm) and expressed as mg GAE/g of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case.

8.3. Results and Discussion

8.3.1. Previous Assays

The cork boiling water provides an oxidation response (pH 4.5) in the SPE-GPH electrode, as shown in Figure 8.1. It can be observed that a well-defined peak appears at+ 0.2 V and a small peak at more positive Ep (+ 0.32 V). The intensity of both peaks increases proportionally with the concentration of the polyphenols in the cork boiling water samples.

Taking into account the complex composition of cork boiling water due to the presence of different phenols/polyphenols and other compounds, voltammograms of different phenols solutions were registered that may be present in the samples in concordance with the literature revised.

In Table 8.1 the data obtained from the peak potentials and peak intensities of each compound are shown.

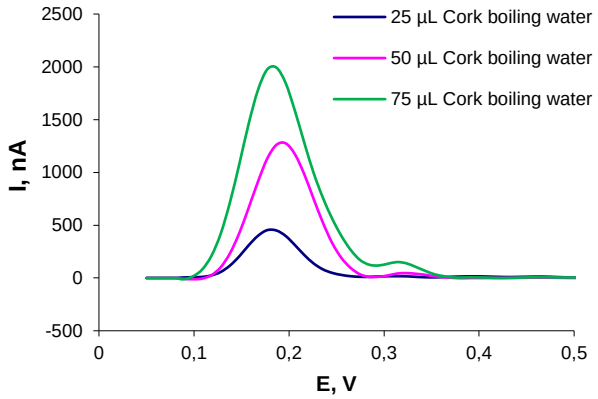

Fig. 8.1. Differential pulse voltammograms (DPV), baseline correction, of different volumes of cork boiling water Conditions: pH 4.5, pulse amplitude: 50 mV and step potential: 10 mV.

Table 8.1. Values of potentials and intensities of the peaks for each phenolics compounds. Conditions: pH of 4.5; 50 mV of pulse amplitude and 10 mV of step potential. Ellagic acid (EA); Ferulic Acid (FER); Gallic Acid (GA); Protocatechuic Acid (PA); Vanillin and other compounds (V); Vanillic Acid (VA); Syringic Acid (SA); Syringic aldehyde (SIR); Sinapaldehyde (SIN) and Coniferaldehyde (CON).

Compound	[C], µM	E_{p1}(V)	I_{p1}(nA)	E_{p2}(V)	I_{p2}(nA)
EA	3.3	0.304	5430		
VA	6.0	0.234	2590	0.518	1030
GA	5.9	0.197	2400		
PA	6.5	0.226	688		
SA	7.2	0.382	983		
V	6.6	0.568	1040		
CON	5.6	0.226	2060	0.431	835
SIN	4.8	0.177	3930	0.304	466
FER	5.2	0.197	2580	0.402	468
SIR	5.1	0.226	1720	0.49	1720

In order to have an estimation of the phenolic compounds of low molecular weight, cork boiling water was also analyzed by HPLC [22]. In Table 8.2 the results obtained are shown.

As can be seen in Figure 8.1 and Table 8.1, the peak that appears at Ep+ 0.2 V in cork boiling water is close to the peak potential of GA and also to other compounds. Also, the second peak observed at + 0.3 V in the voltammograms of the cork boiling water samples is close to EA peak potential. On the other hand, the HPLC analysis (Table 8.2) determines that the majority compounds in the cork boiling water samples are GA and EA, with contents around of 43% and 14 %, respectively.

Table 8.2. HPLC analysis of phenolic compounds of low molecular weight in cork boiling water

Compound	[ppm]	%
GA	26.0	43.4
PA	13.0	21.8
VA	5.7	9.5
V	3.5	5.8
FER	1.9	3.2
CON	1.5	2.6
EA	8.3	13.8

Therefore, taking into account the potentials values of voltammetric signal observed in the cork boiling waters and the results obtained by chromatographic analysis, the GA and EA were selected as possible compounds to express the content of total phenols of low oxidation potential as mg equivalent of one of them per gram of dry cork.

For that, we proceed first to the DPV voltammetric studies of EA and GA by using the screen printed electrodes modified with graphene (SPE-GPH).

8.3.2. Electroanalytical Behavior of Ellagic and Gallic Acids

8.3.2.1. Effect of pH

The influence of pH on Ip and Ep of EA and GA was examined in the range from 1.4 to 6.6. In Figure 8.2 the voltammograms obtained at each pH are shown. The peak intensity of the compounds decreases when pH increases and remaining constant to pH values greater than 4. For further study, a value of pH 4.5 is selected, because minor changes in pH do not produce large variations in peak intensity, even though sensitivity is lost.

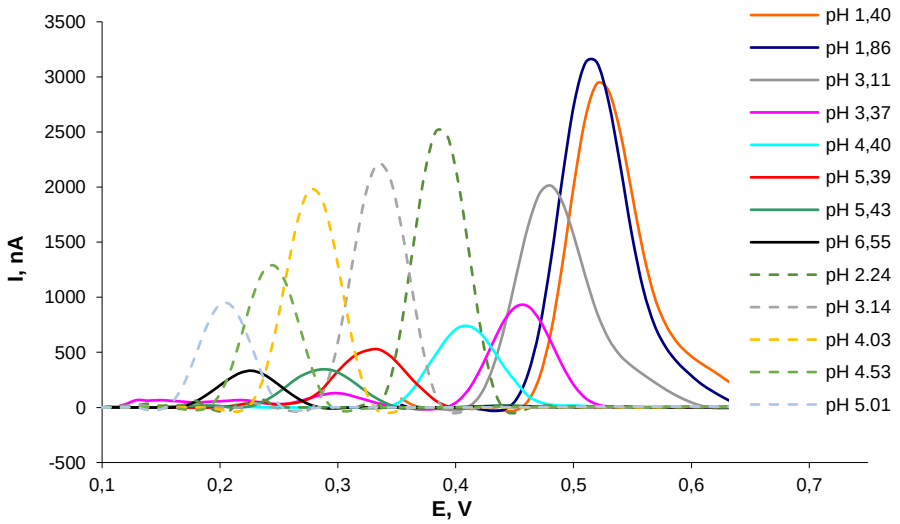

Fig. 8.2. DPV voltammograms of EA (solid line) and GA (dashed line), 0.6 ppm respectively at different pH values (buffers: HAc/NaAcO, $H_3PO_4/H_2PO_4^-$ or $H_2PO_4^-/HPO_4^{2-}$), 50 mV pulse amplitude, 10 mV step potential.

A linear relationship between Ep and pH for EA (Ep (V) = 0.053pH + 0.567) and for GA (Ep(V) =0.063pH + 0.478) were respectively found. These behaviors indicate that protons are involved in the electrode process. On the other hand, the instrumental variables were also optimized. The selected optimal values are: pulse amplitude of 50 mV and step potential of 10 mV (data not shown).

8.3.2.2. Influence of the EA and GA Concentration

The Ip obtained from the application of the optimized DPV methods at different concentration of EA and GA in the range 0.2 to 1.0 ppm are shown in Figure 8.3. As can be seen, a linear relationship was found in the whole range for the two compounds.

The application of a Matlab program to calculate the figures of merit of the linear regression curves, gives values of the detection limits of 0.05 and 0.08 ppm for EA and 0.05 and 0.10 ppm for GA, by using the Winefordner-Long [23] and Clayton [24] methods respectively.

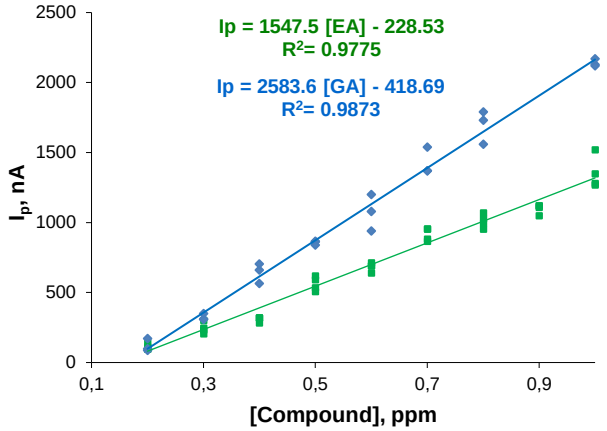

Fig. 8.3. Variation of the peak intensity with the concentration of EA (green) and GA (blue), (pH 4.5, 50 mV pulse amplitude, 10 mV step potential).

8.3.3. Adsorptive Stripping Analysis of Ellagic and Gallic Acids

In the preliminary experiments it was observed that EA and GA were accumulated in SPE-GPH electrode. Therefore, we carried out also studies using differential pulse voltammetry with adsorptive preconcentration.

The influences of pH on Ip and Ep for these compounds were investigated and a similar behavior was observed to that obtained without preconcentration process.

However, the increase of the peak intensity with pre-concentration for the GA is higher than the observed for the EA also with preconcentration.

8.3.3.1. Effect of Accumulation Potential

The study of the influence of the accumulation potential in the peak intensity was carried out in the range from 0to + 0.2 V. Due to any changes in the peak intensity were observed at values lower than + 0.2 V, a value of + 0.05 V was selected for further experiments.

8.3.3.2. Effect of Accumulation Time

The influence of accumulation time (t_{acc}) on the Ip of EA and GA was studied in the range from 15 to 120 seconds (0.02 M HAc/NaOAc pH 4.5 buffer solution). During the accumulation period, an E_{acc} of + 0.05 V was applied.

In Figure 8.4A, a linear relationship between the accumulation time and the peak intensity was observed up to 90 seconds with a concentration of 0.6 ppm EA. In the case of GA (Figure 8.4B), the study of the influence of the accumulation time was performed using a concentration of 0.3 ppm, because of the response at higher concentration (0.6 ppm) was only linear until 30 s of accumulation time. As shown in Figure 8B the linear relationship of 0.3 ppm of GA is maintained until 120 s of accumulation time.

So, it has been chosen for both compounds and for further studies, an accumulation time of 30 s.

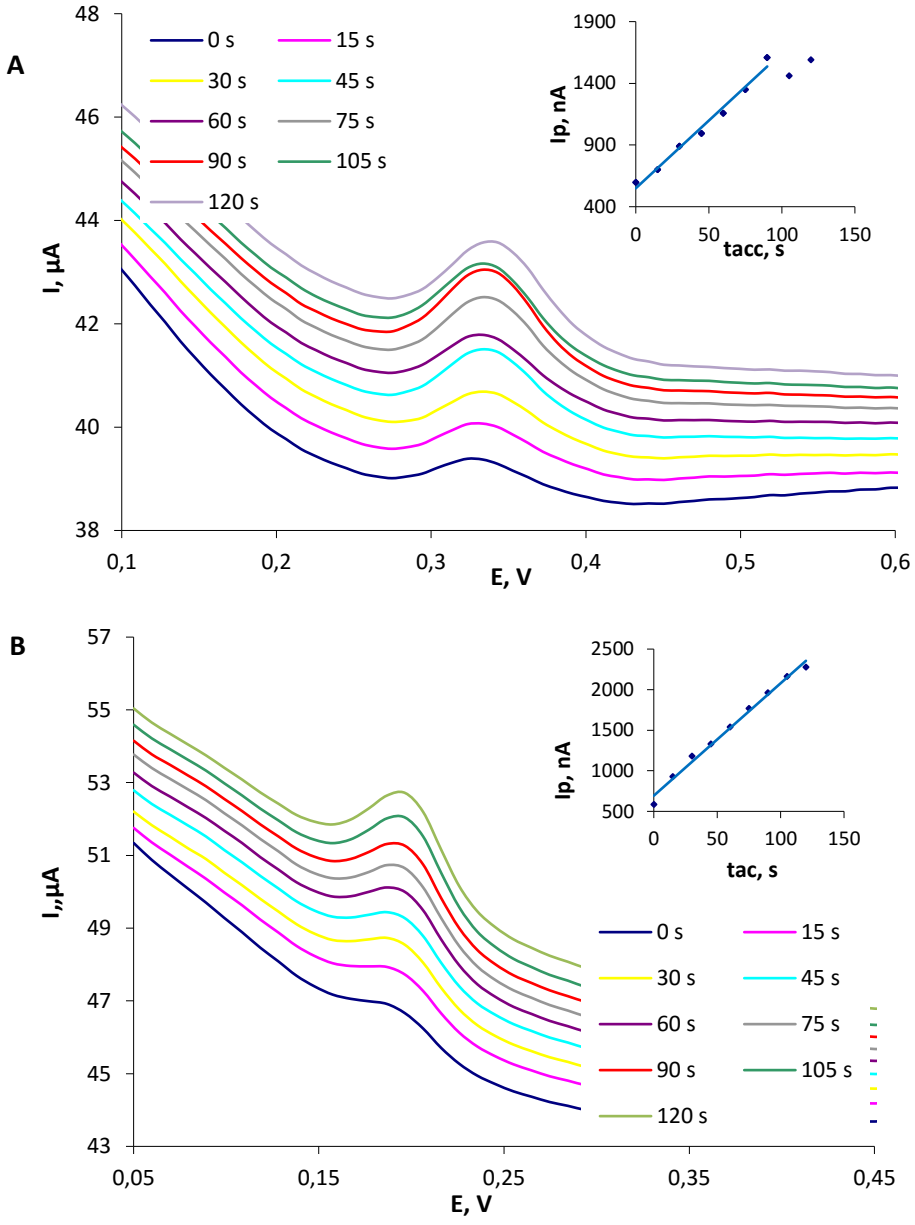

Fig. 8.4. Voltammograms of DPV at different t_{acc} and variation of Ip with t_{acc}: A) 0.6 ppm of EA, B) 0.3 ppm of GA. pH 4.5, 50 mV pulse amplitude; 10 mV step potential; E_{acc}=+0.05 V and t_{eq}=10 s.

8.3.3.3. Influence of the EA and GA Concentration

The influence of concentration on Ip, in the range 0.2 to0.8 ppm of EA and GA are also studied using the optimized experimental conditions (E_{acc} of + 0.05 V, t_{acc} 30 s).

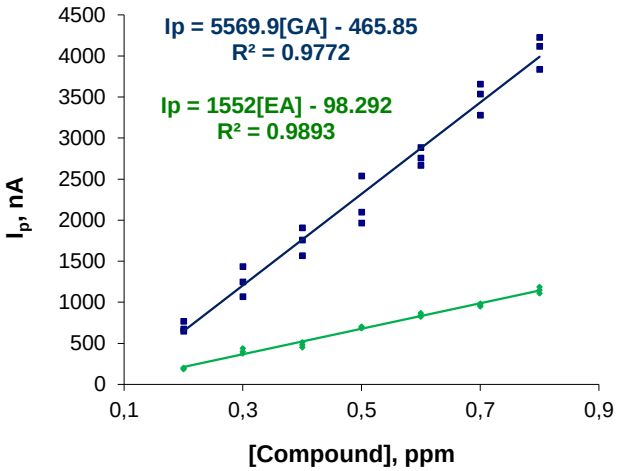

Fig. 8.5. Variation of the Ip with the concentration of EA (green) and GA (blue). Conditions: pH 4.5, 50 mV pulse amplitude,10 mV step potential, + 0.05 V of E_{acc}, 30s of t_{acc}, and 10 s of $t_{eq.}$

As shown in the calibration curve (Figure 8.5), it demonstrates a linear behavior in the whole concentration range studied with slopes (nA/ppm) of 1552 and 5570, and correlation coefficients of 0.9893 and 0.9772 for EA and GA respectively. The detection limits were calculated by Winefordner-Long [23] and Clayton [24] methods, obtaining values of 0.04 and 0.06 ppm for EA, and 0.05 and 0.09 ppm for GA, respectively.

8.3.4. Determination of Total Phenolic Compounds of Low Oxidation Potential in Cork Boiling Waters

The developed methods were applied for the determination of total phenolic content of low oxidation potential, in several cork boiling water samples.

The electroanalytical measurements were carrying out applying the procedure previously optimized and described in the experimental section.

According to the results obtained, the GA were used to estimate the content of total phenolic compound of low oxidation potential in cork boiling water, and the external calibrations were utilized for quantification.

In Figure 8.6, the voltammograms obtained, with and without accumulation step, from different samples of cork boiling water is shown.

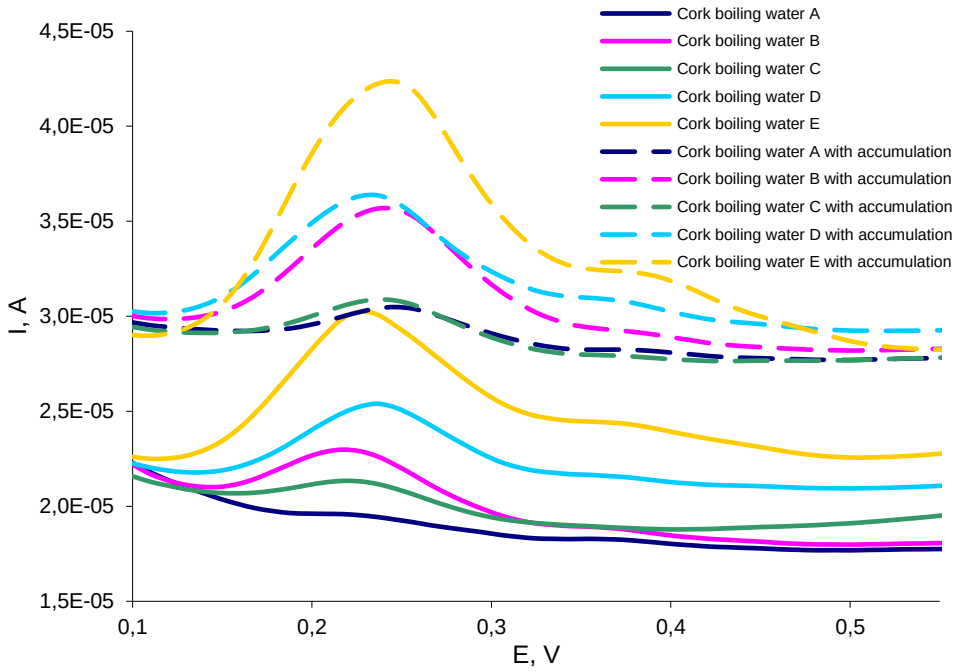

Fig. 8.6. Voltammograms of DPV of cork boiling water samples.

The results obtained in the analysis of total phenolic compounds of low oxidation potential values in different real sample of cork boiling water are shown in Table 8.3, expressed as mg GAE/g dry cork.

Table 8.3. Analysis of total phenolic content of cork boiling water boiled. Electroanalytical and spectrophotometric methods.

Cork boiling water samples	Composition [Cork boiling water], g cork/L	FC [a]		DPV [b]		AdSDPV [c]	
		Average	Std. deviation	Average	Std. deviation	Average	Std. deviation
A	860.8	1.86	0.01	0.08	0.01	0.23	0.02
B	1062.1	3.68	0.02	0.29	0.04	0.46	0.01
C	909.1	2.10	0.01	0.18	0.02	0.27	0.03
D	1760.5	3.17	0.01	0.23	0.01	0.33	0.02
E	1900.0	5.98	0.02	0.41	0.01	0.66	0.04

[a] Folin-Ciocalteau Method, total phenolic content expressed as mg GAE/g dry cork; [b] Differential Pulse Voltammetry Method, total phenolic content, of low oxidation potential compound, expressed as mg GAE/g dry cork; [c] Differential Pulse adsorptive Stripping Voltammetry Method, total phenolic content, of low oxidation potential compound, expressed as mg GAE/g dry cork

In order to validate the proposed voltammetric methods, the results obtained were compared with those determined by using spectrophotometric (Folin-Ciocalteau reagent) (see Table 8.3). As can be seen also in Figure 8.7, a good correlation is observed.

As described also in the Table 8.3 the phenolic content determined by electroanalytical methods are lower than the obtained by the spectrophotometric method. The reason is that the photometric method is less selective. Nevertheless, we determine that the electroanalytical method proposed by using SPE-GPH electrode is capable to estimate the antioxidant power, because it is more selective, fast, and sensitive and does not require any sample pretreatment.

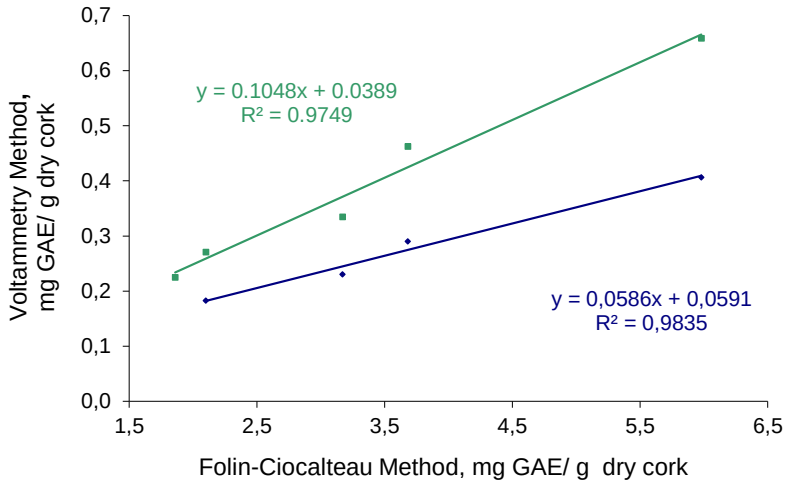

Fig. 8.7. Correlation between the results obtained by the electroanalytical method proposed (blue line: without accumulation and green line: with accumulation) and the results obtained by spectrophotometric method using the Folin-Ciocalteau reagent. Conditions: pH 4.5, pulse amplitude: 50 mV, step potential: 10 mV, E_{acc}=+0.05 V, t_{acc}= 30 s, t_{eq}= 10 s.

8.4. Conclusions

The developed electroanalytical methods, with or without previous accumulation step, allow the determination of total phenols content of low oxidation potential, in cork boiling water (expressed as mg GAE/g dry cork), by using SPE-GPH electrodes. A good correlation was obtained between both electrochemical and spectrophotometric methods, even though the first ones achieve lower results because the present higher selectivity and the quantification is involving only the polyphenols with low oxidation potentials.

However, the more important aspect is to determine the antioxidant capacity by using a technique that has ad-vantages such as: high sensitivity, simplicity, good stability, low cost and portable instrumentation, and low interferences of other non electroactive compounds.

The screen-printed electrode modified with grapheme presents suitable reproducibility, good versatility and favorable kinetic of the electrode process. In this work we demonstrate

for the first time the applicability of voltammetric methods by using SPE-GPH in the electrochemical characterization of industrial cork by-products, especially the cork boiling water.

Additionally, other advantage of the proposed methods is that it is not necessary any treatment of the sample. So, the application of the electroanalytical methods developed by using SPE-GPH can be used for in situ measurements in the cork boiling water samples from cork industry due to the portability of the electrochemical equipment.

On the other hand, the possibility to profit the cork boiling water to obtain the phenolic compounds it is of interest.

8.5. References

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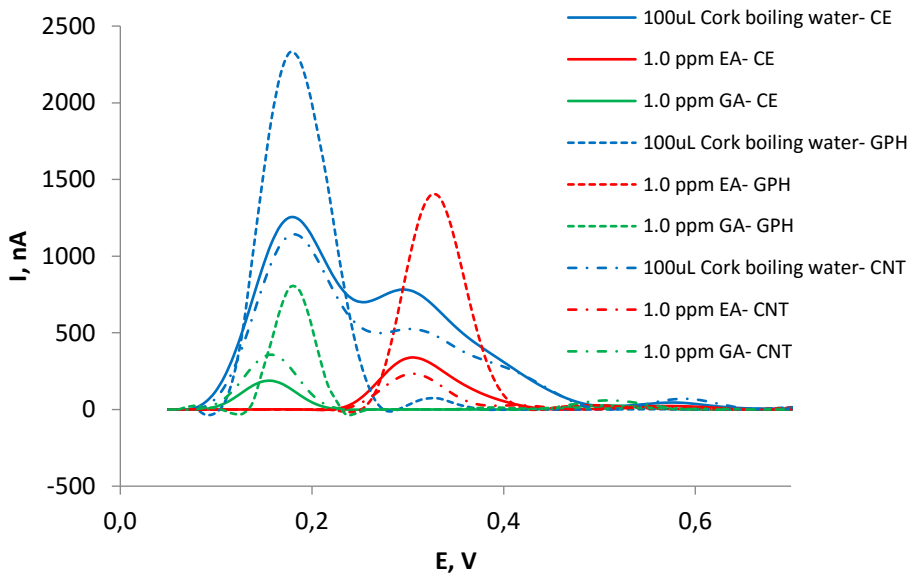
8.6. Supplementary information

In this figure, the electrochemical behavior of EA and GA in different screen-printed carbon electrodes (namely CNTs and unmodified SPCEs) and in the graphene modified screen-printed electrode is shown. Also, the voltammograms obtained in real cork boiling water samples by using the same SPEs appear in the same figure.

As demonstrated, a better sensitivity and peak resolution has been obtained by using screen-printed graphene electrodes in comparison with screen-printed carbon electrodes and screen-printed carbon nanotubes electrodes. So, it justifies the advantages to use the first one.



Differential pulse voltammograms (DPV). Baseline correction, of 100µL cork boiling water, 1.0 ppm Ellagic Acid (EA) and 1.0 ppm Gallic Acid (GA). Screen-Printed electrodes: Simple carbon electrode (CE), electrode modified with graphene (GPH) and Carbon nanotubes electrode (CNT). Conditions: pH of 4.5; 50 mV of pulse amplitude and 10 mV of step potential.

9. Flow injection analysis with electrochemical detection using printed electrodes modified with graphene for determination of total phenolic compounds in cork boiling water samples

La importancia de la presencia de compuestos fenólicos en diferentes matrices, radica en laspropiedades antioxidantes y anticancerígenas que estos compuestos poseen.

La industria preparadora del corcho produce gran cantidad de agua residual, procedente del hervido de las planchas de corcho previo a la fabricación del tapón. Estas aguas residuales son ricas en compuestos fenólicos, los cuales proceden del corcho el cual, durante el proceso de hervido, liberan estos compuestos hidrosolubles que se van acumulando en las calderas de cocido.

En este capítulo se ha desarrollado un método mediante análisis de inyección de flujo con detección electroquímica, utilizando electrodos impresos modificados con grafeno, para la determinación del contenido total de compuestos fenólicos en las aguas de cocido de corcho, expresados como equivalentes de ácido gálico

Se han optimizado las condiciones químicas e instrumentales, seleccionando como valores óptimos: pH 4,5, potencial aplicado en la detección amperométrica fue +0.65 V, flujo de 2 mL/min. Se han establecido las curvas de calibración y se han calculado los principales parámetros de calidad.

Finalmente se ha aplicado el método desarrollado para la determinación de fenoles totales en diferentes aguas de cocido de corcho, comparándose los resultados con los obtenidos mediante la aplicación del método fotométrico comúnmente utilizado. Se ha obtenido una buena correlación entre ambos, si bien el contenido total obtenido es mayor con el método fotométrico debido a su poca selectividad, ya que incluye cualquier compuesto presente con características antioxidante diferente a los fenoles.

Este trabajo fue presentado como Keynote al *XXXV Meeting of Electrochemistry of the Spanish Royal Society of Chemistry – 1st E3 Mediterranean Symposium: Electrochemistry for Environment and Energy,* en Burgos del 14 al 16 de julio de 2014 y está pendiente de remisión para su publicación.

Flow injection analysis with electrochemical detection using printed electrodes modified with graphene for determination of total phenolic compounds in cork boiling water samples

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Abstract

Phenolic compounds are interesting due to their antioxidant properties and anticarcinogenic activity. Different phenolic compounds have been found in cork boiling water samples. In this work, a new method to determine the total phenolic compounds content by FIA with electrochemical detection (screen-printed electrode modified with graphene) has been developed. The most widely method used to determine total content of phenolic compounds is based in spectrophotometry by using the Folin-Ciocalteau reagent, which provides the total content of phenols in the samples, however, this reagent is able to reduce all the species in the sample that have any antioxidant capacity.

In the present work, the determination of phenolic compounds by the flow injection analysis electrochemical detection using screen-printed carbon electrodes modified with graphene was explored. Hydrodynamic curves were obtained and the different instrumental chemical variables were properly optimized (applied potential 0.65 V, step interval 0.12 s, flow rate 2 mL/min, and pH 4.5). The figures of merit were also calculated for the calibration curves obtained. Finally, the application of the optimized methodology was performed in cork boiling water samples and the results obtained were expressed as mg gallic acid equivalents (GAE)/Kg of cork. On the other hand, a good correlation between the results obtained by spectrophotometric method and the method developed in this work has been found.

Keywords: FIA, phenolic compounds, electrochemical detection, screen printed electrodes, graphene

9.1. Introduction

Cork comes from the outer bark of *Quercus suber* L. This bark is removed from the tree periodically, every 9 years approximately depending on the region. Portugal, Spain, Algeria, Morocco, Tunisia, Italy and France (del Pozo et al., 1999) are the countries that have cork forests extend over an area of nearly 2.7 million hectares (Ha). Cork is a vegetal tissue made of dead cells that protect the living parts of the oak's trunk and branches. This versatile material has been used for different purposes since ancient times, which are thermal insulation, decoration, and so on. Currently, its main use is for the manufacturing of caps on wine. One of the first steps, after the extraction of the cork from the tree, is the immersion in boiling water for one hour. The aim is to clean the material, remove any water-soluble substances or microorganisms, and improve its texture and plastic properties (Ávila et al., 1998). The analysis of bioactive compounds (phenolic compounds) presents a great interest due to their antioxidant and anti-carcinogenic properties (Sánchez et al., 2013, Watson et al., 2014).

Some of these phenolic compounds are present in different ratios in several lignocellulosic materials, such as cork, and thereby in waste by-products of the associated industries, which has been object of a several studies in the last few years (Silva et al., 2005). In the group of phenolic compounds some acids as ellagic, vainillic, gallic, protocatechuic, salicylic, ferulic, syringic, or vainillin, coniferaldehyde, synapaldehyde can be highlighted among others.

In the literature published different analytical techniques have been applied for the the determination of phenols in numerous matrixes. The most used method is the spectrophotometric one (Singleton and Rossi, 1965) usually carried out using the Folin–Ciocalteu reference method. This method presents low selectivity; aspect that cannot be so much important considering that, in most cases, the aim is to obtain a total index of polyphenols in each sample for being able to estimate the “antioxidant capacity”. Likewise, the application of electroanalytical techniques has been also explored in this field. They are particularly well suited for the analysis of polyphenols, because the antioxidant properties of these compounds are related to their ability to donate electrons (Kilmartin and Hsu,

2003). As described in the literature, the electroanalytical evaluation of the "total polyphenolic" content has been carried out by using different electrodes, namely carbon, bare or modified glassy carbon, biosensors etc., (Kilmartin and Hsu, 2003; Sánchez et al., 2013; Makhotkina and Kilmartin, 2010; Souza et al., 2011). Recently, the advent and succeed of screen-printing technology have opened new exciting opportunity to apply electrochemical techniques for the analysis of this compounds. Modified or not graphene, carbon nanotubes, are opening new possibilities for the analysis of polyphenols because of the screen-printed electrodes present suitable reproducibility, more versatility and they improve the kinetic of the electrode process.The use of printed electrodes (SPE) offers the possibility to propose rapid and simple methods for the determination of interesting compounds. The SPE are easy to use and have the facility to modify his surface according to different strategies, for example to favor the kinetic electrochemistry or other ends. For that, is interesting their use.

W. Siangproh et al. (Weena et al., 2009) have published a review in 2009 about flow injection analysis with electrochemical detection, by usisng different types of electrodes and several electrochemical detection in different fields. The interest is due to the need or search of automation, in the chemical analysis, and in that the FIA has found a great number of applications.

The goal of this paper is to develop a single, fast and low cost method to determine the total content of phenolic compound in boiling water cork samples. In a previous paper (Guiberteau-Cabanillas et al., 2015), a method to determine by DPV the content of total of phenols of low oxidation potential in cork boiling by using SPE_GPH was proposed. With the purpose to develop a fast method for determination of total contents of phenols, and not only of phenols of low oxidation potential values, in cork boiling water samples, a FIA method, by using SPE modified with graphene has been developed

In addition of to be a fast method of analysis, when the SPE for natural samples are used it is not necessary remove each electrode after each measurement. The reason is the low residence time on the surface electrode. In this situation, no adsorption has been observed. Nevertheless, analysis in bath is necessary to remove the electrode after each measurement when the samples are boiling cork water. FIA analysis, allows using the same

electrode (SPE) 30-40 times in natural samples, as cork boiling water samples. Therefore, the total cost of analysis, using screen printed electrodes and even more the modified ones in bath is more expensive than FIA analysis.

9.2. Experimental

9.2.1. Reagents

All chemicals used were analytical grade reagents. Gallic, ellagic and acetic acids were supplied by Sigma-Aldrich. Sodium acetate, phosphoric acid, monosodium phosphate anhydrous, disodium phosphateanhydrous, sodium hydroxide, sodium carbonate anhydrous, methanol, ethanol, Folin-Ciocalteau reagent and boric acid were supplied by Panreac.

9.2.2. Samples

Standard solutions of ellagic acid (EA) and gallic acid (GA) (200 $mg \cdot L^{-1}$) were prepared, in volumetric flask, by weighing the appropriate amount and dissolving into methanol or ethanol:water (20:80), respectively. More diluted solution was prepared in 25 mL volumetric flask by taking the adequate amount and diluting with Milli-Q water to the mark, in presence of 80 µL of 3.0 M KCl electrolyte solution necessary by using silver pseudo reference screen-printed electrode and the appropriate volume of the buffers solution. The buffers solution of different pH values used to fix the pH of solution and mobile phase were the followings: acetic acid/sodium acetate; Briton-Robinsons; phosphoric acid/ phosphate monopotassium; phosphate monopotassium/phosphate dipotassium; etc.

Cork boiling water samples were obtained after immersing of different cork samples, with varying content, in boiling water for one hour. After that, the cork was immediately filtered. When the filtrate water achieved the room temperature, the final volume was determined.

9.2.3. Pretreatment of the working electrode

In order to obtain better reproducibility of the electrochemical measurements, an initial electrochemical pretreatmentof screen-printed electrode modified with graphene (SPE-GPH) was performed by applying a constant potential of + 1.4 V during 300 s (Moreno et al., 2010) in 0.02 M HAc/NaAcO (pH 4.5), which is the buffer solutions used for further electrochemical studies.

9.2.4. Instruments

Differential pulse voltammetry and amperometric measurements for electrode activation were performed with an Autolab PGSTAT 10 (Eco Chimie B.V., The Netherlands) potentiostat interfaced to an AMD K-6 26 MHz computer system and controlled by Autolab GPES 4.8 software version for Windows 98.

Amperometric flow injection analysis was carried by using bipotenciostat µStat200 (DropSens, Spain). Wall-jet flow cell (DRP-FLWCL, DropSens, Spain) for screen printed electrodes (DropSens, Spain). A peristáltic Pump Rainin Dynamax RP-1 (Oakland, California), with a rotatory head for four adjustable flow channel, flows between 0.1 $mL \cdot min^{-1}$ a 5.0 $mL \cdot min^{-1}$. A valve manual six-port rotary valve Model 1106 (Omnifit Ltd., UK) where the SPCEs were placed. An electrochemical detector µStat200 (DropSense, Spain). Screen-printed carbon electrode modified with graphene (DRP-110GPH, DropSens, Spain) were used with cable connector for SPEs (DRP-CAST DropSens S.L., Oviedo, Spain) to bipotenciostate. The signal has been received by Software DropView versión 2.0 (DropSens, Spain).

The spectrophotometric equipment was a UV/VisVarian Cary-50 spectrophotometer (Palo Alto, California, USA).

9.2.5. Analytical procedures FIA analysis

Flow injection analysis was developed using 0.05 M Acetic acid/sodium acetate buffer solution (pH 4.5) in KCl 0.01 M as working carrier. It was flowed through the electrochemical

cell by a peristaltic pump and the absence of bubbles was checked. When a constant baseline current was reached, solutions were injected into the flow stream via the valve loop (20 µL) and the fiagram was recorded at a fixed potential selected as optimum.

Appropriate concentration samples were prepared in 25 mL volumetric flasks and the appropriate volume of the buffer solutions of 0.02 M HAc/NaAcO pH 4.5 was added. Furthermore, 80 µL of 3.0 M KCl were also added and finally Milli-Q water to the mark. For the analysis of the cork boiling water, different diluted samples were prepared taking different volumes of cork boiling waters at the same chemical conditions that the samples of gallic acid.The samples were injected to the flow system as is indicated above.

The total phenolic content was calculated as GAE from the calibration curve of GA standard solutions (0.1- 8.0 mg L^{-1}) and it was expressed as mg GAE·g^{-1} of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case. The external calibration was used to analysis of total phenolic content in cork boiling waters.

9.2.6. Determination of total phenolic content by spectrophotometric method

The total phenolic content of the cork boiling water was determined by spectrophotometric method (Singleton and Rossi, 1965) using the Folin-Ciocalteau reagent. In a 25 mL volumetric flask, 0.5 mL of Folin-Ciocalteau reagent, 10 mL of aqueous sodium carbonate (75 g·L^{-1}), aliquots of 0.5 mL of each cork boiling waters and Milli Q water were added. Each mixture was kept in the dark and at room temperature for 60 min. After homogenization, the absorbance was measured at 670 nm using a UV-Vis spectrophotometer.

The total phenolic content was calculated as GAE from the calibration curve of gallic acid standard solutions (0 .1- 16.0 mg L^{-1}) and expressed as mg GAE·g^{-1} of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case.

9.3. Results and discussion

The cork boiling water contains different phenolic compounds that give an electrochemical response (oxidation peak in DPV) by using SPE-GPH electrodes. The signal appears at a potential values next, for example, to gallic acid (results not shown). For that, gallic acid was selected in a previous work to determine by DPV the total content of phenols of low oxidation potential values in cork boiling water, as mg of gallic acid equivalent/ kg of dry cork. In this work, a FIA method by electrochemical detection and using screen-printed electrodes modified with graphene has been carried out.

9.3.1. Flow injection analysis

Taking into account that gallic acid are selected to express the content of phenol compounds in cork boiling water, and in order to select the best conditions of FIA analysis, a study of gallic acid (SPE-GPH) solution by FIA analysis and using SPE-GPH, has been carried out . First, the influence of flow rate on Ip, the influence of the number of point taken per second interval, has been carried out. The optimum values as optima selected are the following: flow rate of 2 mL·min^{-1} and 12 points interval (Figure 9.1). It can be emphasized that the flow rate is a very important variable in FIA analysis.

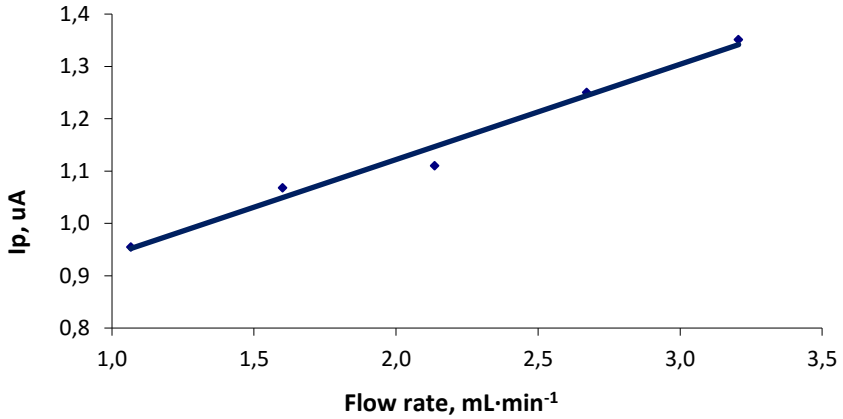

Figure 9.1. Influence of flow rate on Ip (2.0 mg L^{-1} GA; pH 4.5 (0.5M); 80 µL KCl (3M); Potential applied of +0.65 V; Step interval 0.12s)

The gallic acid and other phenolic compounds are present in cork boiling water samples. These compounds give oxidation response (DPV) in the range of potential values between 0.2-0.5V in the range of pH 2-6.

Initially, a constant potential value of +0.65 V was selected to the study of the influence of pH versus the intensity response, by FIA analysis, for solutions containing gallic acid (2 mg L^{-1}) of +0.65 V. In Figure 9.2 the influence of pH by FIA analysis is shown. The intensity of the fiagram peak is constant at pH values between 4.0 and 7.0. A pH value of 4.5 was selected for later experiments.

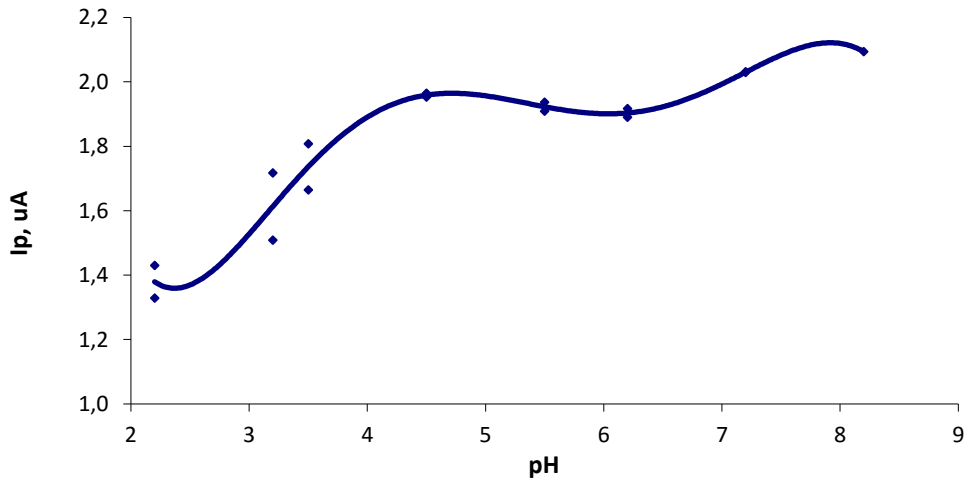

Figure 9.2 .Influence of pH on I. 2.0 mg L^{-1} GA; pH 4.5; E applied +0.65 V; Flow rate 2 mL·min^{-1}.

9.3.2. Hydrodinamyc curves

The hydrodinamic curve has been studied in the range of potential of +0.2 - +0.9 V. A mobile phase of pH 4.5 containing KCl 0.01 M and a flow rate of 2 mL·min^{-1} has been used. The solution of gallic acid (2 mg L^{-1}) at pH 4.5 were injected and the fiagram obtained to different potential values (+0.2 - +0.9 V). In Figure 9.3, hydrodynamic curves are shown. It can be observed that a plateau of Ip in the range of potential between +0.6 - +0.8 V has been found. To the subsequent studies a value of E of +0.65 v has been selected.

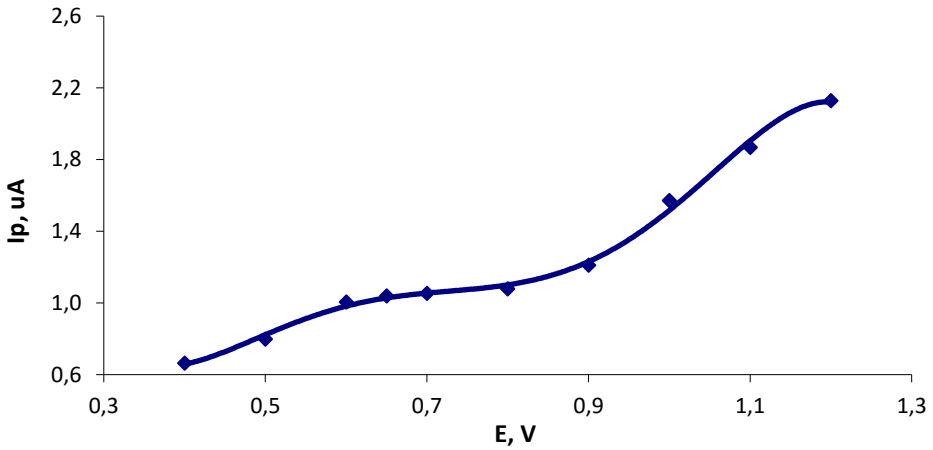

Figure 9.3.Hydrodynamic curve. GA 2.0 mg L^{-1}; pH 4.5 buffers solution; Flow rate 2 mL·min^{-1}; Potential applied +0.65 V.

9.3.3. Calibration curves. Analytical figures of merit

In order to evaluate the repeatability to the signal in FIA analysis system, a series of 12 injections of GA 2.4 mg L^{-1} was performed (Figure 9.4). The relative standard deviation calculated was 2.4%.

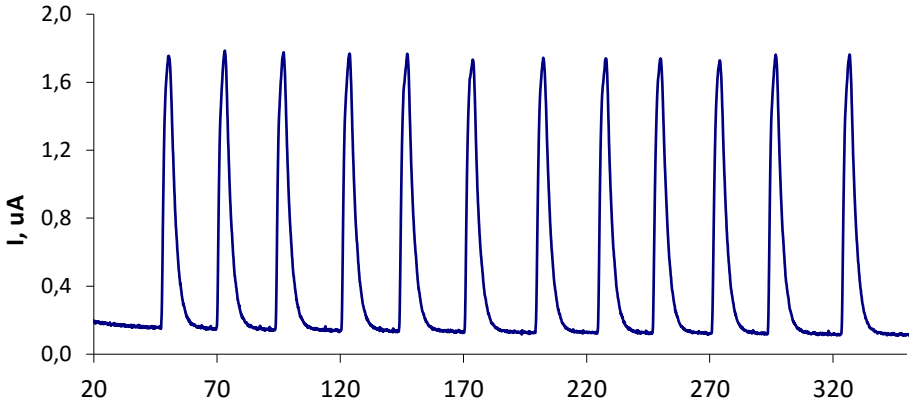

Figure 9.4. Repeatability of 2.4 mg L^{-1} GA; pH 4.5 (0.5M); KCl 80 µL 3 M; Potential +0.65 V.

The influence of gallic acid concentration in the FIA intensity response was carried out by using three repetitive injections, in the range from 0.1 to 8.0 mg L^{-1}. Results indicated that the peak current increased linearly with the increase of concentration of gallic acid have been studied, and it was found to be linear response with the concentration. In Figure 9.5 fiagrams of gallic acid for different concentrations level in triplicate, under conditions optimized, are shown. The calibration plots were stablished by external standard methodology preparing each sample by triplicate and the analytical figures of merit are summarized in Table 9.1. An adequate linearity (98.8%) and limit of detection were obtained. In Figure 9.6 a representation of Ip vs GA concentration are shown.

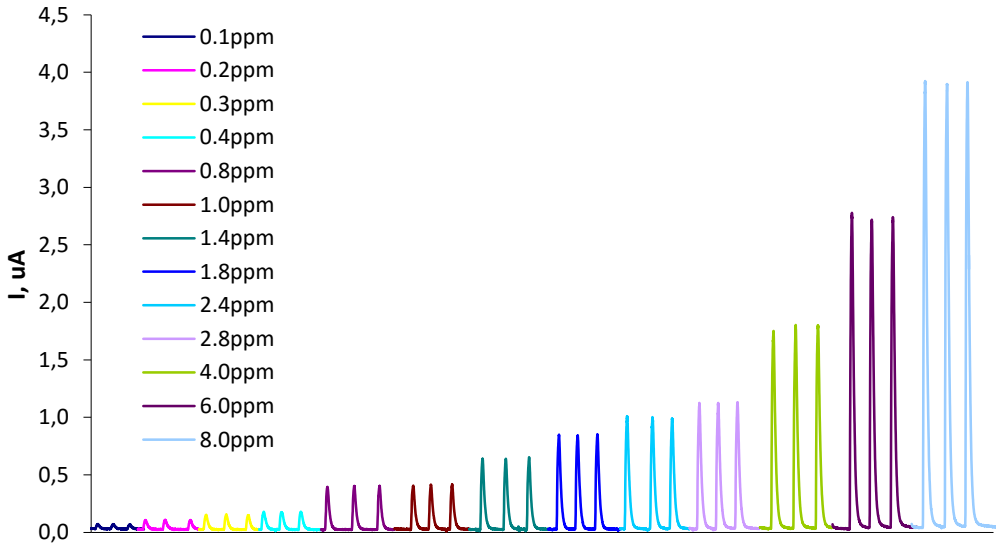

Figure 9.5. Flow injection with amperometric detection results for GA (0.1–8.0 mg L^{-1}); applied potential of +0.65 V; pH 4.5 buffer with tree injections.

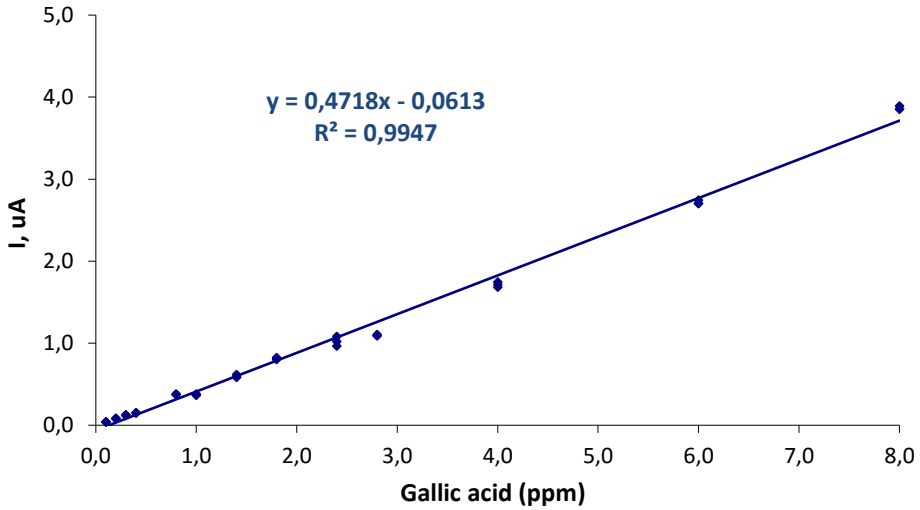

Figure 9.6. Influence of GA concetration. pH 4.5; E applied +0,65 V.

Table 9.1. Analytical figures of merit.

Slope (nA L mg^{-1})	0,4718
Standard deviation of the slope (nA L mg^{-1})	0.0057
Intercept (nA)	-0.0613
Standard deviation of intercept (nA)	0.0184
Standard deviation of regression	0.0830
Analytical sensitivity (mg L^{-1})	0.1759
R^2	0.9947
Linearity (%)	98.80
Detection limits:	
Winefordner- Long method (mg L^{-1})	0.1172
Clayton´s method (mg L^{-1})	0.3636

9.3.4. Analysis of total phenolic compounds in cork boiling water samples

After the calibration, the developed method was applied in the analysis of four different cork boiling water samples of total phenolic content expressed as mg of gallic acid equivalent·kg^{-1} dry cork. Results obtained are shown in Table 9.2.

In order to validate the proposed method, the obtained results were compared with those determined by using spectrophotometric method (Folin-Ciocalteau reagent) (see Table 9.2).

Table 9.2. Analysis of total phenolic content in differents samples of cork boiling water by FIA and Spectrophotometric (FC reagent) methods.

Cork boiling water samples	Spectrophotometric Method (FC)[a]	FIA Method (SPE-GPH-FIA)[b]	Ratio FC/SPE-GPH-FIA
A	1.86 ± 0.01	1.07 ± 0.06	1.73
B	3.68 ± 0.02	1.85 ± 0.04	2.08
C	2.10 ± 0.01	0.99 ± 0.01	2.12
D	3.17 ± 0.01	1.59 ± 0.02	1.99
E	5.98 ± 0.02	2.93 ± 0.01	2.04

[a] "Total phenols" as mg gallic acid equivalent (GAE)·g^{-1} cork ± standard deviation

[b] "Total phenols" as mg gallic acid equivalent (GAE)·kg^{-1} dry cork ± standard deviation

The data obtained with the proposed method were correlated with those obtained by using the spectrophotometric method (Figure 9.7). A good correlation is observed. As described also in the Table 9.2 the phenolic content determined expressed as gallic acid equivalents (GAE)·g^{-1} of cork by FIA methods, proposed in this work, are lower than the obtained by the spectrophotometric method. The reason is that the photometric method is less selective. Nevertheless, FIA analysis, by using SPE-GPH electrode, allows estimating

the antioxidant capacity, because it is more selective. On the other hand, it is a fast and sensitive method, and does not require any sample pretreatment. On the other hand, the ratio found between the content of phenolic compound in cork boiling water samples by FIA and spectrophotometric methods is higher than DPV/Spectrophotometric method in previous studies. Therefore, FIA analyses give more real results due to the application of a higher potential value of detection.

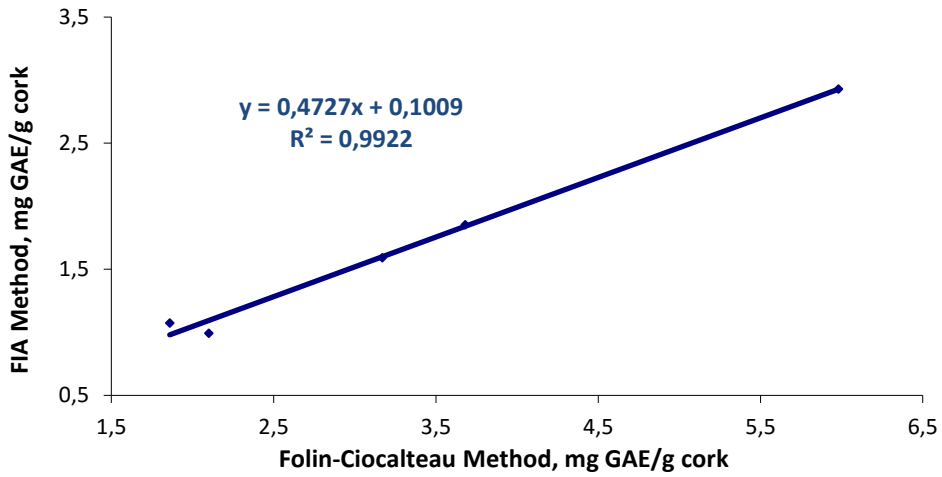

Figure 9.7. Correlation between the results obtained by FIA analysis and the results obtained by spectrophotometric method using the Folin-Ciocalteau reagent. E applied +0,65 V; pH 4.5.

9.4. Conclusion

A FIA method by using SPCE_GPH has been successfully utilized for the determination of total phenolic compound in cork boiling water expresed as mg gallic acid equivalent/kg of dry cork. A good correlation between the proposed method and Folin-Ciocalteau spectrophotometric method has been found.

The proposed FIA method with electrochemical detection (SPE-GPH) does not require any previous treatment of the samples, only water dilution and it not necessary any electrochemical cleaning step of the electrode. For this reason, it is possible their use for

routine analysis. The results obtained in this paper, have clearly revealed several advantages of the association of flow injection analysis and amperometric detection. Sensitivity and selectivity have been enhanced.

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10. Conclusiones

Conclusiones

✓ *Caracterización de los extractos de corcho*

La determinación de fenoles totales en extractos de corcho se encuentra entre 0.9 y 12.0 mg quivalente ácido gálico·g^{-1} de corcho seco. Los extractos que mayor cuantificación presentan son los extractos *B* y *C*, obtenidos por el *método_1* y *método_2*, respectivamente. También por estos mismos métodos de extracción, se obtienen los extractos con menor concentración, extractos *A* y *D*, respectivamente.

En el caso de los taninos totales, las concentraciones obtenidas están comprendidas en un rango de 1.5 a 23.3 mg equivalente ácido tánico·g^{-1} de corcho seco. Los extractos obtenidos por *método_1* y *método_2* son los que tienen mayor concentración de taninos totales, extractos *B* y *C*, respectivamente.

Los valores de la actividad antioxidante hidrofílica de los extractos de corcho se encuentran entre 13.0-152.6 µmol Trolox·g^{-1} de corcho seco, siendo el extracto *E* (*método_3*) el de mayor cuantificación. Respecto a la actividad antioxidante lipofílica, 6.9-93.0 µmol Trolox·g^{-1} de corcho seco, el extracto obtenido por el *método_1* (extracto *B*) es el que posee mayor concentración.

El mayor número de compuestos fenólicos de bajo peso molecular identificados se ha dado en los extractos *A* (*método_1*) y *F* (*método_3*); y la mayor cuantificación de compuestos fenólicos se obtiene en el extracto *C* (*método_2*). Los extractos procedentes de la fase acuosa extraída con dietil éter (extractos *B, H* y J) son los que presentan menor cuantificación. Se deduce que al realizar la extracción con dietil éter, los compuestos fenólicos de bajo peso molecular se transfieren a la fase orgánica mientras que los elagitaninos permanecen en la fase acuosa. Los compuestos fenólicos mayoritarios en los extractos de corcho han sido el ácido elágico, vescalagina, castalagina y ácido gálico.

El extracto de corcho que presenta una mayor concentración de los elagitaninos, vescalagina y castalagina, es el extracto *B* obtenido por el *método_1* de extracción, proporcionando unas concentraciones de 2141.1 y 1441.2 µg ·g^{-1} de corcho seco en

vescalagina y castalagina, respectivamente. Por el contrario, en el mismo método también se obtiene el extracto *A*, en el cual los elagitaninos se encuentran por debajo del límite de detección. Estos resultados confirman que en la extracción posterior con dietil éter, los compuestos fenólicos de bajo peso molecular se encuentran en la fase orgánica mientras que los elagitaninos están en la fase acuosa.

✓ *Aislamiento de elagitaninos*

Del estudio de estabilidad del extracto B, seleccionado para el aislamiento de elagitaninos, se puede concluir que:

- No se aprecia degradación de los elagitaninos (vescalagina y castalagina) al aumentar la temperatura hasta 60°C. Sin embargo, se aprecia una disminución de la concentración de estos compuestos con el tiempo (hasta 24h) a una temperatura dada (60°C). Esta disminución se observa en mayor medida en las primeras horas, manteniéndose después constante su contenido.

- Respecto a la estabilidad de estos compuestos en presencia o ausencia de luz, se advierte mayor degradación cuando los extractos son expuestos a la luz.

- Se percibe una diferencia notable en la degradación de los dos compuestos estudiados, siendo la castalagina más estable.

Se han obtenido unos rendimientos en la purificación de vescalagina y castalagina, a partir de extractos de corcho, de 846.5 ± 28.9 and 912.4 ± 45.1 $\mu g \cdot g^{-1}$ corcho, respectivamente. Se ha verificado la pureza de estos compuestos aislados mediante HPLC y HPLC-DAD-MS.

✓ *Actividad apoptótica de los extractos de corcho*

Se han aislado y purificado extractos de compuestos fenólicos procedentes del corcho. Según los resultados obtenidos por el Departamento de Fisiología Animal de la Universidad de Extremadura. Los resultados en conjunto indican que los extractos acuosos de corcho con contenido fenólico inducen muerte celular por apoptosis en las células leucémicas humana HL-60.

✓ *Análisis electroquímicos de aguas de cocido de corcho*

Las muestras de agua de cocido de corcho presentan señales de oxidación: prepico y pico o pico y postpico dependiendo del tipo de electrodo de trabajo que se utilice (GC o SPE-GPH). Los fenoles presentes (según análisis por HPLC) que tienen un potencial de oxidación más próximo a la señal de oxidación del agua de cocido de corcho (dependiendo del electrodo de trabajo utilizado) y que están en mayor proporción (HPLC) son los ácidos gálico y elágico. Por ello, se ha seleccionado el ácido gálico para expresar el contenido total de fenoles cuando se utiliza electrodos impresos modificados con grafeno, y ácido elágico para electrodos de carbono vitrificado.

Se proponen nuevos metodos electroanalíticos (DPV y AdSDPV), por primera vez para la determinación del contenido total de compuestos fenólicos de bajo potencial de oxidación utilizando electrodos impresos modificados con grafeno (SPE-GPH) presentes en aguas de cocido de corcho (expresándolo como mg equivalente de ácido gálico o elágico/g corcho seco). Se observa una buena correlación entre los resultados obtenidos con los métodos propuestos y los que se obtienen de la aplicación del método espectrofotométrico de Singleton y Rossi, en el que se utiliza el reactivo de Folin-Ciacolteau. No obstante, el contenido absoluto de fenoles totales por el método espectrofotométrico es mayor ya que el método es menos selectivo, debido a que determina también otros compuestos presentes que se oxiden, no sólo los fenoles. Con los metodos propuestos por DPV y AdSDPV, se determina la capacidad antioxidante de las aguas de cocido de corcho con las ventajas de: alta sensibilidad, simplicidad, utilizacion de electrodos desechables e instrumentación que puede utilizarse “in situ” en la industria del corcho. Por otra parte, no hay tratamiento previo de la muestra una vez obtenido las aguas de cocido del corcho.

El electrodo de carbono vitrificado, tambien se ha utilizado para la propuesta de métodos electroanalíticos (DPV y AdSDPV) para la determinación del contenido de fenoles totales presentes en aguas de cocido de corcho. En este caso se utiliza el ácido elágico para expresar el contenido total de fenoles de bajo potencial de oxidación. Hay una buena correlación entre los resultados obtenidos por métodos electroanaliticos con el electrodo de carbono vitrificado y por el método espectrofotométrico.

Se exponen las ventajas e inconvenientes de la utilización de los electros impresos en contraste con los de carbono vitrificado. Se concluye que los electrodos impresos

desachables proporcionan la ventaja de mejor cinética electroquímica, estrategias de modificación mejores, su fácil uso y evita procedimientos de limpieza tediosos como en el caso de los electrodos de carbono vitrificado. Por el contrario, el coste global del análisis es mayor con electrodos impresos comerciales sobre todo modificados ya que en el caso de muestras naturales, en muchas ocasiones, se limita su uso a un solo barrido.

Mediante análisis de inyección de flujo con detetección electroquímica, se ha desarrolado un método para la determinación de fenoles totales presentes en aguas de cocido de corcho, utilizando electrodos impresos modificados con grafeno. Se comparan los resultados con el método espectrofotométrico obteniéndose una buena correlación. En este caso los valores obtenidos entre los dos métodos se acercan más debido a que el potencial en la detección amperométrica es de +0,65 V.

Los resultados obtenidos, acerca del contenido de fenoles totales en diferentes aguas de cocido de corcho, expresados como relación entre los métodos propuestos en esta tesis y los obtenidos por el método espectrofotométrico son: 2.0; 11.0 y 8.0 en FIA, DPV y AdSDPV respectivamente utilizando SPE-GPH (expresando el resultado en equivalentes de ácido gálico); y 14.0 y 16.8 para DPV y AdSDPV con electrodo de carbono vitrificado (expresado como equivalente de ácido elágico), respectivamente. Las mejores relaciones se han obtenido por al análisis de inyección de flujo con detección amperométrica (FIA).

11. Anexo. Artículos publicados

PHYTOTHERAPY RESEARCH
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Quercus Suber L. Cork Extracts Induce Apoptosis in Human Myeloid Leukaemia HL-60 Cells

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***Quercus suber* L. cork contains a diversity of phenolic compounds, mostly low molecular weight phenols. A rising number of reports support with convergent findings that polyphenols evoke pro-apoptotic events in cancerous cells. However, the literature related to the anti-cancer bioactivity of *Q. suber* L. cork extractives (QSE) is still limited. Herein, we aim to describe the antitumor potential displayed by cork extractives obtained by different extraction methods in the human promyelocytic leukaemia cells. In order to quantify the effects of QSE on cancer cells viability, phosphatidylserine exposure, caspase-3 activity, mitochondrial membrane potential and cell cycle were evaluated. The results indicated that the QSE present a time-dependent and dose-dependent cytotoxicity in the human promyelocytic leukaemia cells. Such a noxious effect leads these leukaemia cells to their death through apoptotic processes by altering the mitochondrial outer membrane potential, activating caspase-3 and externalizing phosphatidylserine. However, cells cycle progression was not affected by the treatments. This study contributes to open a new way to use this natural resource by exploiting its anti-cancer properties. Moreover, it opens new possibilities of application of cork by-products, being more efficient in the sector of cork-based agriculture. Copyright © 2015 John Wiley & Sons, Ltd.**

Keywords: polyphenols; cork extracts; apoptosis; cancer cells.

Abbreviations: QSE, *Quercus suber* L. cork extractives.; PBS, phosphate buffered saline; PS, Phophatidyl-serine; ROS, reactive oxygen species; TMRM, Metramethylrhodamine methyl ester; PI, propidium iodide; FITC, Fluorescein isotiocyanate; ABTS, 2,2-azino-bis (3-ethylbenzthiazoline sulfonate)

INTRODUCTION

It is broadly known that plants cannot rely on motion as animals do; however, they contain a wide variety of molecules that confer them a chemical compensation. Secondary metabolites play heterogeneous roles such as defence against predators, pigmentation, reproduction, growth and many other functions are given mainly by plant phenolic compounds, the most widespread family of molecules in plants. Phenolic compounds can be found in plants at their leaves, bark, roots, root exudates, flowers, fruits and so on (Giovannini and Masella, 2012). *Quercus suber* L., widely known as cork oak is natively spread across southwest Europe and northwest Africa. Spain is the second cork producer in the world, where Extremadura's production corresponds to the 50% of the Spanish cork; therefore, this sector reaches a huge meaning for the aforesaid region. Cork, the bark of *Q. suber*, has been used by different cultures along the history because of its peculiar characteristics such as elasticity, low permeability or heat-resistance giving it a large variety of applications. Production of wine stoppers is the main application of this material, apart from having the highest economical repercussion. Moreover, *Q. suber* cork contains a diversity of phenolic compounds, mostly low molecular weight phenols and tannins such as ellagitannins, which has polyphenolic composition. More than a few ellagitannins has been found in *Quercus sp.*, for instance, roburin A and E, grandinin, vescalagin and castalagin, moreover others with related structures (Mayer *et al.,* 1967; Mayer *et al.,* 1971; Nonaka *et al*., 1990; Dos Remedios *et al.,* 2003; Giovannini and Masella, 2012). Lipophilic and phenolic compounds are not chemically linked, and it is simply extracting them by polar and non-polar solvents (Fortes *et al.,* 2004; Silva *et al*., 2005). Therefore, it was expected that raw extractives of *Q. suber* L. cork extractives (QSE) conserve a considerable amount of compounds contained in unprocessed bark (Santos *et al*., 2010). The main composition QSE are based on phenolic, aliphatic and triterpenic components, both in cork and cork by-products throughout industrial processing (Sousa *et al*., 2006). In line with this, a great antioxidant potential of QSE, in the range of ascorbic acid, has been reported proposing nutraceutical applications (Santos *et al*., 2010). The nutraceutical perspectives of cork consist in its low molecular weight compounds. Phenolic components of cork, as ellagitannins, have been described to induce apoptosis in tumour cells (Fernandes *et al.,* 2009; Quideau, 2009; Giovannini and Masella, 2012). In addition, other molecules contained in cork have been described to have beneficial effects in health;

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likewise, castalagin and vescalagin have been shown to possesses a substantial inhibitory properties in colon cancer (Fridrich *et al.,* 2008). Several phenolic compounds present in cork have been tested displaying an inhibitory effect in a dose-dependent manner (Fernandes *et al.,* 2009). Previous works showed cytotoxic effects of casuarin and castalagin in promyelocytic leukaemia cells (Yang, 2000). More than few reports support with convergent findings that polyphenols evoke pro-apoptotic events in cancerous cells, such as activation of caspase-3 by ellagic acid and quercitin (Mertens-Talcott and Percival, 2005), promotion of reactive oxygen species (ROS) generation and elicitation of intrinsic by woodfordin I (Liu *et al.,* 2004), among others (Giovannini and Masella, 2012).

Apoptosis is a process accurately controlled by a cell death signalling to keep the homeostasis of adult tissues. There are two canonical pathways by which apoptosis signal is transmitted, outer pathways or receptor dependent and inner pathway or mitochondrial dependent. During inner apoptotic pathways, mitochondria suffer a loss of their membrane potential, permeabilizing their membrane and allowing the free ion flow and releasing pro-apoptotic factors. Uncontrolled apoptosis plays an essential role in different pathologies, both by over-activation such as neurodegenerative diseases and by under-activation such as cancer. As mentioned earlier, a growing number of studies report the anti-cancer bioactivity of polyphenols in different cancer cell models (Giovannini and Masella, 2012; Cerella *et al.,* 2013). Scarce are the studies about the anti-cancer bioactivity of QSE. Herein, we describe the anti-tumor potential displayed by cork extractives obtained by different methods in a leukaemia cell line. This study will contribute in the near future to design nutraceutics with anti-tumor and antioxidant properties from raw extractives of *Q. suber* L. cork.

MATERIAL AND METHODS

Materials

Human promyelocytic leukaemia (HL-60) 15-12 cell lines (ECACC N° 88120805) were purchased from The European Collection of Cell Cultures (ECACC) (Dorset, UK). RPMI 1640 medium, fetal bovine serum, penicillin/streptomycin and N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin were obtained from Lonza (Porriño, Spain). Tetramethylrhodamine methyl ester (TMRM) was obtained from Molecular Probes (Eugene, Oregon, USA). Annexin binding buffer, propidium iodide (PI) and annexin V-fluorescein isotiocyanate (FITC) were obtained from Immunostep (Salamanca, Spain). All other reagents were of analytical grade.

Cell culture

Human promyelocytic leukaemia cells, a human promyelocytic leukaemia cell line, were cultured in RPMI 1640 complete medium (Lonza) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were grown in a humidified air/CO_2 (5%) atmosphere at 37 °C and maintained in an exponential growth phase for all experiments. Cells were routinely plated at a density of 3×10^5 cells/mL into fresh flasks and the viability was >95% in all experiments as assayed by the trypan-blue exclusion method.

Cork samples

Reproduction cork planks ('refuge' grade) were provided by several local companies of San Vicente de Alcántara (Badajoz, Spain). Cork samples were reclassified according to industrial criteria. An average sample composed by fragments of several planks from different industries was made.

Phenolic extraction

Cork samples, free of outer bark, were ground and sieved (0.25–0.50 mm particle size). About 20 g of the cork powder sample was submitted to a soxhlet extraction with dichloromethane for 6 h to remove the lipophilic components. Then, the solid cork residue was divided into two fractions (I and II), which followed distinct extraction pathways. Extracts A, B and C were obtained according to the procedure of Santos *et al.* (Santos *et al*., 2010).

On the other hand, other reproduction cork planks ('refuge' grade), from the same local companies, were ground and sieved (0.5–1.0 mm particle size), free of outer bark too. About 20 g of the cork powder sample was extracted with 200 mL of aqueous acetone (Me_2CO: water, 7:3) in the dark and under nitrogen at room temperature with magnetic stirring for 24 h. The extracts were filtered, evaporated under reduced pressure (to remove Me_2CO) and then freeze dried, according to the procedure of Zhentian *et al*. (1999), yielding extract F.

Total phenolic content

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method (Singleton and Rossi, 1965). The 10 mg extracts of A, B, C and F were dissolved in 10 mL aqueous methanol (MeOH: water, 50:50). Folin–Ciocalteau reagent containing 0.5 mL and 10 mL of aqueous sodium carbonate (75 g/L) were added to an aliquot of 0.5 mL of each extract, to a final volume of 25 mL. Each mixture was kept for 60 min at room temperature in the dark. After homogenizing, the absorbance was measured at 670 nm, using a UV/Vis Varian Cary-50 spectrophotometer (Palo Alto, CA, USA). The total phenolic content was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (0.5–16.0 μg/mL) and expressed as microgram of gallic acid equivalent per gramme of dry extract. The analyses were carried out in triplicate and the average value was calculated in each case.

Antioxidant capacity

Total antioxidant capacity was evaluated by means of a colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions. This assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2-azino-bis (3-ethylbenzthiazoline sulfonate) (ABTS) to ABTS by metmyoglobin. The capacity of the antioxidants in the sample to prevent ABTS oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and quantified as millimolar Trolox equivalents.

Toxicity assay

Propidium iodide is fluorescence molecule, which passes across damaged plasma membranes and intercalates double-stranded DNA. PI was used as a dye to amplify the fluorescence of non- vital cells. After treatments with QSE and vehicle control (1% dimethyl sulfoxide (DMSO)), cells were stained with PI, and the later fluorescence monitoring allowed the evaluation of toxicity of the treatments regarding the time and dose. Each sample was tested 5–8 times in independent experiments.

Annexin-V/propidium iodide apoptosis assay

After treatments with QSE and vehicle control (1% DMSO), cells were washed twice with phosphate buffered saline (PBS) and centrifuged at 500 g for 5 min; then the supernatant was discarded, and the pellet was resuspended in 95 μL annexin V-binding buffer containing annexin V-FITC at a density of 10^6 cells/mL. Cells were analyzed by flow cytometry (Cytomycs FC-500; Beckman-Coulter, Hialeah, FL, USA) after addition of PI. Each sample was tested 3–5 times in independent experiments. Annexin V binds to those cells that express phosphatidylserine (PS) on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows for live cells (unstained with either fluorochrome) to be discriminated from early apoptotic cells (stained only with annexin V), late apoptotic or necrotic cells (stained with both annexin and PI) (Bejarano *et al.,* 2011).

Measurement of caspase-3-like activity

The measurement of DEVD-AMC (Asp-Glu-Val-Asp 7-amido-4-methylcoumarin) (Sigma, Madrid, Spain) cleavage was performed using a modified version of a fluorometric assay. Treated cells with QSE and 1% DMSO (control) were pelleted and washed once with PBS. After centrifugation, cells were resuspended in PBS at a concentration of 2×10^6 cells/100 μL; 25 μL of the suspension were added to a microtiter plate and mixed with the appropriate peptide substrate dissolved in a standard reaction buffer (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 % sucrose, 5 mM dithiothreitol (DTT), 0.001% NP-40 (Nonidet P-40) and 0.1 % 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), pH 7.25). Cleavage of the fluorogenic peptide substrate was monitored by aminomethyl coumarin (AMC) liberation in an automatic microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) using 355 nm excitation and 460 nm emission wave-lengths.

Mitochondrial membrane potential

The quantification of mitochondrial membrane depolarization was carried out by measuring the fluorescence intensity of the membrane-permeant cationic probe TMRM. The resulting signal of TMRM-stained cells thus represents only the probe accumulated in intact mitochondria. A collapse on mitochondrial membrane is indicated by a decrease in output fluorescence. Briefly, treated cells with QSE and 1% DMSO (control) (1×10^6 cells/mL) were washed once with PBS and loaded with 1 μM TMRM by incubation at 37 °C for 30 min. The fluorescence intensity of TMRM was measured in an automatic microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). Excitation wavelength was set at 443 nm and emission wavelength at 575 nm. Treatments were carried out in triplicate. The data are presented as fold increase over the pretreatment level (experimental/control).

Cell cycle

After treatment, treated cells with QSE and 1% DMSO (control) (approximately 1×10^6) were washed with PBS and fixed in 70 % ethanol for 30 min at 4 °C. The cells were again rinsed with PBS and resuspended in 500 μL of PBS containing 2.5 mg/mL PI and 50 mg/mL RNase. The sample were kept in the dark at 4 °C for 30 min and analyzed by flow cytometry with excitation at 488 nm and emission at measured at 560–640 nm (FL2 mode) (Cytomycs FC-500; Beckman-Coulter). Cells undergoing apoptosis stain with PI and exhibit a reduced DNA content with a peak in the hypodiploid region (Bejarano *et al.,* 2009). The percentage of every phase was represented, and the percentage of apoptosis was taken as the fraction containing hypodiploid DNA.

RESULTS

After extraction and determination of total antioxidant capacity and phenolic content of the different QSE (Table 1), we firstly analyzed the toxicity of every QSE on HL-60 leukaemia cell line. It is noteworthy to mention that each QSE has been extracted by different methods. Therefore, it could be expected that different concentrations among the different kinds of phenolic molecules are present on QSE. The treatments were applied keeping the phenolic constant the phenolic content. Toxicity was studied through the loss of plasma membrane integrity and the consequent PI permeability. Despite not all the extractives have the same toxicity regarding to their phenolic content, PI fluorescence manifests a clear dose-dependence in all types assayed getting their maximal effect at the maximal concentration treated 30 μg phenol/mL during 24 h (Fig. 1A). Likewise, the evaluation of kinetic signature of toxicity exhibits a rising toxic behaviour along the analyzed times. Coherently, both curves of dependence indicate

Table 1. Phenolic content and antioxidant capacity of cork extractives. The total phenolic content was calculated as gallic acid equivalent from the calibration curve of gallic acid and expressed as g gallic acid equivalent. The capacity of the antioxidants in the sample to prevent 2,2-azino-bis (3-ethylbenzthiazoline sulfonate) oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and quantified as millimolar Trolox equivalents.

Type of extract	μg GAE/g extract	μmol Trolox/mg extract
A	0.227 ± 0.010	6.43 ± 0.29
B	0.303 ± 0.015	7.25 ± 0.36
C	0.296 ± 0.006	6.55 ± 0.14
F	0.316 ± 0.013	8.42 ± 0.34

that the QSE type A possesses the most toxic effect on HL-60 cells at 30 μg phenol/mL (Fig. 1B). Unlike other types, the shortest time and the lowest phenolic concentration were enough for type A to carry out a significant increase of toxicity values (Fig. 1).

To determine the apoptogenic capacity of the QSE in HL-60, we analyzed the fluorescence of annexin V-FITC after treating cells with 30 μg phenol/mL for 24 h. The FITC fluorescence values evidenced an increase of the PS exposure at the outer leaflet of the plasma membrane after treatments. Such an apoptotic event indicates that the toxicity shown by QSE induces cell death mainly by apoptosis (Fig. 2). Moreover, no FITC negative cells were found during the analysis, fact which supports the absence of other types of cells death (Fig. 2).

To make firm the results that indicate apoptogenic properties of the QSE on leukaemic cells, caspase-3 activity was analyzed. The signal of specific fluorogenic substrate revealed that the four QSE promote the caspase-3 activation significantly at 30 μg phenol/mL for 24 h (Fig. 3). These outcomes agree with PS exposure reinforcing that QSE trigger apoptotic cell death in HL-60 cells.

In order to study the mitochondrial alterations under the stated QSE, TMRM assay was carried out. Interestingly, a high percentage of HL-60 cells suffered a loss of mitochondrial potential when were treated individually with the four QSE containing 30 μg phenol/mL for 24 h (Fig. 4). This indicates a mitochondrial damage, which could be involved in the apoptotic signal caspase-3 activation upstream.

Last but not the least, cell cycle was analyzed. The profile of DNA content shows that phases containing checkpoints, such as S, G1 and G2/M, did not increased their percentage. Therefore, cell cycle did not stop its progress because of the treatments. On the contrary, hypodiploid phase outcomes agree with previous values; extracts evoked an important increment of sub-G1 phase at the expense of the remainder phases, where accordingly, the type A also manifested the strongest effect on DNA fragmentation.

DISCUSSION

Plant polyphenols are being considered among the most promising anticancer chemopreventives (Giovannini and Masella, 2012). Actually, the risk of cancer has been shown to be relieved upon high dietary polyphenol intakes (Knekt *et al.,* 2002). Radical scavenging properties of polyphenols avoid lipid peroxidation and DNA damages, events which are closely linked with carcinogenesis. Malignant cells have accumulated mutations because of DNA damages becoming much more resistant to apoptosis than healthy cells. A growing number of works report that plant polyphenols reset apoptotic pathways in tumor cells (Orlikova and Diederich, 2012). The mechanisms through which these compounds act restarting apoptosis on tumor cells are heterogeneous. Certain phenolic compounds activate cell death acting as agonist of cell death receptors, such as luteolin (Horinaka *et al.,* 2005). Others perform their apoptotic action by triggering the intrinsic pathway, such as theaflavins and thearubigins, the major polyphenols of black tea, which has been reported to alter Bcl-2/Bax ratio, up-regulated by p53 and triggering mitochondrial apoptosis (Halder *et al.,* 2008). However, some phenols such as epigallocatechin-3-gallate, curcumin, quercetin and resveratrol have been shown triggering simultaneously intrinsic and extrinsic pathways, where Bid links both apoptotic pathways (Orlikova *et al*., 2012). In this study, we show the molecular events involved in the apoptotic effects induced by raw extracts obtained from *Q. suber* L. cork on human promyelocytic leukemia

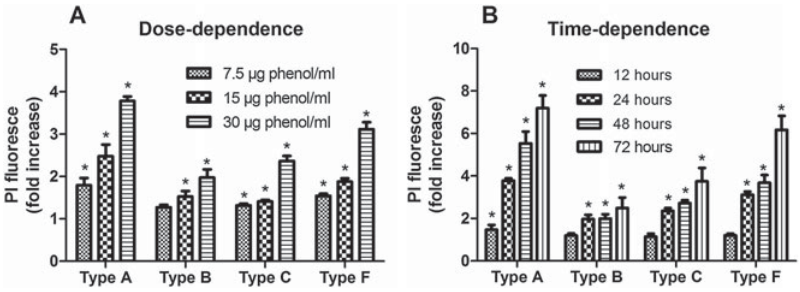

Figure 1. Cork extracts induce a dose-dependent and time-dependent decrease on viability of leukaemia cells. Human promyelocytic leukaemia cells were incubated for 24 h with increasing concentrations of phenols contained in cork extracts (A) or with cork extracts at a final concentration of 30 μg phenols/mL for several periods of time (B). Vehicle (1% dimethyl sulfoxide) was used as control. Viability was analyzed as described in the Material and Methods section. Values are presented as means ± SD of 5–8 separate experiments, which were carried out in triplicate and expressed as fold increase (experimental/control). $*P < 0.05$, compared with control values. PI, propidium iodide.

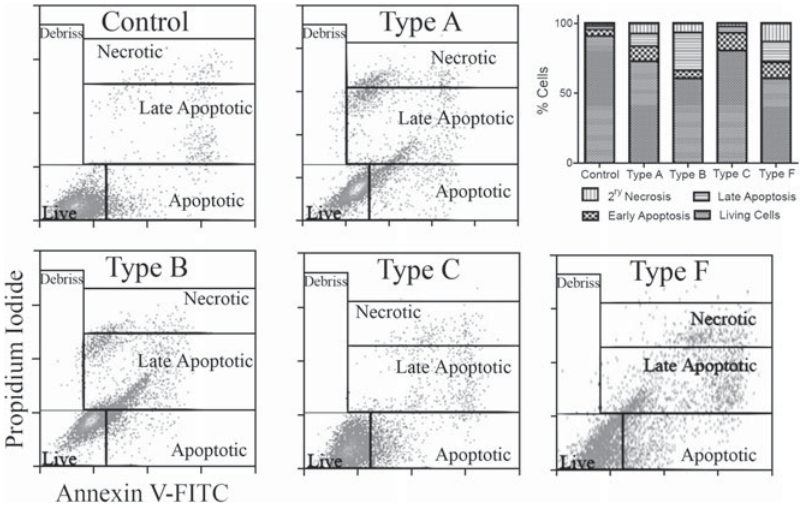

Figure 2. Cork extracts induce apoptosis in human promyelocytic leukaemia cells (HL-60). HL-60 were treated for 24 h with *Quercus suber* L. cork extractives containing 30 μg phenols/mL. Vehicle (1% dimethyl sulfoxide) was used as control. Apoptosis was analyzed as described in the Material and Methods section. Cytometer images shown were selected as the most representative. The results are represented as the distribution of the apoptosis phase's percentage, and they are representative of 3–5 independent experiments.

HL-60 cells. PI staining evidenced a dose-dependent and time-dependent mortality of HL-60 cells treated with the QSE, which was mediated by apoptotic cell death as phosphatidylserine externalization evidenced. Predictable outcomes taking into account that phenolic acids are outstandingly present in cork (reported by Conde *et al.,* 1997; Cadahía *et al.,* 1998; Conde *et al.,* 1998; Sousa *et al*., 2006). In particular, in our current system HL-60 cells, cork phenolic molecules separately have been presented as potential therapeutic agents for the treatment of leukaemia or with preventive properties. Such is the case that, on HL-60 cells, cork phenolic molecules such as ellagic acid has been described as a potent inducer of apoptosis (Hagiwara *et al.,* 2010); gallic acid was shown inducing apoptosis taking it place through both physiological and cell damage pathways (Yeh *et al*., 2011); results with caffeic acid suggest an apoptosis associated with mitochondrial dysfunction

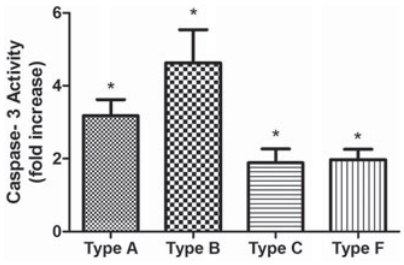

Figure 3. Cork extracts induce activation of caspase-3. Human promyelocytic leukaemia cells were treated for 24 h with *Quercus suber* L. cork extractives containing 30 μg phenols/mL. Vehicle (1% dimethyl sulfoxide) was used as control. Bars represent the means ± SD of 6–8 separate experiments and expressed as fold increase (experimental/control). $*P < 0.05$, compared with control values.

(Chen *et al.,* 2001); HL-60 cells underwent apoptosis under protocatechuic acid treatment by unbalancing the Bcl-2/Bax ratio (Tseng *et al*., 2000); also, castalagin has been reported to exert cytotoxic effects by inducing DNA fragmentation and apoptotic cell death. Despite a rising number of studies showing anticancer properties of plant extracts, there is a great scarcity of literature describing the physiological events triggered by QSE on cancer cells. Accordingly to similar researches carried out with polyphenol-rich extracts (Benarba *et al.,* 2012; Li *et al.,* 2013), QSE fostered caspase-3 activation, a key protease in most of apoptotic processes whose activation represents the point of no return in apoptosis signalling.

Preservation of mitochondrial potential is essential to keep at bay the release of mitochondrial pro-apoptotic factors. QSE-treated cells suffered disruption of mitochondrial functions as results revealed. This fact is in agreement with previous findings performed on cancer cells treated with both polyphenol-rich extracts (Benarba *et al.,* 2012; Li *et al.,* 2013) and cork phenolic molecules separately (Tseng *et al*., 2000; Chen *et al.,* 2001; Alfredsson *et al.,* 2014). Therefore, QSE could collaborate with the mitochondrial apoptotic pathway by altering the permeability of the mitochondria and releasing the apoptosis transducers. As evidenced by results, each QSE shows its particular behaviour for each assay performed. Such a fact could be expected given that each QSE likely contains different relative composition among phenolic compounds. The relative concentration of phenolic compounds of QSE is not only determined by the extraction method but also it depends on the phenolic composition of the raw material. The location where cork is generated will affect the phenolic content of cork. The soil type, the weather conditions, the action phytofagous and so on are closely bound to the final composition of raw materials. Keeping in mind in Table 1 that neither the starting richness

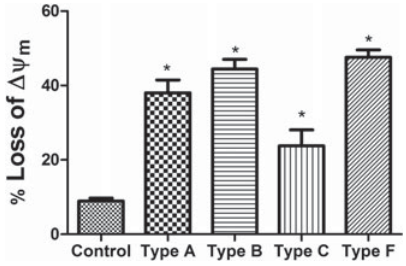

Figure 4. Cork extracts reduce the mitochondrial membrane potential in leukemia human promyelocytic leukaemia cells (HL-60). HL-60 metramethylrhodamine methyl ester-loaded cells were treated for 24 h with *Quercus suber* L. cork extractives containing 30 μg phenols/mL. Vehicle (1% dimethyl sulfoxide) was used as control. Values are presented as percentage ± SD of 6–8 separate experiments and expressed as fold increase (experimental/control). $*P < 0.05$, compared with control values.

of phenolic compounds in QSE nor their antioxidant capacity does not show a correlation to their proapototic properties. Given that the phenolic composition of cork is not fixed, this entails that reproducing exactly the same results is unlikely. Nonetheless the main message of this study is that QSE keep proapoptotic effect on HL-60 cells to a greater or lesser extent.

On the other hand, some of the main compounds present in QSE have been reported to induce alterations in cell cycle kinetics (Agarwal *et al.,* 2006; Kuriyama *et al.,* 2013; Zhang, 2014). Surprisingly, in our study, cells did not suffer a block in the cell cycle, given that the ratio among phases did not undergo any change. In this regard, the literature about the effects of isolated QSE polyphenols on cycle of tumor cells remains still limited. The last but not least, the DNA quantitative analysis exhibited additional information. Figure 5 shows a substantial increase in the sub-G1 phase after a treatment with QSE at the expens of the rest of phases, what is indicative of DNA fragmentation, and is also coherent with the presence of apoptotic events described earlier. Dividing cells are able to interrupt the cycle progression in phase G1, S or G2 checkpoints to repair DNA damages before continuing. Depending on the gravity of the damage, the cell decides whether repairing or initiating intracellular apoptosis signaling in response to damage will remove potentially hazardous cells (Hanahan and Weinberg, 2000). Therefore, we hypothesize that the leukaemia

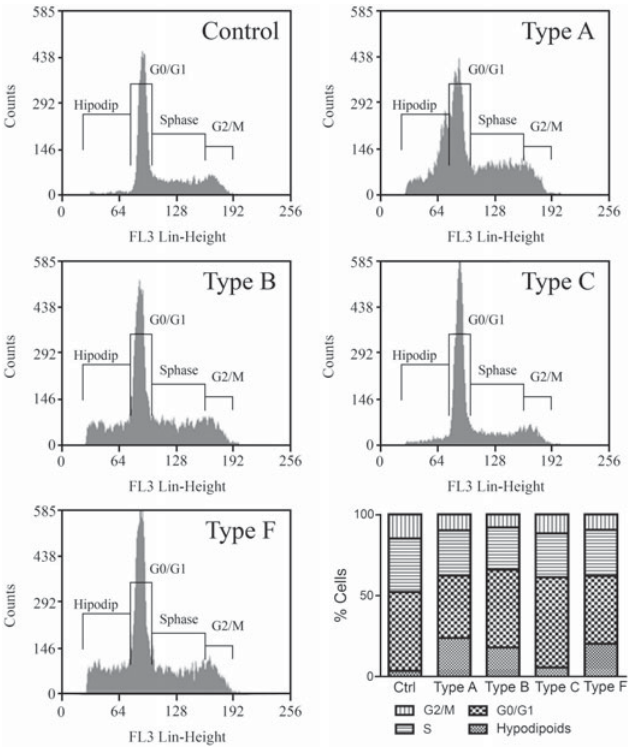

Figure 5. Cork extracts did not affect cell cycle progression. Human promyelocytic leukaemia cells were treated for 24 h with *Quercus suber* L. cork extractives containing 30 μg phenols/mL. Vehicle (1% dimethyl sulfoxide) was used as control. Cytometer images shown were selected as the most representative. The results are represented as the distribution of the cell-cycle phase's percentage and are representative of 6–8 independent experiments.

cells sensitive to the treatment are directly prompted to apoptosis, as revealed in DNA content analysis (Fig. 5).

In summary, cork is a renewable resource, which has reached an outstanding social and economic meaning in Spain, particularly in Extremadura. More than 75 % of cork produced all around the world is used to made cork stoppers for wine, generating a lot of by-products, which are mainly used as biofuel or compost. This study explores a door to new possibilities of application of cork by-products, being more efficient the sector of cork-based agriculture. The present study reports the QSE-induced apoptosis in HL-60 cells altering the mitochondrial outer membrane potential, activating of caspase-3 and externalizing phosphatidylserine. Nonetheless, additional studies regarding anti-tumor properties are needed, by analyzing isolated QSE compounds to be checked in a wide number of tumor cell lines. Anti-cancer and anti-tumor properties of cork extractives open a new way to exploit this natural resource.

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Conflict of Interest

The authors declare that there is not conflict of interest in this study.

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Electroanalytical Behavior of Gallic and Ellagic Acid Using Graphene Modified Screen-Printed Electrodes. Method for the Determination of Total Low Oxidation Potential Phenolic Compounds Content in Cork Boiling Waters

Agustina Guiberteau-Cabanillas,*[a] Belén Godoy-Cancho,[b] Elena Bernalte,[a] Miriam Tena-Villares,[a] Carmen Guiberteau Cabanillas,[c] and Manuel A. Martínez-Cañas[b]

Abstract: New electroanalytical methods have been proposed for the estimation of the total content of phenolic compounds of low oxidation potential in cork boiling water, expressed as gallic acid equivalent/g dry cork, by using screen-printed electrode modified with graphene. The results obtained have been compared with spectrophotometric method (Folin-Ciocalteau) and a good correlation has been found between them. The detection limits obtained by Winefordner-Long and Clayton methods were 0.04 and 0.06 ppm for ellagic acid (EA), and 0.05 and 0.09 ppm for gallic acid (GA), respectively. The sensitivity, simplicity, selectivity, fast and low cost of the developed methodologies is also demonstrated.

Keywords: Gallic acid · Ellagic acid · Screen-printed electrodes · Phenolic compounds · Cork boiling water

1 Introduction

The analysis of bioactive compounds (phenols and polyphenols) is of great interest for its antioxidant antimutagenic, antithrombotic, anti-inflammatory and anti-carcinogenic properties [1,2]. Some of these phenols or polyphenols, in particular the acids ellagic, vanillic, gallic, protocatechuic, salicylic, ferulic and syringic, or vanillin, coniferaldehyde and synapaldehyde are present in different amounts in several lignocellulosic materials, such as cork, and thereby in waste by-products of the associated industries, which has been object of a several studies in the last few years [3].

Cork comes from the outer bark of *Quercus suber L*, commonly known as oak. This bark is removed from the tree periodically, every 9 years approximately depending on the region. Cork forests extend over an area of nearly 2.7 million hectares (Ha) in seven countries: Portugal, Spain, Algeria, Morocco, Tunisia, Italy and France [4].

Cork is a vegetal tissue made of dead cells that protect the living parts of the oak's trunk and branches.

It is a versatile material that has been used for different purposes since ancient times, among which are thermal insulation, decoration, and its main use actually for the manufacturing of caps on wine. For these applications, cork has to be previously pretreated. One of the first steps after the extraction of the cork from the tree, is the immersion in boiling water for one hour. The aim is to clean the material, remove any water-soluble substances or microorganisms, and improve its texture and plastic properties [5].

Further, this cork boiling waters are characterized by high levels of phenolic compounds [6], as tannic, gallic, protocatechuic, syringic, ellagic acid, vanillic and protocatechuic aldehyde [7]; and also vanillic and ferulic acids [8]. In fact, the isolation and characterization of phenolic compounds in cork is of interest to the pharmaceutical, food industry, etc. In the literature, several analytical methods have been reported for the determination of the "total phenols compounds" in different kind of samples. The spectrophotometric method [9], based on the reaction with the Folin–Ciocalteau reagent is the most used. This method is characterized by low selectivity because the reagent does not only measure phenols, and will react with any reducing substances.

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Nevertheless, since Blasco et al. [10] introduce the "electrochemical index" concept, which could be defined as the total phenolics concentration obtained by means of electrochemical techniques for predominant and representative phenolic classes. The electroanalytical methodologies began to play an important role and gain future prospects in the determination of the total phenolic content, considering always the inherent selectivity and sensitivity of electrochemical techniques versus spectrophotometric protocols.

As described in the literature over the past decade, these methods are particularly well suited for the analysis of polyphenols, because the antioxidant properties of these compounds are related to their ability to donate electrons. Thus, the electroanalytical analysis of the "total polyphenol" content has been carried out by using different electrodes i.e. carbon, bare or modified glassy carbon, biosensors, etc. [1,11–13]. For example, several electroanalytical methods have been developed by using a glassy carbon working electrode and they have been applied to determine the content of phenolic compounds in wine [10,14–17], tea [18] and juices [18,19]. Also, other types of electrodes, such as glassy carbon modified with carbon nanotubes [1] or biosensors (tyrosinase biosensor based on glassy carbon electrode modified with gold nanoparticle [20]) have been also used for these purposes.

Recently, the advantage and succeed of screen-printing technology has opened new exciting opportunity to apply electrochemical techniques for the analysis of this compounds. On the other hand, the screen-printed electrode without modification or modified with graphene, carbon nanotubes are opening new possibilities for the analysis of phenols/polyphenols because the screen-printed electrodes present suitable reproducibility, more versatility and improvement of the kinetic of the electrode process.

In the present work, the electroanalytical behavior of the gallic and ellagic acids is performed by using, for the first time, screen-printed electrode modified with graphene. The optimization of the instrumental and chemical variables is carried out, and the figures of merits of the methods are also established. The proposed voltammetric methods are applied to cork boiling water samples, which gives oxidation peaks at potential values next to the mentioned polyphenols, in order to determine the total low oxidation potential phenolic compounds content.

2 Experimental

2.1 Apparatus

All voltammetric measurements were performed using a computer controlled potentiostat μAutolab (ECO Chemie, Holland) and Metrohm (Herisau, Switzerland) 663 VA stand. Screen-printed carbon electrode modified with graphene (DRP-110GPH) were used with cable connector for SPEs (DropSens S.L., Oviedo, Spain)

The spectrophotometric equipment was a UV/Vis Varian Cary-50 spectrophotometer (Palo Alto, California, USA).

2.2 Reagents

All chemicals used were of analytical grade. Gallic, ellagic protocatechuic, vanillic, syringic and ferulic acids, syringic, coniferyl and sinapyl aldehyde, and vanillin were supplied by Sigma-Aldrich. Acetic acid was provided by Sigma-Aldrich, while sodium acetate, phosphoric acid, monosodium phosphate anhydrous, disodium phosphate anhydrous, sodium hydroxide, sodium carbonate anhydrous, methanol, ethanol, Folin–Ciocalteau reagent and boric acid were supplied by Panreac.

2.3 Samples

A standard of ellagic acid (EA) and gallic acid (GA) solutions (200 mg/L) was prepared, in volumetric flask, by weighing the appropriate amount and dissolving into methanol or ethanol: water (20 : 80), respectively.

More diluted solution was prepared in 25 mL volumetric flask by taking the adequate amount and diluting with Milli-Q water to the mark, in presence of 80 μL of 3.0 M KCl electrolyte solution necessary to the electroanalytical determination by using silver pseudo reference screen-printed electrode.

Cork boiling water samples were obtained after immersing of different cork samples, with varying content, in boiling water for one hour. After that, the cork was immediately filtered. When the filtrate water achieved the room temperature, the final volume was determined.

2.4 Pretreatment of the Working Electrode

In order to obtain better reproducibility of the electrochemical measurements, an electrochemical pretreatment of screen-printed electrode modified with graphene (SPE-GPH) was performed by applying a constant potential of +1.4 V during 300 s [21] in 0.02 M HAc/NaAcO (pH 4.5), which is the buffer solutions used for further electrochemical studies.

2.5 General Procedure. Differential Pulse Voltammetry (DPV)

Samples of the appropriate concentration of GA and EA were prepared in 25 mL volumetric flasks and the appropriate volume of the buffer solutions of 0.5 M HAc/NaAcO, $H_3PO_4/H_2PO_4^-$, $H_2PO_4^-/HPO_4^{2-}$ or 0.04 M Britton–Robinson buffer were added to obtain the desired pH. Furthermore, 80 μL of 3.0 M KCl were also added and finally Milli-Q water to the mark. The samples were introduced into the voltammetric cell and were analyzed by Differential Pulse Voltammetry (DPV). All measurements were performed at room temperature, and the DPV voltammograms at 50 mV of pulse amplitude and

10 mV of step potential. After each scan, the cleaning process of SPE-GPH was carried out, as following: firstly, the SPE-GPH electrode was immersed in Milli Q water, and secondly in buffer solutions to check the effectiveness of the process, ensuring that no signals appear in the voltammograms.

For the analysis of the cork boiling water, different diluted subsamples were prepared taking different volumes of cork boiling waters from 25 to 200 μL and Milli-Q water was added to the mark in 25 mL volumetric flask. Buffers solution and 80 μL of 3.0 M KCl were also added.

2.6 DPV Determination of Total Phenolics Compounds of Low Oxidation Potential in Cork Boiling Waters

The determination of total phenolic content of low oxidation potential, present in the cork boiling water, expressed as gallic acid equivalents (GAE), was carried out by two different procedures:

–Without accumulation process: In a 25 mL volumetric flask, 75–100 μL of cork boiling water, 1 mL of 0.5 M buffer HAc/NaAcO (pH 4.5), 80 μL of 3.0 M KCl and Milli-Q water to the mark were added. Then, the sample was introduced into the electrochemical cell and the DPV voltammogram was recorder.

–With accumulation process: In a 25 mL volumetric flask, 25–50 μL of cork boiling water, 1 mL of 0.5 M buffer HAc/NaAcO (pH 4.5), 80 μL of 3.0 M KCl and Milli-Q water to the mark were added. Then, the prepared sample was introduced into the electrochemical cell recording its DPV voltammograms, 30 s of accumulation time, and +0.05 V of accumulation potential and 10 s equilibrium time.

The total phenolic content of low oxidation potential values was calculated as GAE from the calibration curve of GA standard solutions (0.2–1.0 ppm) and it was expressed as mg GAE/g of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case.

2.7 Total Phenolic Content by Spectrophotometric Method

The total phenolic content of the cork boiling water was determined by spectrophotometric method using the Folin–Ciocalteau reagent [9]. In a 25 mL volumetric flask, 0.5 mL of Folin-Ciocalteau reagent, 10 mL of aqueous sodium carbonate (75 g/L), aliquots of 0.5 mL of each cork boiling waters and Milli Q water were added. Each mixture was kept in the dark and at room temperature for 60 min. After homogenization, the absorbance was measured at 670 nm using a UV/Vis spectrophotometer. The total phenolic content was calculated as GAE from the calibration curve of gallic acid standard solutions (0.5–16.0 ppm) and expressed as mg GAE/g of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case.

3 Results and Discussion

3.1 Previous Assays

The cork boiling water provides an oxidation response (pH 4.5) in the SPE-GPH electrode, as shown in Figure 1. It can be observed that a well-defined peak appears at +0.2 V and a small peak at more positive E_p (+0.32 V). The intensity of both peaks increases proportionally with the concentration of the polyphenols in the cork boiling water samples.

Taking into account the complex composition of cork boiling water due to the presence of different phenols/polyphenols and other compounds, voltammograms of different phenols solutions were registered that may be present in the samples in concordance with the literature revised.

In Table 1 the data obtained from the peak potentials and peak intensities of each compound are shown.

In order to have an estimation of the phenolic compounds of low molecular weight, cork boiling water was also analyzed by HPLC [22]. In Table 2 the results obtained are shown.

As can be seen in Figure 1 and Table 1, the peak that appears at E_p +0.2 V in cork boiling water is close to the peak potential of GA and also to other compounds. Also, the second peak observed at +0.3 V in the voltammograms of the cork boiling water samples is close to EA peak potential. On the other hand, the HPLC analysis (Table 2) determines that the majority compounds in the cork boiling water samples are GA and EA, with contents around of 43 % and 14 %, respectively.

Therefore, taking into account the potentials values of voltammetric signal observed in the cork boiling waters and the results obtained by chromatographic analysis, the GA and EA were selected as possible compounds to express the content of total phenols of low oxidation potential as mg equivalent of one of them per gram of dry cork.

For that, we proceed first to the DPV voltammetric studies of EA and GA by using the screen-printed electrodes modified with graphene (SPE-GPH).

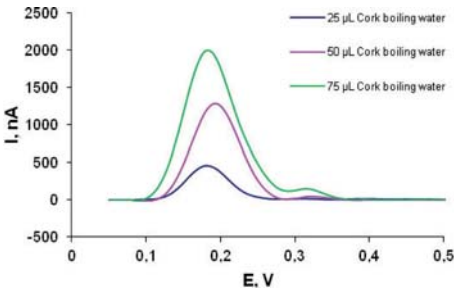

Fig. 1. Differential pulse voltammograms (DPV), baseline correction, of different volumes of cork boiling water Conditions: pH 4.5, pulse amplitude: 50 mV and step potential: 10 mV.

Table 1. Values of potentials and intensities of the peaks for each phenolics compounds. Conditions: pH of 4.5; 50 mV of pulse amplitude and 10 mV of step potential. EA: ellagic acid; FER: ferulic acid; GA: gallic acid; PA: protocatechuic acid; V: vanillin and other compounds; VA: vanillic acid; SA: syringic acid; SIR: syringic aldehyde; SIN: sinapaldehyde; CON: coniferaldehyde.

Compound	[C] (mM)	E_{p1}(V)	I_{p1}(nA)	E_{p2}(V)	I_{p2}(nA)
EA	3.3	0.304	5430	–	–
VA	6.0	0.234	2590	0.518	1030
GA	5.9	0.197	2400	–	–
PA	6.5	0.226	688	–	–
SA	7.2	0.382	983	–	–
V	6.6	0.568	1040	–	–
CON	5.6	0.226	2060	0.431	835
SIN	4.8	0.177	3930	0.304	466
FER	5.2	0.197	2580	0.402	468
SIR	5.1	0.226	1720	0.490	1720

Table 2. HPLC analysis of phenolic compounds of low molecular weight in cork boiling water.

Compound	[ppm]	%
GA	26.0	43.4
PA	13.0	21.8
VA	5.7	9.5
V	3.5	5.8
FER	1.9	3.2
CON	1.5	2.6
EA	8.3	13.8

3.2 Electroanalytical Behavior of Ellagic and Gallic Acids

3.2.1 Effect of pH

The influence of pH on I_p and E_p of EA and GA was examined in the range from 1.4 to 6.6. In Figure 2 the voltammograms obtained at each pH are shown.

The peak intensity of the compounds decreases when pH increases and remaining constant to pH values greater than 4. For further study, a value of pH 4.5 is selected, because minor changes in pH do not produce large variations in peak intensity, even though sensitivity is lost.

A linear relationship between E_p and pH for EA (E_p (V) = −0.053pH + 0.567) and for GA (E_p (V) = −0.063pH + 0.478) were respectively found. These behaviors indicate that protons are involved in the electrode process.

On the other hand, the instrumental variables were also optimized. The selected optimal values are: pulse amplitude of 50 mV and step potential of 10 mV (data not shown).

3.2.2 Influence of the EA and GA Concentration

The I_p obtained from the application of the optimized DPV methods at different concentration of EA and GA in the range 0.2 to 1.0 ppm are shown in Figure 3. As can be seen, a linear relationship was found in the whole range for the two compounds.

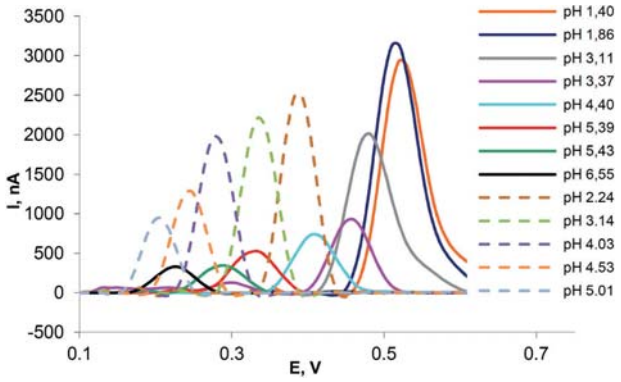

Fig. 2. DPV voltammograms of EA (solid line) and GA (dashed line), 0.6 ppm respectively at different pH values (buffers: HAc/NaAcO, $H_3PO_4/H_2PO_4^-$ or $H_2PO_4^-/HPO_4^{2-}$), 50 mV pulse amplitude, 10 mV step potential.

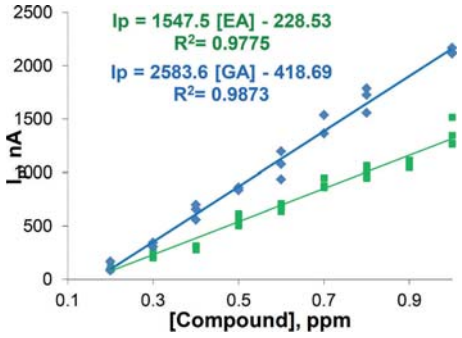

Fig. 3. Variation of the peak intensity with the concentration of EA (green) and GA (blue), (pH 4.5, 50 mV pulse amplitude, 10 mV step potential).

The application of a Matlab program to calculate the figures of merit of the linear regression curves, gives values of the detection limits of 0.05 and 0.08 ppm for EA and 0.05 and 0.10 ppm for GA, by using the Winefordner–Long [23] and Clayton [24] methods respectively.

3.3 Adsorptive Stripping Analysis of Ellagic and Gallic Acids

In the preliminary experiments it was observed that EA and GA were accumulated in SPE-GPH electrode. Therefore, we carried out also studies using differential pulse voltammetry with adsorptive preconcentration.

The influences of pH on I_p and E_p for these compounds were investigated and a similar behavior was observed to that obtained without preconcentration process.

However, the increase of the peak intensity with preconcentration for the GA is higher than the observed for the EA also with preconcentration.

3.3.1 Effect of Accumulation Potential

The study of the influence of the accumulation potential in the peak intensity was carried out in the range from 0 to +0.2 V. Due to any changes in the peak intensity were observed at values lower than +0.2 V, a value of +0.05 V was selected for further experiments.

3.3.2 Effect of Accumulation Time

The influence of accumulation time (t_{acc}) on the I_p of EA and GA was studied in the range from 15 to 120 seconds (0.02 M HAc/NaOAc pH 4.5 buffer solution). During the accumulation period, an E_{acc} of +0.05 V was applied.

In Figure 4A, a linear relationship between the accumulation time and the peak intensity was observed up to 90 seconds with a concentration of 0.6 ppm EA. In the case of GA (Figure 4B), the study of the influence of the

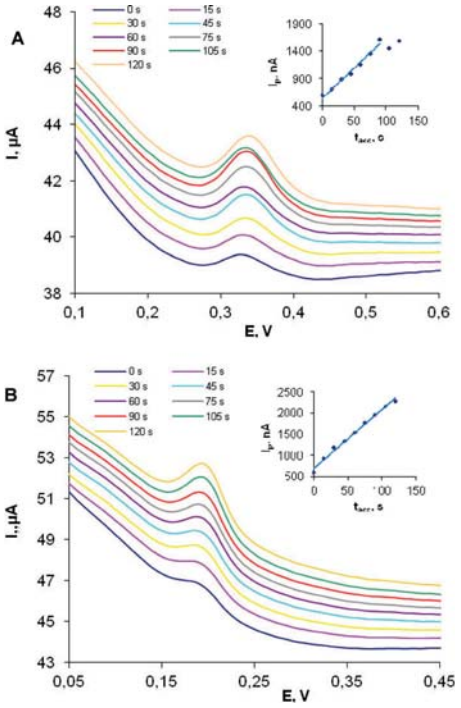

Fig. 4. Voltammograms of DPV at different t_{acc} and variation of I_p with t_{acc}: A) 0.6 ppm of EA, B) 0.3 ppm of GA. pH 4.5, 50 mV pulse amplitude; 10 mV step potential; E_{acc} = +0.05 V and t_{eq} = 10 s.

accumulation time was performed using a concentration of 0.3 ppm, because of the response at higher concentration (0.6 ppm) was only linear until 30 s of accumulation time. As shown in Figure 4B the linear relationship of 0.3 ppm of GA is maintained until 120 s of accumulation time.

So, it has been chosen for both compounds and for further studies, an accumulation time of 30 s.

3.3.3 Influence of the EA and GA Concentration

The influence of concentration on I_p, in the range 0.2 to 0.8 ppm of EA and GA are also studied using the optimized experimental conditions (E_{acc} of +0.05 V, t_{acc} 30 s).

As shown in the calibration curve (Figure 5), it demonstrates a linear behavior in the whole concentration range studied with slopes (nA/ppm) of 1552 and 5570, and correlation coefficients of 0.9893 and 0.9772 for EA and GA respectively. The detection limits were calculated by

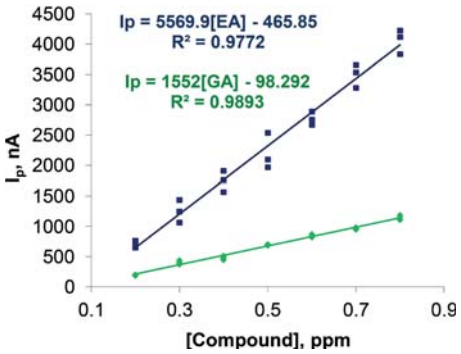

Fig. 5. Variation of the I_p with the concentration of EA (green) and GA (blue). Conditions: pH 4.5, 50 mV pulse amplitude, 10 mV step potential, +0.05 V of E_{acc}, 30 s of t_{acc}, and 10 s of t_{eq}.

Winefordner–Long [23] and Clayton [24] methods, obtaining values of 0.04 and 0.06 ppm for EA, and 0.05 and 0.09 ppm for GA, respectively.

3.4 Determination of Total Phenolic Compounds of Low Oxidation Potential in Cork Boiling Waters

The developed methods were applied for the determination of total phenolic content of low oxidation potential, in several cork boiling water samples.

The electroanalytical measurements were carrying out applying the procedure previously optimized and described in the experimental section.

According to the results obtained, the GA were used to estimate the content of total phenolic compound of low oxidation potential in cork boiling water, and the external calibrations were utilized for quantification.

In Figure 6, the voltammograms obtained, with and without accumulation step, from different samples of cork boiling water are shown.

The results obtained in the analysis of total phenolic compounds of low oxidation potential values in different real sample of cork boiling water are shown in Table 3, expressed as mg GAE/g dry cork.

In order to validate the proposed voltammetric methods, the results obtained were compared with those determined by using spectrophotometric (Folin–Ciocalteau reagent) (see Table 3). As can be seen also in Figure 7, a good correlation is observed.

As described also in the Table 3 the phenolic content determined by electroanalytical methods are lower than the obtained by the spectrophotometric method. The reason is that the photometric method is less selective. Nevertheless, we determine that the electroanalytical method proposed by using SPE-GPH electrode is capable to estimate the antioxidant power, because it is more selective, fast, and sensitive and does not require any sample pretreatment.

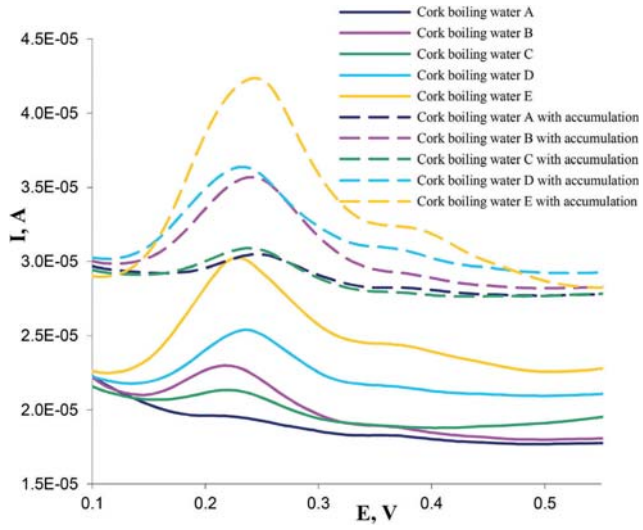

Fig. 6. Voltammograms of DPV of cork boiling water samples.

Table 3. Analysis of total phenolic content of cork boiling water boiled. Electroanalytical and spectrophotometric methods.

Cork boiling water samples	Composition [Cork boiling water], g cork/L	FC [a]		DPV [b]		AdSDPV [c]	
		Average	Std. deviation	Average	Std. deviation	Average	Std. deviation
A	860.8	1.86	0.01	0.08	0.01	0.23	0.02
B	1062.1	3.68	0.02	0.29	0.04	0.46	0.01
C	909.1	2.10	0.01	0.18	0.02	0.27	0.03
D	1760.5	3.17	0.01	0.23	0.01	0.33	0.02
E	1900.0	5.98	0.02	0.41	0.01	0.66	0.04

[a] Folin–Ciocalteau method, total phenolic content expressed as mg GAE/g dry cork. [b] Differential pulse voltammetry method, total phenolic content, of low oxidation potential compound, expressed as mg GAE/g dry cork. [c] Differential pulse adsorptive stripping voltammetry method, total phenolic content, of low oxidation potential compound, expressed as mg GAE/g dry cork.

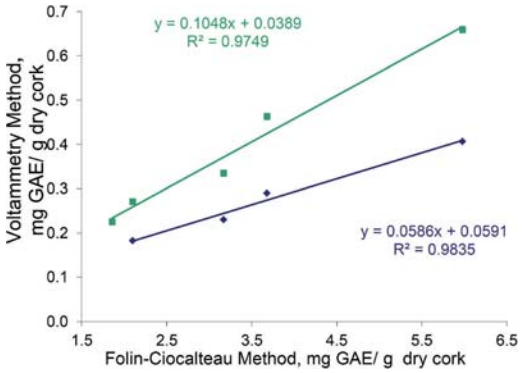

Fig. 7. Correlation between the results obtained by the electroanalytical method proposed (blue line: without accumulation and green line: with accumulation) and the results obtained by spectrophotometric method using the Folin–Ciocalteau reagent. Conditions: pH 4.5, pulse amplitude: 50 mV, step potential: 10 mV, $E_{acc} = +0.05$ V, $t_{acc} = 30$ s, $t_{eq} = 10$ s.

4 Conclusions

The developed electroanalytical methods, with or without previous accumulation step, allow the determination of total phenols content of low oxidation potential, in cork boiling water (expressed as mg GAE/g dry cork), by using SPE-GPH electrodes. A good correlation was obtained between both electrochemical and spectrophotometric methods, even though the first ones achieve lower results because the present higher selectivity and the quantification is involving only the polyphenols with low oxidation potentials.

However, the more important aspect is to determine the antioxidant capacity by using a technique that has advantages such as: high sensitivity, simplicity, good stability, low cost and portable instrumentation, and low interferences of other non electroactive compounds.

The screen-printed electrode modified with graphene presents suitable reproducibility, good versatility and favorable kinetic of the electrode process. In this work we demonstrate for the first time the applicability of voltammetric methods by using SPE-GPH in the electrochemical characterization of industrial cork by-products, especially the cork boiling water.

Additionally, other advantage of the proposed methods is that it is not necessary any treatment of the sample. So, the application of the electroanalytical methods developed by using SPE-GPH can be used for in situ measurements in the cork boiling water samples from cork industry due to the portability of the electrochemical equipment.

On the other hand, the possibility to profit the cork boiling water to obtain the phenolic compounds it is of interest.

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