NUEVAS METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE ANTIOXIDANTES ALIMENTARIOS

NEW ANALYTICAL METHODOLOGIES FOR FOOD ANTIOXIDANT DETERMINATION

Tesis Doctoral
Álvaro Andreu Navarro
Noviembre 2011

TÍTULO:*Nuevas metodologías analíticas para la determinación de antioxidantes alimentarios*

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LOS DIRECTORES

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Catedrática del Departamento de Química Analítica de la Universidad de Córdoba

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Trabajo presentado para aspirar al Grado de Doctor en Ciencias

EL DOCTORANDO,

Fdo. **Álvaro Andreu Navarro**
Licenciado en Ciencias Químicas

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CERTIFICAN: Que la Tesis Doctoral "NUEVAS METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE ANTIOXIDANTES ALIMENTARIOS" ha sido desarrollada en los Laboratorios del Departamento de Química Analítica de la Universidad de Córdoba y del Institut für Analytische Chemie, Chemo- und Biosensorik de la Universidad de Regensburg, y que a nuestro juicio, reúne todos los requisitos exigidos a este tipo de trabajo.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en la ciudad de Córdoba, a 20 de Octubre de 2011.

Agustina Gómez Hens

Juan Manuel Fernández Romero

TÍTULO DE LA TESIS: NUEVAS METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE ANTIOXIDANTES ALIMENTARIOS

DOCTORANDO/A: ÁLVARO ANDREU NAVARRO

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El doctorando D. Álvaro Andreu Navarro cursó brillantemente los estudios del Master en Química Fina Avanzada, obteniendo excelentes calificaciones en las asignaturas del mismo. El trabajo fin de Máster se publicó en la revista Journal of Agricultural and Food Chemistry, de referencia en el área de conocimiento.

La temática de la Tesis se encuadra dentro de las tendencias de la Química Analítica ya que aborda nuevas metodologías analíticas para la determinación de antioxidantes alimentarios mediante sistemas automáticos de análisis cromatográficos y no cromatográficos. Se han establecido nuevos métodos para la determinación de polifenoles mediante separación cromatográfica y derivatización post-columna, junto con detectores fotométricos y fluorimétricos. También se ha desarrollado un método quimiométrico para la clasificación de vinos utilizando la información obtenida en uno de los métodos cromatográficos propuestos. En la segunda parte de la Tesis se describen dos métodos no cromatográficos basados en el uso de la técnica de mezcla de flujo detenido y de nanopartículas de oro, lo que ha permitido ampliar el campo de aplicación analítica de estas nanopartículas. Es de destacar que se ha demostrado la utilidad práctica de todos los métodos desarrollados mediante su aplicación al análisis de distintos tipos de alimentos.

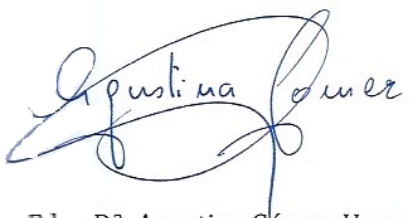
La utilización de una variedad de técnicas de separación, detección y quimiométricas, y la resolución de los distintos problemas surgidos a lo largo del trabajo experimental desarrollado han contribuido a la formación investigadora del doctorando, lo que constituye un objetivo básico del Doctorado. Las investigaciones realizadas han dado lugar a seis artículos científicos, cinco de ellos ya publicados y el sexto enviado para su publicación. También se han presentado cinco comunicaciones a Congresos nacionales e internacionales.

La estancia realizada por el doctorando en el Grupo de Investigación del profesor O. Wolfbeis (Universidad de Resenburgo, Alemania) durante el desarrollo de la Tesis Doctoral ha completado satisfactoriamente su formación investigadora ya que le ha permitido ampliar sus conocimientos sobre la síntesis y caracterización de nanopartículas de oro y la utilización de la técnica de resonancia del plasmón de superficie, la cual está muy relacionada con estas nanopartículas.

Por todo ello, consideramos que la investigación desarrollada y recogida en esta Memoria, reúne todos los requisitos necesarios en cuanto a originalidad, innovación y calidad, por lo que autorizamos la presentación de la Tesis Doctoral de D. Álvaro Andreu Navarro.

Córdoba, 20 de octubre de 2011

Firma del/de los director/es



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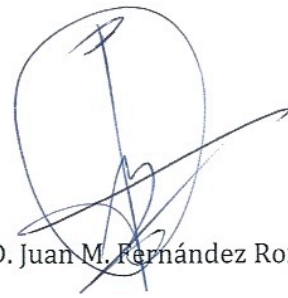

Fdo.: D. Juan M. Fernández Romero

Mediante la defensa de esta Memoria se pretende optar a la obtención de la mención "Doctorado Europeo" habida cuenta de que el doctorando reúne los requisitos exigidos para tal mención:

1. Cuenta con los informes favorables de dos doctores pertenecientes a Instituciones de Enseñanza Superior de países europeos distintos a España.

2. Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a un centro de enseñanza superior de otro país europeo.

3. Parte de la defensa de la Memoria se realizará en la lengua oficial de otro país europeo.

4. El doctorando ha realizado una estancia en el Institut für Analytische Chemie, Chemo- und Biosensorik de la Universidad de Regensburg, Alemania, de tres meses de duración, que ha contribuido a su formación y permitido desarrollar parte del trabajo experimental de esta Memoria.

Agradezco a la Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía la concesión de una beca predoctoral adscrita al Proyecto de Excelencia P06-FQM-01356 que me ha permitido dedicar estos últimos 4 años al desarrollo de esta Tesis.

Gracias a todos aquellos que
me han dirigido, ayudado,
apoyado y acompañado a lo
largo de esta Tesis Doctoral

A mi madre, porque gracias a su esfuerzo me ha dado la posibilidad de llegar hasta aquí

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OBJETO

El objetivo genérico de las investigaciones que se recogen en esta Memoria ha sido el desarrollo de nuevas metodologías analíticas cromatográficas y no cromatográficas que contribuyan a mejorar el control de la calidad alimentaria. Los estudios realizados se han centrado en la determinación de antioxidantes naturales y sintéticos en alimentos. Para alcanzar este objetivo se han realizado las investigaciones siguientes:

- Estudio de la aplicabilidad de nuevas reacciones de derivatización postcolumna en cromatografía de líquidos utilizando la luminiscencia sensibilizada de terbio(III) y la fluorescencia de larga longitud de onda como sistemas de detección para la determinación de compuestos fenólicos.

- Utilización de métodos quimiométricos en análisis clasificatorio mediante cromatografía de líquidos y detección fotométrica y fluorimétrica.

- Investigación de la capacidad de diversos aditivos antioxidantes alimentarios para originar nanopartículas de oro y su aplicación para la determinación de estos aditivos utilizando la dispersión de la radiación como sistema de detección.

- Estudio de la utilidad del uso conjunto de la fluorimetría de larga longitud de onda, la oxidación enzimática y nanopartículas de oro para la determinación del contenido de polifenoles en alimentos.

- Aplicación de los métodos propuestos al análisis de alimentos con objeto de demostrar su utilidad práctica.

AIM

The general aim of the research included in this study has been the development of new analytical chromatographic and non chromatographic methodologies that contribute to improve the control in food quality. The researches have focused on the determination of natural and synthetic antioxidants in foods. The following investigations have been developed to achieve this goal:

- Study of the applicability of new post-column derivatization reactions in liquid chromatography using terbium(III) sensitized luminescence and long wavelength fluorescence as detection systems.

- Use of chemometric methods in classification analysis by liquid chromatography using photometric and fluorimetric detection.

- Investigation of the ability of several food antioxidant additives to form gold nanoparticles and its application to the determination of these additives using resonance light scattering as detection system.

- Study of the usefulness of the combined use of long wavelength fluorimetry, enzymatic oxidation and gold nanoparticles for the determination of polyphenols in foods.

- Application of the proposed methods in food analysis to demonstrate their practical usefulness.

INTRODUCCIÓN

En esta introducción se comentarán algunos aspectos básicos relacionados con las especies químicas que se han utilizado como analitos en las investigaciones realizadas y las principales herramientas instrumentales y metodológicas que han permitido el desarrollo de los métodos determinativos presentados. Puesto que la Memoria está dedicada al establecimiento de nuevos métodos para la determinación de antioxidantes alimentarios, naturales y sintéticos, se describen brevemente las principales propiedades y el modo de actuación de los compuestos más representativos de este grupo, el interés por controlar su presencia en los alimentos y algunas características de los métodos determinativos previamente descritos. En lo referente a las herramientas utilizadas, se comentan sucintamente los antecedentes más destacables sobre la aplicabilidad analítica de la luminiscencia sensibilizada de lantánidos, la fluorescencia de larga longitud de onda y la dispersión de la radiación como sistemas de detección. También se incluyen algunos aspectos actuales sobre análisis clasificatorio y sistemas de flujo.

ANTIOXIDANTES ALIMENTARIOS

Los antioxidantes alimentarios son compuestos químicos que en pequeñas cantidades evitan o retrasan la oxidación de sustancias fácilmente oxidables, tales como los lípidos [1]. La oxidación de lípidos en los alimentos es un proceso complejo en el que frecuentemente intervienen radicales libres y que puede producirse durante la recolección, procesamiento y almacenamiento de los alimentos [2]. Factores como calor, luz, oxígeno, enzimas, metales de transición, metaloproteínas y microorganismos pueden actuar como iniciadores o promotores de este proceso dando lugar a alteraciones en el aroma y sabor del alimento, a la pérdida de ácidos grasos esenciales, de vitaminas liposolubles y de otros compuestos bioactivos, y a la formación de especies potencialmente tóxicas.

Existen diversos compuestos que se encuentran de forma natural en los alimentos y que presentan propiedades antioxidantes, protegiendo a los tejidos de procesos oxidativos a través de la dieta. Los principales antioxidantes naturales son los polifenoles (flavonoides y ácidos polifenólicos) junto con vitamina C (ácido ascórbico), vitamina E

(tocoferoles) y carotenoides [3]. Algunos de estos compuestos se utilizan como aditivos alimentarios aunque, debido a su escasa estabilidad, la industria alimentaria también utiliza desde los años cuarenta antioxidantes sintéticos que son compuestos fenólicos, tales como butilhidroxianisol (BHA), butilhidroxitolueno (BHT) y galato de propilo (GP). No obstante, su uso actualmente está restringido a ciertos alimentos y en cantidades limitadas, debido principalmente, a sus potenciales efectos cancerígenos.

El mecanismo de actuación y la eficacia de los antioxidantes dependen de sus características físicas y químicas y del sistema implicado [2]. Estas sustancias pueden actuar a distintos niveles en el proceso oxidativo de los lípidos: 1) evitando la formación de radicales o interaccionando con ellos, 2) actuando como complejantes de iones metálicos como Fe(II) y Fe(III), lo que impide su efecto catalítico en la formación de lipoperóxidos, 3) inhibiendo a enzimas prooxidativas, 4) descomponiendo los peróxidos y otras especies con oxígeno reactivo (ROS, reactive oxygen species), y 5) interfiriendo reacciones en las que están implicados radicales peroxilo ($LOO^{\cdot}$) o alcoxilo ($LO^{\cdot}$).

La evaluación de la capacidad y la actividad antioxidante de un compuesto es un proceso complejo ya que deben estudiarse los posibles mecanismos de actuación en diferentes condiciones, teniendo en cuenta las propiedades multifuncionales que puede presentar [1]. A veces, al realizar investigaciones de este tipo se han obtenido resultados contradictorios, lo que se ha atribuido a factores tales como características del ensayo, la naturaleza del sustrato utilizado y la influencia de otros componentes del sistema. Hay que tener en cuenta que el estudio de estos factores utilizando como modelo un sistema simple puede ser sencillo, pero la situación es más problemática en un sistema heterogéneo como es la matriz de un alimento o el organismo humano. Un método simple para evaluar la eficacia de un antioxidante es mediante un ensayo comparativo, utilizando el denominado "factor antioxidativo" (AF): I_A/I_O, donde I_A e I_O son los periodos de inducción obtenidos para una grasa o aceite en presencia y ausencia, respectivamente, de un antioxidante. El valor de AF, por tanto, es mayor cuanto más eficaz es el antioxidante.

El desarrollo de métodos para la determinación de antioxidantes en alimentos es una línea de investigación bastante activa, como se muestra en

la **Figura 1**, donde puede observarse el crecimiento exponencial de las publicaciones sobre esta temática en los últimos años. A continuación se describen brevemente las características más destacables de estos compuestos y los principales métodos descritos para su determinación.

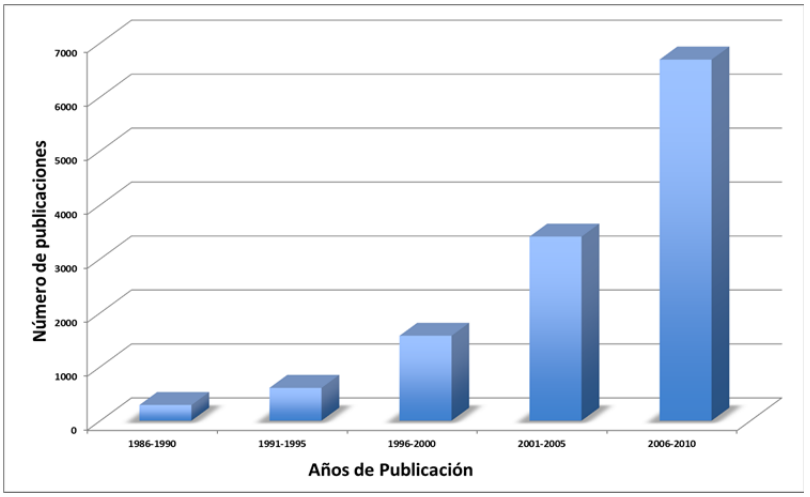


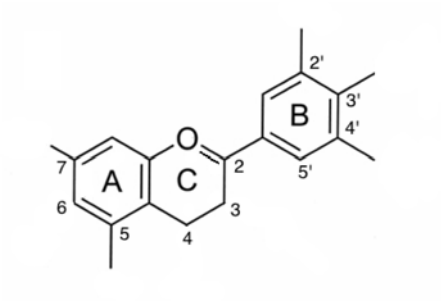
Figura 1. Distribución a lo largo del tiempo de las publicaciones relacionadas con métodos para la determinación de antioxidantes. (Base de datos SCOPUS).

1. Antioxidantes naturales

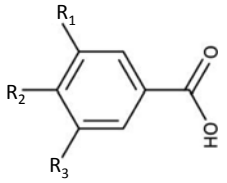
Uno de los grupos más representativos de los antioxidantes naturales lo constituyen los compuestos fenólicos, al que pertenecen los flavonoides y los ácidos fenólicos junto con taninos, estilbenos y lignanos [4]. Estos compuestos son metabolitos secundarios de las plantas y se consideran responsables de la calidad sensorial y nutricional de frutas y verduras, donde se encuentran ampliamente distribuidos, así como aceite de oliva y te, entre otros alimentos. La potencial toxicidad de los antioxidantes sintéticos ha dado lugar a que aumente el interés por estos compuestos de origen natural, a los que también se les atribuyen propiedades antimicrobianas, antivirales y antiinflamatorias.

En la **Figura 2** se muestran las estructuras de los principales componentes de los polifenoles, de los que se han descrito más de 8.000

Flavonoides

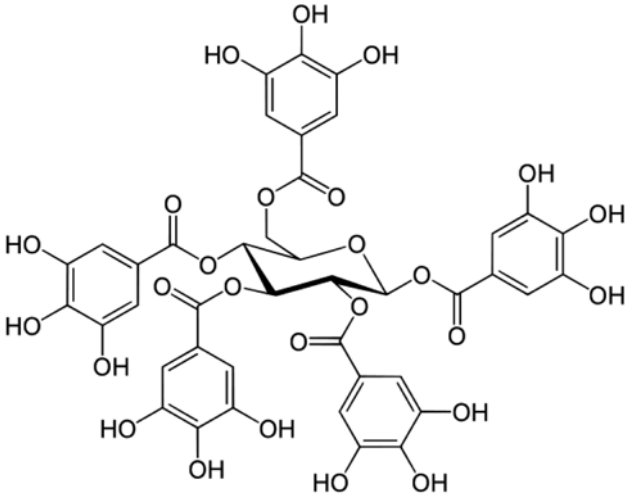


Ácidos hidroxibenzoicos

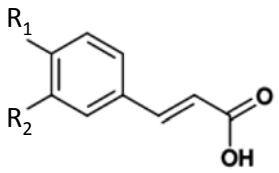


R_1=R_2=R_3=OH: Ácido gálico
R_1=R_2=OH, R_3= - : Ácido protocatecuico

Gallotaninas
(ej. pentagalloyglucosa)



Ácidos hidroxicinámicos



R_1=OH: Ácido cumárico
R_1=R_2=OH: äcido cafeico

Estilbenos (ej. Resveratrol)

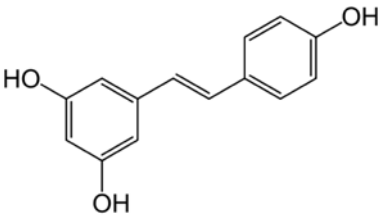
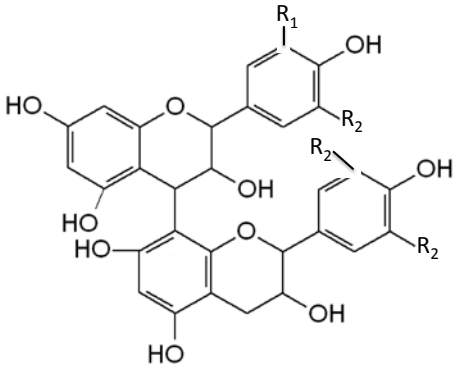


Figura 2. Estructuras químicas de los principales clases de compuestos fenólicos.

Proantocianidinas (R_1, R_2=H, OH)
(ej. Dímero de procianidina tipo-B, R_1=OH, R_2=H)



Lignanos
(ej. Secoisolariciresinol)

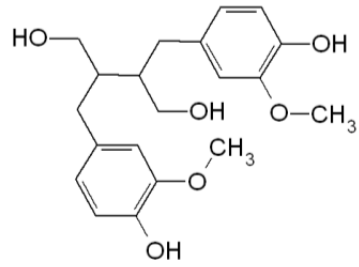


Figura 2. Continuación

compuestos. Uno de los subgrupos más numerosos lo constituyen los flavonoides, los cuales pueden encontrarse bien en forma libre o como glucósidos. Existen más de 4000 flavonoides, de los que unos 500 se encuentran en forma libre [5]. El esqueleto de los flavonoides está formado por tres anillos fenólicos denominados A, B y C (**Figura 3**). El anillo A está condensado con el anillo C, el cual tiene unido en la posición 2 al anillo B como sustituyente. Se dividen en varias clases según su estado oxidativo y sus sustituyentes. Como muestra la **Figura 3**, el anillo C puede ser un pirano heterocíclico (antocianidinas y catequinas o flavanoles) o una pirona (flavonas, flavonoles, flavononas e isoflavonas). A los flavonoides de este último grupo, los cuales tienen un grupo carbonilo en el C-4 del anillo C, se les denomina frecuentemente 4-oxo-flavonoides.

Los ácidos fenólicos constituyen, junto con los flavonoides otro grupo importante de compuestos fenólicos procedentes principalmente de las plantas [6]. Las estructuras de los principales componentes de este grupo se muestran en la **Tabla 1.** Entre ellos se encuentran los que tienen la estructura del ácido hidroxicinamico, como los ácidos cafeico, p-cumárico y ferúlico, y los que tienen la estructura del ácido hidroxibenzoico, como los ácidos vainíllico, protocatecuico, gentísico y

siríngico . Estos compuestos realizan diversas funciones en la vida de las plantas y afectan a sus características sensoriales.

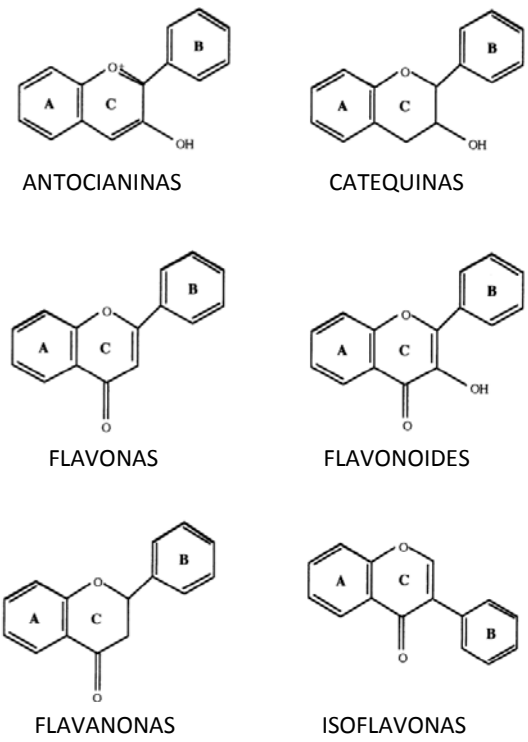
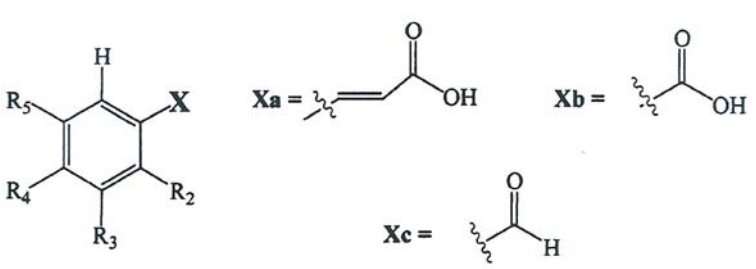


Figura 3. Estructuras químicas de las principales clases de flavonoides.

Los polifenoles tienen numerosas aplicaciones industriales ya que se utilizan como conservantes y colorantes naturales en alimentos y en la producción de pinturas, papel y cosméticos. Actualmente tienen un gran interés los procesos de tratamiento de residuos de industrias alimenticias ya que éstos pueden contener cantidades importantes de compuestos fenólicos. Por ejemplo en los residuos de las extractoras de aceitunas existe entre el 1 y el 1,8 % de contenido fenólico, según la variedad de aceituna [7]. También se han descrito las hojas del olivo como fuente de estos compuestos [8] y los desechos de la industria de los cítricos y de otras frutas [9].

Tabla 1. Estructuras de los ácidos fenólicos más abundantes de origen natural



R_2	R_3	R_4	R_5	X	Nombre Común
H	H	H	H	a	ácido cinámico
-OH	H	H	H	a	ácido o-cumárico
H	H	-OH	H	a	ácido p-cumárico
H	-OH	H	H	a	ácido m-cumárico
H	$-OCH_3$	-OH	H	a	ácido ferúlico
H	$-OCH_3$	-OH	$-OCH_3$	a	ácido sinápico
H	-OH	-OH	H	a	ácido cafeico
H	H	H	H	b	ácido benzoico
-OH	H	H	H	b	ácido salicílico
H	H	-OH	H	b	ácido p-hidroxibenzoico
H	$-OCH_3$	-OH	H	b	ácido vaínillico
H	$-OCH_3$	-OH	$-OCH_3$	b	ácido siringico
H	-OH	-OH	H	b	ácido protocatecuico
-OH	H	H	-OH	b	ácido gentisico
-OH	-OH	-OH	-OH	b	ácido gálico
H	$-OCH_3$	$-OCH_3$	H	b	ácido verátrico
H	$-OCH_3$	-OH	$-OCH_3$	c	siringaldehído
H	$-OCH_3$	-OH	H	c	vainillina

Entre las principales fuentes de compuestos fenólicos en la dieta humana se encuentran los vinos, las bebidas de zumo de frutas y el té. En la **Tabla 2** se muestra el contenido de polifenoles totales en algunas de estas bebidas. El vino contiene una gran variedad de flavonoides (flavonol, flavan-3-ol y antocianinas) y no flavonoides (ácidos fenólicos, alcoholes fenólicos, estilbeno y ácido hidroxicinnamico) [10]. El perfil de polifenoles varía de los vinos tintos a los blancos debido a la variedad de uva y al proceso de vinificación. El elevado contenido en polifenoles de los vinos

tintos ha dado lugar a que se les considere más protectores de la salud que otras bebidas alcohólicas [11].

Tabla2. Contenido aproximado de compuestos fenólicos en bebidas

Tipo de bebida	Contenido aproximado de compuestos fenólicos
zumos comerciales	
Manzana	339 ± 43[a]
Uva	535 ± 11[a]
Naranja	755 ± 18[a]
Piña	358 ± 3[a]
Zumos naturales	
Uva (roja)	1728[a]
Uva (blanca)	519[a]
Tés	
Te negro	80.5 – 134.9[b]
Te verde	65.8 – 106.2[b]
Te verde	61 – 200[b]
Vinos tintos	
Argentino	1593 – 1637[a]
Brasileño	1947 – 1984[a]
Español	1869[a]
Francés	1847 – 2600[a]
Vinos blancos	
Argentino	216[a]
Brasileño	256 – 353[a]
Francés	245[a]
Español	292[a]

[a] mg L^{-1}; [b] mg g^{-1} . Referido a ácido gálico

Existe una bibliografía muy extensa sobre los efectos beneficiosos de los compuestos fenólicos en la salud [1,2,4,5,12,13]. Estudios epidemiológicos han puesto de manifiesto que la dieta rica en polifenoles se correlaciona con el aumento de la longevidad y la disminución en la incidencia de las enfermedades cardiovasculares en la población, aun cuando la ingestión de grasas sea elevada [14]. Además de las propiedades antioxidantes de los polifenoles, se ha demostrado que estos compuestos presentan numerosas propiedades, actuando como antivirales, anti-bacterianos, anti-inflamatorios, vasodilatadores, anti-cancerígenos y anti-isquémicos. También inhiben la formación de lipoperóxidos y la agregación plaquetaria y mejoran la permeabilidad y la fragilidad capilar.

Los polifenoles previenen el daño de los componentes celulares producido por reacciones químicas en las que están implicados radicales libres. El oxígeno existente en el organismo es una fuente importante de radicales libres, tales como los radicales hidroxilo ($^{\cdot}OH$) e hidroperoxilo ($HO_2^{\cdot}$), el anión superóxido ($O_2^{\cdot-}$), y de peróxido de hidrógeno [15]. Estas especies pueden interaccionar con lípidos, enzimas y otras proteínas y ácidos nucleicos, siendo la oxidación de ácidos grasos insaturados presentes en las membranas de las células el proceso oxidativo más frecuente en el organismo. Los radicales libres de oxígeno formados durante el metabolismo pueden alcanzar niveles citotóxicos dañando las membranas de las células, provocando su ruptura, y a otros componentes celulares o extracelulares tales como enzimas, lipoproteínas y otras proteínas, colesterol y material genético del núcleo. Todo ello puede dar lugar a una amplia variedad de patologías, tales como procesos inflamatorios, arterioesclerosis, carcinogénesis y envejecimiento, entre otras.

También hay que indicar que, a pesar de sus efectos beneficiosos, los polifenoles pueden actuar en ciertas circunstancias como prooxidantes, al igual que otros antioxidantes, favoreciendo la oxidación de ciertos compuestos. Por ejemplo, la actividad prooxidante de los flavonoides es directamente proporcional al número de grupos hidroxilo ya que se ha demostrado que los mono- y dihidroxiflavonoides no presentan esta propiedad de forma detectable, mientras que la presencia de múltiples grupos hidroxilo, especialmente en el anillo B, aumenta significativamente la producción de radicales hidroxilo a través de la reacción de Fenton [14, 16,17]. En general, existe un consenso sobre la necesidad de profundizar en el efecto de los polifenoles en el organismo humano debido a que se han dado opiniones contradictorias [17].

El interés que existe por la relación entre los compuestos fenólicos y las propiedades organolépticas, antioxidantes y nutricionales de los alimentos, ha dado lugar al desarrollo de una gran variedad de métodos analíticos en los que se incluyen las correspondientes etapas de extracción, separación y cuantificación [4]. La extracción de estos compuestos de la matriz de la muestra es una etapa crítica, aunque no existe un método estándar con este fin. Las dos técnicas más utilizadas son la extracción con disolventes orgánicos y la extracción con fluidos supercríticos, aunque en

ambos casos se produce la co-extracción de otros compuestos tales como azúcares, ácidos orgánicos y proteínas, por lo que se necesitan procesos posteriores de purificación como, por ejemplo, extracción en fase sólida.

La extracción con disolventes puede ser líquido-líquido, en caso que la muestra sea líquida, o sólido-líquido, si la muestra está en estado sólido. La eficacia de la extracción depende de las condiciones experimentales. Por ejemplo, el contenido fenólico en almendras es tres veces superior cuando la extracción se realiza a 50º C que cuando se utilizan 25ºC. Los extractantes más utilizados son metanol o etanol acidificados. Aunque el primero es más eficaz, en la industria alimentaria suele utilizarse más el etanol para evitar la toxicidad del metanol. Además de estos extractantes, se han utilizados otros como acetato de etilo, dietil eter, acetona, n-hexano e isooctano, entre otros. Normalmente se usa una extracción secuencial con una primera etapa agua-disolvente orgánico para separar, por ejemplo, los ácidos fenólicos solubles. Los ácidos fenólicos que están enlazados a las células mediante enlaces ésteres o glucosídicos no se extraen directamente en disolventes orgánicos, siendo necesario liberarlos previamente mediante hidrólisis básica y/o ácida. En general se utiliza hidrólisis básica con hidróxido sódico entre 2 y 10 M, con un tiempo de incubación de hasta 16 h y, a veces, bajo corriente de nitrógeno [18]. Después de la hidrólisis básica puede utilizarse hidrósis ácida para completar la liberación de los compuestos fenólicos. También se ha descrito la liberación de compuestos fenólicos mediante enzimas. La extracción con fluidos supercríticos, utilizando dióxido de carbono, es una alternativa útil para evitar el uso de disolventes orgánicos [19] aunque es necesario utilizar un modificador como metanol para aumentar la polaridad de medio. También se han descrito la extracción con ultrasonidos [20] y la extracción asistida con enzimas [21]. Se ha propuesto un autoanalizador para el fraccionamiento y cuantificación en continuo de los polifenoles presentes en vinos mediante extracción en fase sólida y el detector de dispersión de luz evaporativo [22].

La cromatografía de líquidos es la técnica más utilizada para la separación de compuestos fenólicos [4,23,24]. Normalmente se usa una columna C_{18} en fase invertida con un sistema binario de disolventes que consiste en agua acidificada y un disolvente orgánico polar. Es frecuente que sea necesaria la preconcentración y purificación previa, especialmente

en el caso de muestras con matrices complejas. Esta etapa puede realizarse utilizando partición líquido-líquido con un disolvente inmiscible o extracción en fase sólida. Para la utilización de esta técnica pueden usarse sorbentes como C_{18}, copolímeros de stireno-divinilbenceno o distintos tipos de resinas [25,26]. La fotometría es la técnica que más se ha empleado como sistema de detección en estas separaciones cromatográficas, aunque la espectrometría de masas (MS) es la más útil con este fin [27].

La cromatografía de gases (GC) también se ha utilizado para la separación e identificación de compuestos fenólicos, siendo necesaria la derivatización mediante metilación, trifluoracetilación o conversión a derivados trimetilsililo para formar compuestos volátiles, y la utilización de MS como sistema de detección [28,29]. No obstante, la preparación de las muestras en GC es bastante problemática ya que hay que eliminar los lípidos del extracto, liberar los fenólicos de sus enlaces ésteres y glicosídicos y proceder a derivatización.

La electroforesis capilar es otra técnica alternativa para la separación y determinación de compuestos fenólicos debido a su eficacia en la separación, rapidez del proceso y bajo consumo de muestra y reactivos, aunque una limitación frente a las técnicas cromatográficas es los relativamente altos límites de detección obtenidos [30]. Se han descrito algunos métodos para la determinación de antocianinas y flavonoides [31,32].

Existe una variedad de métodos fotométricos que permiten obtener una estimación aproximada del contenido total de polifenoles en una muestra, pero no dan información cuantitativa de los componentes individuales. El método más representativo de este grupo es el ensayo Folin-Ciocalteu [33] que utiliza ácido gálico como polifenol estándar. Como se discute en el Capítulo 5 de esta Memoria, la principal limitación de estos métodos es su escasa selectividad, dando valores elevados de polifenoles totales ya que otras especies reductoras presentes en la muestra contribuyen a la señal analítica.

Finalmente, cabe indicar que se ha descrito el uso de diversas técnicas espectroscópicas, tales como NMR, MS y NIR para la elucidación de estructuras y la caracterización de compuestos fenólicos aislados de la

matriz de la muestra, pero sin separación previa de los componentes individuales [4].

2. Antioxidantes sintéticos

Como se indicó anteriormente, los antioxidantes sintéticos son un grupo de compuestos fenólicos cuyos componentes más representativos son BHA, BHT y GP. Aunque estos compuestos han sido ampliamente utilizados como aditivos alimentarios, su uso está muy limitado en la actualidad debido a sus potenciales propiedades tóxicas. Por ejemplo, el estudio de la citotoxicidad del BHA en tejidos de animales [34] ha demostrado que este compuesto induce la apoptosis de las células mediante la liberación directa de citocromo C.

Se han descrito numerosos métodos para la separación identificación y cuantificación de estos compuestos en alimentos [35]. La separación de la matriz de la muestra se ha llevado a cabo principalmente mediante extracción con disolventes, siendo el metanol uno de los más utilizados. La cromatografía de líquidos es la técnica más utilizada para la separación entre los distintos antioxidantes, usando generalmente la espectroscopía UV como sistema de detección y, en menor proporción, la MS [35,36]. También se han propuesto algunos métodos mediante GC con el detector de ionización de llama o con MS para la cuantificación [35].

FLUORIMETRÍA DE LARGA LONGITUD DE ONDA

El uso de fluoróforos de larga longitud de onda (LWFs) puede constituir una alternativa útil para mejorar la selectividad de las determinaciones analíticas frente a los fluoróforos convencionales que presentan sus características luminiscentes en la zona UV-visible. La principal propiedad de los LWFs es que su emisión se produce a longitudes de onda superiores a 600 nm, donde prácticamente no existen procesos de absorción o emisión de compuestos presentes en la matriz de la muestra. Otras ventajas que ofrecen estos fluoróforos es que se reducen considerablemente los problemas asociados con la posible degradación de la muestra, con interferencias debidas a señales Raman y con procesos de

inhibición no radiantes ya que, normalmente, la fluorescencia de los LWFs tiene una vida media muy corta [37]. La utilidad de los LWFs se ha demostrado en numerosas aplicaciones, principalmente en el análisis biológico, en el que la fluorescencia de la matriz de la muestra en la región visible del espectro es frecuentemente una fuente de interferencias cuando se utilizan fluoróforos convencionales.

Para que un compuesto emita fluorescencia a larga longitud de onda, el primer requisito es que su estructura sea rígida, con numerosos enlaces conjugados o anillos aromáticos fusionados. El inconveniente que presenta un sistema con una elevada conjugación es que puede alterarse fácilmente cuando se somete a excitación luminosa. Otras limitaciones que frecuentemente presentan estos compuestos son su pequeño desplazamiento Stokes, su escasa solubilidad incluso en disolventes orgánicos y su facilidad para oxidarse. No obstante, a pesar de estos inconvenientes, existe un gran interés por el uso analítico de estos compuestos, habiéndose sintetizado nuevos LWFs que evitan, al menos parcialmente, estas limitaciones.

El grupo que puede considerarse más representativo de LWFs lo forman los fluoróforos tipo cianina cuya estructura básica consiste en dos anillos aromáticos o heterocíclicos unidos por una cadena polimetina con dobles enlaces conjugados, como muestra la **Figura 4**. Las bandas de excitación de estos fluoróforos se encuentran en la zona de 600-900 nm, pero pueden conseguirse desplazamientos a mayores longitudes de onda introduciendo un grupo vinilo (-CH=CH-) en la cadena polimetina. Algunos inconvenientes de estos compuestos son su escasa reactividad para enlazarse a los analitos, su bajo rendimiento cuántico y su facilidad para formar agregados en medio acuoso, lo que causa una rápida disminución de su fluorescencia [38]. No obstante, el comportamiento de estos compuestos como reactivos analíticos puede mejorarse en presencia de surfactantes o de disolventes orgánicos. También se ha utilizado la modificación de su estructura molecular, introduciendo grupos alquilsulfonato, para mejorar su solubilidad en agua, su rendimiento cuántico y su estabilidad fotoquímica, o bien, la introducción de grupos reactivos, tales como isotiocianatos, lo que permite la formación de enlaces covalentes.

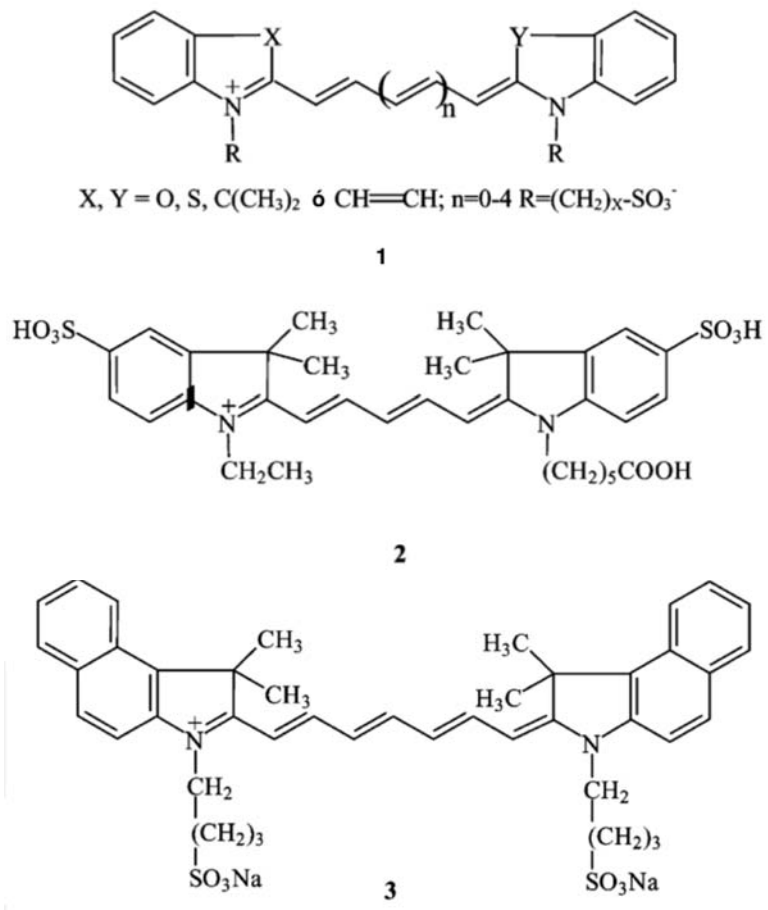


Figura 4. Estructura de los fluoróforos tipo cianina. (1) Estructura básica, (2) Cy5, (3) Verde de indocianina.

Dentro de los fluoróforos cianina utilizados con fines analíticos cabe citar el Cy5 y el verde de indocianina (ICG), también denominado IR-125, cuyas estructuras se muestran en la **Figura 4**. El Cy5 ha sido ampliamente utilizado en CE [39-42] y en el desarrollo de sensores [43-50], mientras que el ICG fue inicialmente utilizado en el campo de la imagen clínica debido a su escasa toxicidad [51]. Aunque este compuesto es muy poco fluorescente en medio acuoso, su fluorescencia aumenta al enlazarse a algunos compuestos, tales como proteínas [38,51]. En esta memoria se ha utilizado el ICG para desarrollar un método para la determinación de polifenoles totales.

Otro grupo de LWFs lo constituye los fluoróforos tipo oxacinas, cuya estabilidad fotoquímica es mejor que la de los fluoróforos cianina. En la **Figura 5** se muestran las estructuras de algunas oxacinas representativas, tales como el violeta de cresilo, azul Nilo y Oxacina 750, donde puede observarse que presentan estructuras más compactas que las de los fluoróforos cianina. La utilidad analítica de estos compuestos ha sido ampliamente descrita [52-62]. El violeta de cresilo se ha utilizado en un método cromatográfico descrito en esta Memoria para la determinación de flavonoides. También cabe citar como LWFs utilizados con fines analíticos a dos derivados rodamina, la Rodamina 800 y el Rojo Texas, cuyas estructuras se muestran en las **Figura 6**, y a la naftofluoresceina, las ficobiliproteinas y a los derivados escuarina, aunque su uso como reactivos analíticos ha sido relativamente limitado en los últimos años [63-66].

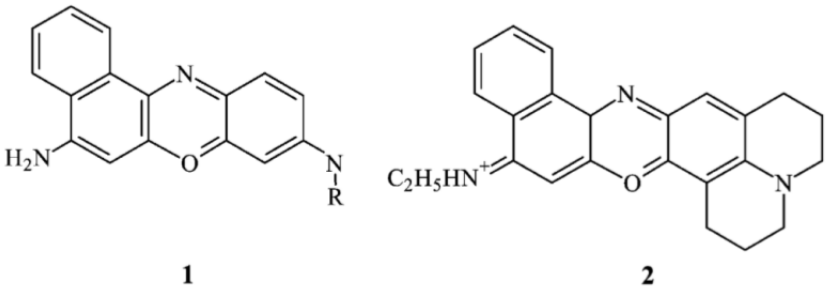


Figura 5. Estructura de los fluoróforos tipo oxacina. (1) Violeta de Cresilo (R: -H_2) y Azul Nilo (R:-($C_2H5)_2$), (2) Oxacina 750.

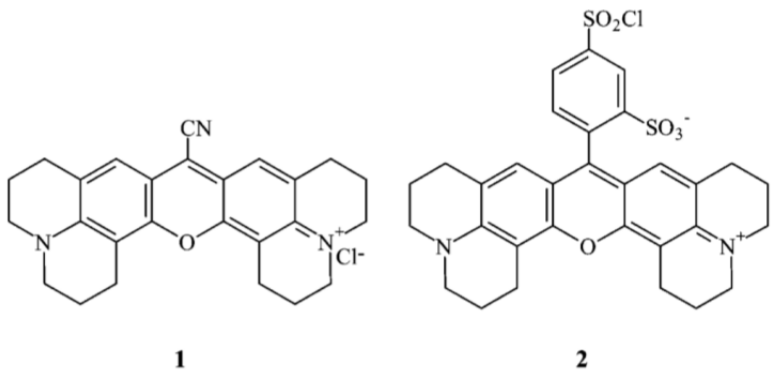


Figura 6. Estructura química de: (1) Rodamina 800, (2) RojoTexas.

Un grupo especial de LWFs lo forman algunos complejos de rutenio(II), cuya vida media de la fluorescencia es mucho mayor que la de los LWFs orgánicos. En general emiten en el intervalo 610-650 nm, aunque sus propiedades espectroscópicas y químicas varían según el ligando. Los complejos de rutenio(II) se han utilizado principalmente para el desarrollo de nuevos sistemas sensores. Por ejemplo, se han descrito sensores de pH basados en quelatos con características luminiscentes sensibles al pH y en la medida de la intensidad luminiscente [67-69] o de la duración de la luminiscencia [70-72].

Los LWFs han sido muy utilizados en procesos de transferencia de energía (fluorescence resonance energy transfer, FRET) con fines analíticos. En estos procesos, normalmente, un compuesto fluorescente actúa como dador de energía no radiante a un compuesto aceptor, que puede ser fluorescente o no fluorescente, originándose la disminución de la emisión fluorescente de la molécula dadora. Debe existir un considerable solapamiento entre el espectro de emisión del dador y el de absorción del aceptor, no siendo necesario que exista una interacción física entre ambos si la duración de la fluorescencia del dador es mayor que la del proceso de transferencia de energía. Se han descrito diversas aplicaciones analíticas de estos procesos utilizando quelatos de Ru(II) o de lantánidos como dadores de energía [65,73-78]. Como ejemplo cabe citar la preparación de nanoesferas luminiscentes como marcadores para multidetección usando un quelato de rutenio(II) (dador) disuelto con varios fluoróforos cianina (aceptores) en un polímero poliacrilonitrilo [73]. Las nanoesferas emiten doble luminiscencia, una procedente del dador y otra de aceptor, variando la eficacia de FRET al variar la concentración de aceptor, lo que puede monitorizarse midiendo el máximo de emisión y la caída de la luminiscencia.

El uso de LWFs como reactivos derivatizantes en cromatografía de líquidos ha sido bastante restringido. Se ha propuesto el azul Nilo para la derivatización precolumna en la determinación de varios compuestos carboxílicos como los ácidos benzoico, acético, fenilacético y hexanoico [57]. La aplicabilidad práctica del sistema se demostró mediante la determinación de ácido fenilacético en plasma [58]. La Rodamina 800 se ha utilizado como aditivo de la fase móvil para la cuantificación de ácido valproico en plasma utilizando detección indirecta de la fluorescencia [79].

Esta metodología permite obtener una respuesta universal cuando el fluoróforo forma parte de la fase móvil y presenta cierta afinidad por la fase estacionaria de forma que, al inyectar los analitos en la columna se obtiene una respuesta del detector debida a la ruptura del equilibrio de distribución del fluoróforo entre las fases móvil y estacionaria. FRET también se ha utilizado en un sistema postcolumna para determinar biotina en plasma humano usando una ficoeritrina conjugada a biotina y Cy5 unida a estreptavidina [64].

Los LWFs han encontrado mayor aplicación en CE [39-41,80,81]. Se han descrito diversos LWFs modificados para la derivatización con grupos amino de aminoácidos y proteínas. Por ejemplo, existen diversos derivados comerciales de LWFs para el marcaje de grupos amino, como el derivado de Cy5 utilizado para la determinación de plaguicidas aminoácidos fosforados en suelos mediante derivatización off-line previa a la separación mediante CE [82]. Por el contrario, la disponibilidad de LWFs como reactivos derivatizantes de grupos carboxilo es más restringida. Cabe citar un colorante cianina polimetina propuesto para unirlo a ácidos grasos, con un grupo amino aromático que se enlaza covalentemente a los analitos [83]. También existen algunas aplicaciones en las que los LWFs se han utilizado como marcadores no covalentes para la determinación de proteínas mediante CE [84,85]. Esta alternativa es más simple y rápida que el marcaje covalente, donde normalmente se requiere un control estricto del pH. Además, su uso en CE minimiza la limitación encontrada en cromatografía de líquidos en la que el exceso de fluoróforo puede quedar retenido en la zona hidrofóbica de la columna [86]. Como ejemplo del uso del marcaje no covalente en CE cabe citar un estudio comparativo para proteínas con colorantes escualeno en las modalidades pre-columna y en columna, encontrando mejores resultados en el segundo caso [85].

También se ha descrito el uso de LWFs en cromatografía electrocinética micelar (MEKC) mediante detección de fluorescencia indirecta, opción que es útil cuando los analitos no poseen propiedades fluorescentes adecuadas o un grupo funcional que pueda enlazarse a un fluoróforo. El mecanismo de detección implica la perturbación del complejo fluoróforo-micela por el analito, debiendo ser diferente la intensidad de la fluorescencia que presenta el fluoróforo en la fase micelar a la que presenta en fase acuosa. Esta técnica se ha utilizado, por ejemplo, para determinar

explosivos en suelo mediante un colorante cianina [87] usando un microchip con una longitud de separación de 65 mm.

LUMINISCENCIA SENSIBILIZADA DE LANTÁNIDOS

Los iones lantánidos, especialmente terbio(III) y europio(III) forman quelatos con ligandos multidentados, principalmente con grupos dadores de oxígeno cargados negativamente, que presentan especiales propiedades luminiscentes en disolución debido a un proceso de transferencia de energía intramolecular desde el estado triplete excitado del ligando al ion lantánido. Constituye un proceso de luminiscencia sensibilizada muy rápido que, normalmente, no está controlado por difusión ya que el lantánido está unido al ligando mediante enlace coordinado [88].

Aunque podría considerarse que la luminiscencia sensibilizada de lantánidos es un tipo especial de fosforescencia sensibilizada a temperatura ambiente, existen diferencias básicas entre ambos procesos debido a que el primero es intramolecular y el segundo es intermolecular. En este último, una especie con una estructura capaz de presentar un cruce entre sistemas eficaz desde el estado singlete excitado al triplete excitado, puede transferir su energía desde este estado a un compuesto como biacetilo o 1,4-dibromonaftaleno, el cual se excita pasando al estado triplete, con la consiguiente emisión de fosforescencia. Debido a que se trata de un proceso intermolecular en disolución, el oxígeno puede inhibir fácilmente los estados tripletes del dador y del aceptor por lo que es esencial la desoxigenación del medio. Por el contrario, el carácter intramolecular de la luminiscencia sensibilizada de lantánidos hace que el proceso sea menos susceptible a la inhibición colisional del estado triplete del dador por el oxígeno. Algunas de las razones que justifican la aplicabilidad analítica de esta técnica son las siguientes:

1) Los iones lantánidos presentan una buena estabilidad y su toxicidad es baja ya que tienen una escasa absorción en el tracto gastrointestinal e incluso, aunque se inyecten, no pueden penetrar en las células.

2) Pueden formar quelatos muy luminiscentes debido a los procesos anteriormente indicados de transferencia de energía desde el ligando al lantánido. Estos procesos dan lugar a que los quelatos presenten un gran desplazamiento Stokes evitando problemas de solapamiento entre las bandas de excitación y emisión, tales como los que presentan fluoróforos convencionales como la fluoresceína y que obliga a reducir el ancho de banda de las rendijas, con la consecuente reducción de la señal fluorescente.

3) La emisión del lantánido presenta una gran selectividad espectral, con bandas de emisión muy estrechas, debido a que este proceso se produce a través de los niveles resonantes del ion lantánido.

4) También presentan muy buena selectividad temporal debido a la relativamente larga duración de la emisión de algunos quelatos lantánidos, lo que permite realizar la medida analítica mediante el modo tiempo resuelto, cuando la emisión fluorescente de interferentes fluorescentes se ha anulado. Esta característica también mejora la sensibilidad ya que la medida se puede realizar mediante la integración de la señal durante un tiempo determinado.

5) La emisión se produce a longitudes de onda relativamente largas, a diferencia de las bandas que presentan la fluorescencia de fondo de la muestra, las cuales aparecen de menores longitudes de onda.

Para que se produzca una transferencia de energía eficaz desde el ligando-analito al ión lantánido deben cumplirse los siguientes requisitos: 1) las transiciones no radiantes del ligando ($S_1 \rightarrow S_0$ o $T_1 \rightarrow T_0$) deben se mínimas; 2) la energía del nivel de resonancia del ión lantánido debe ser ligeramente inferior a la del estado triplete excitado del ligando, de forma que exista una elevada probabilidad de que se produzca la transferencia de energía; y 3) no deben producirse transiciones no radiantes del ión lantánido excitado.

Existen diversos factores, tales como el uso de agentes sinergéticos, tensoactivos, pH, co-luminiscencia y átomos pesados, que contribuyen a que se cumplan estos requisitos [89]. Por ejemplo, debido al elevado número de coordinación del terbio(III), al formar quelatos con ciertos ligandos pueden quedar sitios de coordinación libres que pueden ser ocupados por moléculas de agua, lo que favorece la inhibición de la luminiscencia debido a procesos de desactivación no radiantes. Este efecto negativo se evita utilizando un segundo ligando que desplaza a las moléculas de agua de la esfera de coordinación del ión lantánido, ejerciendo un efecto sinergético en la luminiscencia sensibilizada. Con este fin se utilizan aminopolicarboxilatos y el óxido de tri-n-octil fosfina (TOPO) ya que tienen fuertes propiedades coordinantes y pueden llenar los huecos vacios de la esfera de coordinación del lantánido. La presencia de tres cadenas octilo dadoras en la molécula de TOPO protege tanto al terbio(III) como al ligando de posibles colisiones que originen desactivaciones no radiantes. La presencia de tensoactivos en el sistema también protege al quelato de procesos de inhibición.

Otro proceso que puede mejorar la intensidad de la luminiscencia sensibilizada es el fenómeno de la co-luminiscencia debida a un aumento de la excitación del ión lantánido favorecida por la absorción de quelatos que no emiten luminiscencia pero que transfieren la energía absorbida al quelato del lantánido. Este proceso se ha utilizado en esta Memoria para la derivatización postcolumna de flavonoides mediante la formación de quelatos de aluminio y terbio.

Las especiales características de la luminiscencia sensibilizada de terbio han dado lugar a un elevado número de métodos para la determinación de compuestos orgánicos basados en la formación de quelatos estables con analitos-ligandos que contienen átomos de oxígeno cargados negativamente [88]. También se ha descrito su utilidad en bioensayos, tales como el inmunoensayo [90], existiendo ensayos comerciales como el sistema DELFIA [91].

Otro campo de aplicación analítica de la luminiscencia sensibilizada de terbio ha sido su uso como sistema de detección en cromatografía de líquidos mediante reacciones de derivatización pre- o postcolumna. En la **Tabla 3** se muestran algunas de las aplicaciones descritas [92-99], donde

puede observarse que se consiguen límites de detección muy bajos en el análisis de muestras con matrices bastante complejas. En esta Memoria se describen dos métodos en los que se utiliza el terbio(III) como derivatizante postcolumna con resultados muy satisfactorios como se discute posteriormente.

Tabla3. Aplicaciones de los iones de terbio como reactivos derivatizantes en cromatografía de líquidos.

Analito	Fase Móvil	LOD	Muestra	Referencia
Ochratoxin A	RP	3 μM	Queso	[92]
Orotato	RP	1 nM	Orina	[93]
Glutatión	RP	20 nM	Orina	[94]
L-cisteina	RP	0.15 μM	Orina	[95]
Compuestos fenólicos	RP	0.016 – 0.3 μg mL^{-1}	Vino	[96]
Ciprofloxacina	RP	0.1 μM	Biológica	[97]
Flumequina	RP	1.1 ng mL^{-1} 15 ng mL^{-1}	Pollo	[98]
Fluoroquinolonas	RP	2 – 95 ng mL^{-1}	Leche	[99]

DISPERSIÓN DE LA RADIACIÓN

La dispersión de la radiación es un fenómeno óptico que ha sido ampliamente utilizado como sistema de detección en los métodos analíticos, aunque recientemente ha aumentado el interés por su uso desde la introducción de nanomateriales en dichos métodos.

Según el tamaño de las partículas (d) que interaccionan con los fotones de la radiación y de la longitud de onda (λ) de la radiación incidente, pueden distinguirse diferentes fenómenos de dispersión. La dispersión Rayleigh ($d < 0.5\ \lambda$) la originan partículas cuyo diámetro medio es menor que la longitud de onda de la radiación, produciéndose una dispersión prácticamente simétrica en todas las direcciones. Al aumentar el tamaño de la partícula, la radiación dispersada se desplaza en el sentido de la radiación transmitida, dando lugar a las dispersiones Tyndall ($d \sim \lambda$) y Mie ($d >> \lambda$). Existen otros fenómenos en los que, además de la dispersión,

se produce un cambio en la frecuencia de la radiación, como ocurre en el efecto Raman.

Las dos técnicas clásicas basadas en el fenómeno de la dispersión sin cambio de frecuencia son la turbidimetría y la nefelometría, cuya principal diferencia se encuentra en la forma de realizar las medidas de dispersión. En turbidimetría se obtiene la relación entre la intensidad de la radiación transmitida y la intensidad de la radiación incidente, mientras que en nefelometría se mide la radiación dispersada que forma un ángulo (≤ 90º) con respecto a la radiación incidente. En general, los límites de detección que se consiguen con nefelometría son más bajos. Estas técnicas son ampliamente utilizadas en el laboratorio clínico como sistemas de detección en métodos inmunoquímicos para la determinación de proteínas, tales como ferritina [100] y fibrina soluble [101]. El fenómeno de dispersión de la radiación también se ha utilizado como sistema de detección en diversas determinaciones cromatográficas [102-105].

La técnica de dispersión de radiación resonante (RLS) utiliza un espectrofluorímetro convencional en el que se realiza el barrido simultáneo de los monocromadores de excitación y emisión con un $\Delta\lambda = 0$ [106], obteniendo una señal intensa en las proximidades de la zona de máxima absorción de una especie que presente propiedades absorbentes. La radiación dispersada en estas condiciones puede considerarse que sigue la teoría Rayleigh en la que se considera que todos los electrones en una partícula oscilan con la misma fase y frecuencia que la onda electromagnética incidente y su oscilación colectiva da lugar a un gran momento dipolar eléctrico oscilante que origina radiación dispersada. No obstante las señales RLS obtenidas con un espectrofluorímetro pueden incluir, además de la dispersión Rayleigh, otros tipos de dispersión de radiación como las dispersiones Mie y Tyndall.

Esta técnica es muy simple y se ha aplicado a la determinación de sustancias que forman agregados moleculares con un cromóforo, por ejemplo, heparina con azul de metileno [107], albúmina con rojo de pirogalol [106] y ácidos nucleicos con morina [109]. Actualmente tiene un gran interés para la monitorización de fenómenos de agregación utilizando nanopartículas de oro o de plata, cuyo tamaño es menor de 20 veces la longitud de onda de la radiación incidente. Estas nanopartículas presentan

absorción en la región visible, produciéndose un desplazamiento batocrómico al aumentar su tamaño debido a la agregación. La utilidad de este fenómeno se ha puesto de manifiesto en inmunoensayo y en estudios de hibridación de ácidos nucleicos [110]. En esta Memoria se utiliza la RLS como sistema de detección para la determinación de antioxidantes mediante la formación de nanopartículas de oro.

SISTEMAS DE FLUJO

En las investigaciones que se presentan en esta Memoria se utilizan sistemas de análisis en flujo para las determinaciones basadas en derivatización postcolumna en cromatografía de líquidos y un sistema de mezcla de flujo detenido en las determinaciones sin separación cromatográfica. Por ello, aunque brevemente, se comentan las principales características de ambos sistemas.

La técnica de análisis en flujo, conocida como FIA (flow injection analysis), forma parte de las técnicas automáticas de análisis con flujo no segmentado, en las que las determinaciones se realizan de forma continua sin utilizar burbujas de aire para la separación entre las distintas zonas transportadas a lo largo del sistema. Normalmente se utilizan muestras líquidas que se insertan directamente en el sistema dinámico (**Figura 7 A**) a través del cual son transportadas, pudiendo incluir reacciones químicas o bioquímicas y sistemas de separación y/o preconcentración. Se utiliza un detector continuo, provisto generalmente de una célula de flujo, para la obtención de las señales transitorias características de la técnica (**Figura 7 B)**. Los métodos desarrollados mediante FIA en esta versión simple son métodos cinéticos de tiempo fijo y, por tanto, ni el equilibrio físico (homogenización de la porción del fluido) ni el químico (equilibrio de la reacción) se han alcanzado en el momento de la detección, por lo que el tiempo de operación ha de ser muy reproducible.

Las características más destacables de la técnica son:

- Versatilidad: Entre otros usos cabe destacar su utilización como simple interfaz entre muestra e instrumento, con intercalación tanto de etapas químicas o bioquímicas como de separación y/o

preconcentración o de ambas, la implantación de sensores químicos y/o bioquímicos en flujo, su acoplamiento con cromatógrafos, o con detectores ICP-AES, ICP-MS, FTIR, MS y, su uso para el seguimiento de procesos industriales.

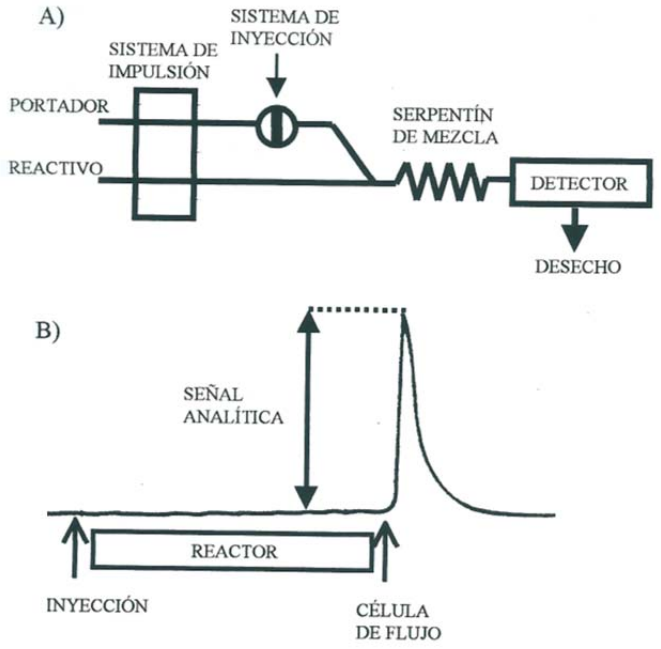


Figura 7. Unidades básicas de un sistema FI (A) y señales transitorias típicas de la técnica (B).

- Rapidez: Es una consecuencia de la ausencia de contaminación entre muestras inyectadas. Las características geométricas de los sistemas favorecen la dispersión radial en detrimento de la axial, y como resultado se obtiene la eliminación de la fase de lavado entre muestras sucesivamente inyectadas. Se obtienen frecuencias de muestreo en condiciones normales de 60 – 100 h^{-1}.
- Precisión: Estudios sobre este parámetro analítico han permitido establecer como más común, un valor de la desviación estándar relativa de los métodos FIA inferior al 2% [111]

- Coste: Bajo, ya que con estos sistemas se suele trabajar a bajas presiones lo que permite prescindir de unidades (bombas, válvulas, etc) de alta presión de un precio mucho mayor.

La técnica de mezcla de flujo detenido [112,113] permite de una forma muy simple la automatización de las medidas cinéticas. Como se muestra en el esquema de la **Figura 8**, los reactantes de un sistema químico se distribuyen entre dos jeringas, denominadas jeringas de impulsión, y son conducidos a gran velocidad hacia la cámara de mezcla que, normalmente, es también la celda de observación del instrumento. El flujo se detiene bruscamente mediante una tercera jeringa, denominada jeringa de parada, permitiendo la obtención rápida de la curva cinética señal-tiempo, cuando el sistema ha alcanzado el estado estacionario. Mediante un soporte informático adecuado se obtienen datos cinéticos, tales como la velocidad inicial de la reacción, amplitud de señal y, en su caso, periodo de inducción.

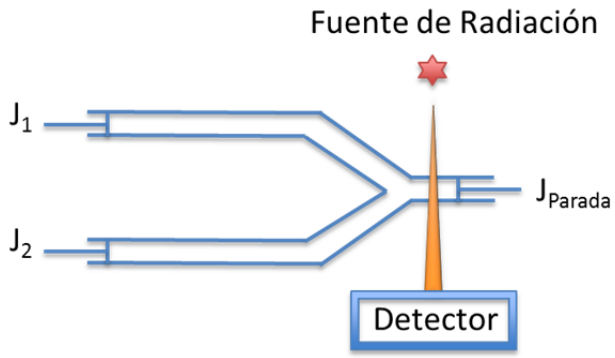


Figura 8. Esquema de la técnica de flujo detenido

Algunas características de esta técnica se resumen a continuación: 1) mezcla rápida, completa y automática de los reactantes, 2) bajo consumo de los mismos, 3) aplicable a sistemas rápidos y lentos, 4) gran precisión, 5) minimización de interferencias, 6) obtención rápida de resultados, y 7) automatización de la etapa de medida. Estas características permiten obtener una gran velocidad de muestreo, lo que justifica su utilización en análisis de rutina. En algunos sistemas, la etapa de medida requiere sólo unos segundos e, incluso, décimas de segundo. En sus inicios, la técnica de mezcla de flujo detenido se orientó principalmente hacia el estudio de la cinética y mecanismos de reacciones rápidas. No obstante, su utilidad en

análisis de rutina ha sido suficientemente demostrada [114-116], siendo su campo de aplicación extraordinariamente amplio.

QUIMIOMETRÍA

El termino quimiometría fue utilizado por primera vez en 1972 por el científico sueco Wold, cuando hizo uso de diferentes procedimientos estadísticos para el tratamiento de datos químicos. En esa década, junto con el químico analítico Kowalski y otros investigadores americanos crearon la International Chemical Society (ICS) en cuyo ámbito, canalizaron el rápido y amplio desarrollo de la quimiometría como herramienta de utilidad en el procesado de datos químicos. Según la ICS, la quimiometría es la disciplina científica que permite aplicar herramientas estadísticas y matemáticas para diseñar y/o seleccionar experimentos y procedimientos de medida, así como para extraer la máxima cantidad de información química a partir de los datos disponibles [117-120]. Su utilidad se ve especialmente reflejada dentro de la Química Analítica, donde la identificación, clasificación e interpretación de los datos proporcionados por las técnicas más actuales de instrumentación analítica pueden llegar a ser una tarea especialmente abrumadora.

La instrumentación analítica actual proporciona una ingente cantidad de información, generalmente agrupada en forma de matrices multidimensionales de datos que a su vez se podrá correlacionar con la concentración de los componentes de una muestra, denominada objeto. Cada muestra puede correlacionarse con un sinfín de medidas, designadas como variables. La implantación del análisis multivariante como técnica de tratamiento de datos conlleva a una mejora sustancial en la calidad de la información analítica de la muestra en estudio, o sobre la similitud o no entre muestras en estudios de clasificación. Frecuentemente los datos multivariantes pueden ser tratados con fines de discriminación y/o de clasificación entre muestras. Cada objeto se caracteriza por un conjunto de medidas que a su vez pueden representarse como un vector en un espacio multidimensional, por lo que el análisis multivariante puede ayudar a visualizar de forma efectiva diferentes objetos. La quimiometría incluye también metodologías para el análisis exploratorio de datos, el diseño experimental y el modelado de la información. Muchas de las aplicaciones

desarrolladas implican metodologías multivariantes para la clasificación de poblaciones.

Desde comienzos de los años 80 el número de artículos y revisiones sobre quimiometría ha sido muy extenso y está ampliamente distribuido en el ámbito de las publicaciones científicas. Se han descrito métodos quimiométricos que inciden en las distintas etapas del proceso analítico, incluyendo muestreo, optimización de variables, filtrado de señales, ensayos de simulación, calibración multivariante y análisis clasificatorio. A continuación se hará una descripción muy genérica de algunas de las técnicas quimiometricas utilizadas en el desarrollo de esta Tesis Doctoral en lo referente al pre-tratamiento de datos y al empleo de metodologías para el análisis clasificatorio.

Una práctica habitual en el ámbito de la quimiometría es el pre-tratamiento de los datos como paso fundamental previo a la utilización posterior de otras técnicas, ya sean de regresión o de clasificación. En análisis multivariante cada muestra es considerada como un vector o como una matriz de datos en la que en muchas ocasiones pueden producirse efectos no deseados que distorsionan la secuencia adecuada de la información suministrada [121]. Otro ejemplo significativo de distorsión que requiere etapas de pre-tratamiento lo constituye la presencia de huecos en los vectores o matrices de datos, como por ejemplo en el tratamiento de registros cromatográficos. Usualmente, la presencia de estos huecos en la secuencia de datos se corresponde con ausencias significativas en una observación concreta o en la obtención de valores de concentraciones de analitos por debajo de los límites de detección de la metodología aplicada. La utilización de vectores o matrices de datos con distorsiones ajenas al objeto de estudio conlleva generalmente a erróneas aplicaciones de las técnicas quimiométricas y por tanto a conclusiones no deseadas. En estas situaciones, el pre-tratamiento de los datos minimiza estas distorsiones y posibilitan la construcción de modelos quimiométricos más simples y robustos.

Las técnicas de pre-procesado se pueden dividir en dos grandes grupos: aquellas que usan directamente unos valores de referencia disponibles para el pre-procesado de datos y aquellas que no los usan [122-128]. Estas últimas se pueden dividir a su vez en otros dos sub-grupos:

métodos de corrección de la dispersión y métodos de derivatización. Los métodos de pre-procesado dependiente de referencia comprenden una serie de metodologías basadas en ajustes ortogonales de los datos respecto a la referencia seleccionada.

Dentro de las técnicas más comunes para la corrección de la señal cabe destacar las siguientes:

1) El centrado sobre la media, que consiste en cambiar el origen de la nueva escala de variables por la media de la variable antes del centrado. Este tratamiento no modifica la varianza de los datos experimentales.
2) El autoescalado, que es un centrado seguido de una normalización y que se utiliza cuando la magnitud de las señales o la relación señal/ruido varía considerablemente de una variable a otra, siendo especialmente importante en la exploración de datos multivariante.
3) La corrección de la línea de base, que se utiliza cuando existen variaciones significativas en la línea de base entre series de datos como por ejemplo en series espectrales. La aplicación más simple supone el uso de algoritmos de substracción de señales con respecto a determinadas zonas donde la intensidad de la señal es mínima. Se obtienen nuevas matrices con datos corregidos que propician la anulación de los efectos de distorsión.
4) El suavizado y filtrado de señales, que intenta reducir el ruido aleatorio existente en la señal instrumental mediante ajuste por desplazamiento de la media de un pequeño intervalo de valores. La metodología de suavizado más usada es la de Savitzky-Golay, en la que se usa un modelo de ajuste de mínimos cuadrados con una función polinómica aplicada sobre una ventana móvil de datos.
5) Las técnicas de derivación de señales se fundamentan en que la diferenciación matemática permite acentuar las diferencias contenidas en la matriz de datos. Por ello, el cálculo de la primera y la segunda derivada es un procedimiento útil para eliminar ruidos de fondo. Las metodologías de derivación de señal más usadas se deben a Savitzky-Golay y a Norris-Williams,

que permiten un suavizado de las señales en base a la derivación de las señales a través de la aplicación de aproximaciones con algoritmos polinómicos. En determinadas circunstancias, la técnica de normalización de los datos experimentales previa al tratamiento posterior mediante análisis multivariante es una técnica útil para el compensado de fluctuaciones externas al proceso de medida.

6) Las técnicas de corrección multiplicativa de señal (MSC) y de variación estándar normalizada (SNV) son metodologías de pre-tratamiento en las que se suavizan los efectos de dispersión de la radiación y de diferencias debidas al tamaño de las partículas en suspensión, especialmente útiles cuando se procesan espectros de reflectancia IR en suspensiones.

7) El objetivo de la metodología denominada corrección ortogonal de la señal (OSC) es corregir la matriz de datos originales eliminando información ortogonal en la construcción de una matriz de respuesta.

Como se ha indicado anteriormente, las técnicas analíticas modernas generan gran cantidad de información cualitativa y cuantitativa, por lo que con frecuencia es necesario aplicar métodos formales capaces de resaltar similitudes y diferencias entre series de objetos. Las técnicas de reconocimiento de pautas facilitan la resolución de aspectos tales como la identificación de las relaciones entre objetos químicamente caracterizados [129-136].

Los objetivos fundamentales del análisis de reconocimiento de pautas se centran en la identificación de relaciones y/o vínculos entre objetos agrupados o clasificados según la similitud de sus datos experimentales. Dentro de las técnicas de reconocimiento de pautas existen diferentes modelos de trabajo, que se puede dividir en dos grandes grupos: 1) el análisis exploratorio y 2) los métodos de clasificación, que a su vez se clasifican en dos modelos: (a) no supervisados, que se utilizan para decir si un conjunto de pautas (variables) se pueden dividir en grupos, y (b) supervisados, en los que se conocen las clases en que puede dividirse una muestra, y el objetivo es la clasificación de una muestra de clase desconocida por sus pautas. Como ejemplo de métodos no supervisados cabe destacar el análisis de grupos o "cluster" (CA), mientras que en el

segundo grupo se encuentran el análisis discriminante lineal (LDA) y cuadrático (QDA), método de los K vecinos más próximos (KNN), de modelado suave de clases análogas independientes (SIMCA) y las redes neuronales artificiales (ANN).

Las técnicas de análisis exploratorio de datos se utilizan para poner de manifiesto y resaltar información contenida en una matriz de datos multidimensional. La observación directa de los datos experimentales, así como la utilización de herramientas estadísticas simples son insuficientes para describir en toda su magnitud las estructuras presentes. Las técnicas de exploración de datos consisten fundamentalmente en dos tipos de técnicas: análisis de componentes principales (PCA) y análisis factorial (FA), siendo esta última la utilizada en el desarrollo de esta Memoria. Ambas técnicas se requieren para definir estructuras que ocupan más de tres dimensiones, para identificar tendencias ocultas presentes en los datos experimentales y estudiar las fuentes o causas de varianza a que obedecen, para obtener variables latentes del sistema y, sobre todo, para obtener la reducción de las dimensiones, de modo que la información relevante contenida en la matriz multidimensional pueda quedar reflejada sobre otras dos o tres dimensiones oblicuas obtenidas como combinaciones lineales de las variables originales.

El PCA es una técnica multivariante que permite transformar un grupo inicial de variables correlacionadas en otro de variables ortogonales denominadas componentes principales (PC), de modo que se reduce el número de datos cuando existe correlación. La idea subyacente del PCA es encontrar componentes principales que sean combinaciones lineales de las variables originales y que permitan describir cada muestra. Puesto que las primeras componentes principales recogen la mayor parte de la variación del conjunto de datos, estos se pueden representar en dos dimensiones (las de sus componentes principales) en lugar de utilizar las totales de origen. El PCA es una herramienta estadística y de proyección valiosa que persigue maximizar la información de la varianza presente en una matriz de datos y representarla en el menor número de dimensiones posibles. Matemáticamente, estas operaciones se consideran transformaciones simples de algebra lineal, donde los nuevos ejes de coordenadas, denominados “egienvectors” (vectores propios, o componentes principales) y que son combinaciones lineales entre las variables originales, cumplen

además con la condición de ser ortogonales entre ellas. Los componentes principales son variables latentes, esto es, que modelan las principales tendencias presentes en la matriz de datos experimentales. Otro aspecto muy importante a tener en cuenta en el PCA es su capacidad para la reducción del número de variables necesarías para representar el sistema, proporcionando un nuevo conjunto de variables que describen propiedades encubiertas hasta ese momento, revelando las tendencias predominantes de los datos.

El análisis factorial (FA) es una técnica multivariante encuadrada dentro del grupo de técnicas de simplificación o reducción de la dimensión y que desde el punto de vista estadístico no difiere del PCA. Sin embargo, desde un punto de vista químico-analítico, FA adquiere una mayor relevancia conceptual, ya que los factores se consideran entes físicos, mientras que los componentes principales son entidades abstractas. La conversión de una entidad abstracta a otra de naturaleza física o química recibe el nombre de "rotación" o "transformación". FA es a menudo utilizado para la transformación de componentes principales "abstractos" a factores químicos bien conocidos.

Por otra parte, los métodos de reconocimiento de pautas supervisados consisten en elaborar modelos matemáticos a partir de un conjunto de muestras que forman parte del conjunto de calibración y que pertenecen a diferentes categorías conocidas. Los modelos establecidos pueden utilizarse para la posterior clasificación de objetos dentro de las clases correspondientes. En estos métodos se encuentran las técnicas de clasificación y las de modelado, que a su vez se dividen en:

1) Técnicas probabilísticas. Entre otras, cabe señalar el análisis discriminante lineal (LDA) y el cuadrático (QDA), siendo técnicas de clasificación. Entre las técnicas probabilísticas no paramétricas se incluye la técnica UNEQ (modelo de clases no iguales).

2) Técnicas basadas en medidas de distancias entre objetos para la clasificación (K-vecinos más próximos, KNN), entre objetos para el modelado y entre objetos y modelos para la clasificación y modelado (SIMCA).

3) Técnicas de clasificación basadas en la experiencia, en las que las especificaciones se adquieren mediante un procedimiento de prueba y corrección: redes neuronales artificiales (ANN) y máquinas de aprendizaje lineal o cuadrático.

De todas ellas se comentará la técnica de análisis lineal discriminante (LDA), que es una técnica multivariante que trata de clasificar a individuos en grupos previamente determinados. Los objetivos concretos que pretenden cubrirse con este tipo de análisis son dos. El primero de ellos consiste en determinar a partir de la información inicial sobre una serie de individuos, de los cuales se conoce el grupo o población a la que pertenecen, si los individuos están perfectamente definidos en función de las variables del método. El segundo es clasificar a individuos, distintos de los manejados en la información inicial, en uno de los grupos existentes.

En las últimas décadas, la quimiometría ha desempeñado una función destacada como herramienta para los estudios dirigidos a la clasificación de los vinos según su variedad, procedencia geográfica, tipo de tratamiento recibido en su manipulación y otras tecnologías, ya que posibilita definir sus características organolépticas en concordancia con su composición química y así establecer esas diferentes formas de clasificación [137-142]. La legislación ha realizado un gran esfuerzo con respecto al etiquetado y denominación de origen de alimentos y bebidas, en respuesta a las exigencias y demanda de los consumidores y la sociedad. La mejora en el rendimiento económico que supone una mayor información y etiquetado de los productos con respecto a su localización y procedencia, y la prevención de fraudes en el etiquetado, producción y comercialización son las tres causas que han propiciado el desarrollo de la quimiometría en el ámbito enológico. Todo ello ha llevado al desarrollo de metodologías que proporcionen cada vez más información sobre los vinos como respuesta de satisfacción de consumidores, productores y exigencias legislativas. Todas estas exigencias han hecho del sector vitivinícola un ejemplo en la aplicación de la quimiometría en la mejora de la información analítica sobre la composición de los vinos y el aseguramiento de la autenticidad de los mismos [143,144].

Se han implementado diferentes técnicas quimiométricas para la discriminación de los vinos en base a distintos criterios como, por ejemplo, la tecnología empleada en la vinificación, de acuerdo con su región de procedencia, tipo y variedad. Se han utilizado varias técnicas de reconocimiento de pautas para la clasificación de los vinos mediante análisis de componentes principales (PCA) [145,146], análisis discriminante lineal (LDA) [147] y canónico (CDA) [148], redes neuronales artificiales [149], K-vecinos más próximos (KNN) [150] y análisis de grupos (CA) [151]. Por otra parte, se han desarrollado metodologías de calibración multivariante mediante PLS [152,153] y CAIMAN [154]. Existe también un gran número de técnicas utilizadas para la clasificación en grupos de vinos con objeto de evaluar su autenticidad mediante la determinación de analitos volátiles, elementos traza y minerales, aminoácidos y compuestos fenólicos.

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CAPITULO 1

HERRAMIENTAS ANALÍTICAS

El desarrollo de las investigaciones recogidas en la presente Memoria ha sido posible gracias al empleo de diversas herramientas analíticas, considerando como tales todos los elementos utilizados en las mismas, desde patrones, reactivos, disoluciones y aparatos empleados, hasta la instrumentación más compleja. En los siguientes apartados de este capítulo se enumeran dichas herramientas junto con sus características más relevantes.

1. Estándares y reactivos

Todos los reactivos y disolventes empleados fueron de calidad analítica. Se detallan a continuación los estándares de los analitos, reactivos y disolventes empleados.

1.1. Estándares de fenoles y otros antioxidantes

En la siguiente tabla se relacionan los compuestos químicos utilizados como analitos en la Memoria, su pureza y la casa comercial que las ha suministrado.

Compuesto	Pureza	Suministro
Ácido ascórbico	≥ 99,0%	SIGMA
Ácido cafeico	≥ 98,0%	SIGMA
Ácido ferúlico	≥ 99,0%	SIGMA
Ácido gálico	97,5 -102,5%	SIGMA
Ácido p-cumárico	≥ 98,0%	SIGMA
Ácido p-hidroxibenzoico	≥ 97,0%	SIGMA
Ácido protocatecuico	≥ 97,0%	SIGMA
Ácido salicílico	≥ 99,0%	ALDRICH
Ácido siríngico	≥ 95,0%	FLUKA
Ácido vainillico	≥ 97,0%	SIGMA
Butilhidroxianisol	≥ 98,5%	SIGMA
Butilhidroxitolueno	≥ 99,0%	SIGMA
Catequina	≥ 99,0%	SIGMA
Catecol	≥ 99,0%	SIGMA
Citrato sódico	≥ 99,0%	SIGMA
Galato de dodecilo	≥ 99,0%	SIGMA
Epicatequina	≥ 97,0%	FLUKA
Fenol	≈ 99,0%	SIGMA-ALDRICH
Hesperidin	≥ 80,0%	FLUKA
Hidroquinona	≥ 99,0%	ALDRICH
Hidroxihidroquinona	≥ 99,0%	ALDRICH
Kaempferol	≥ 97,0%	SIGMA
Naringenin	≥ 95,0%	FLUKA
Naringin	≥ 95,0%	FLUKA
Galato de octilo	≥ 99,0%	SIGMA
Phloroglucinol	≥ 99,0%	ALDRICH
Pyrogalol	≥ 99,0%	SIGMA-ALDRICH

Galato de propilo	≥ 98,0%	SIGMA
Quercetina	≥ 98,0%	ALDRICH
Resorcinol	≥ 99,0%	SIGMA-ALDRICH
Rutina	≥ 94,0%	SIGMA
Siringaldehido	≥ 97,0%	FLUKA
Trans-resveratrol	≥ 99%	SIGMA
Vainillina	≥ 97%	ALDRICH

Para su correcta conservación, las disoluciones de todos los estándares se mantuvieron protegidas de la luz y a una temperatura de 4ºC. Las disoluciones estándares se prepararon bien en medio acuoso o bien en el mínimo volumen de etanol necesario para su disolución, enrasando con agua hasta el volumen final. Las diferentes disoluciones de trabajo fueron preparadas diariamente mediante dilución de las anteriores en agua destilada.

1.2. Disolventes

Se han empleado diversos disolventes durante el desarrollo de las investigaciones, tales como metanol, acetonitrilo, etanol, n-hexano etc.

Estos disolventes se emplearon con diferentes finalidades:

1) Preparación de las disoluciones estándares de los analitos.
2) Limpieza y acondicionamiento de las columnas cromatográficas.
3) Como componentes de las fases móviles utilizadas en los sistemas cromatográficos.

1.3. Reactivos

Disoluciones reguladoras: Normalmente ha sido necesario el ajuste del pH de las muestras con el objeto de conseguir la acidez o basicidad requerida para el desarrollo de las reacciones utilizadas. Para ello se han utilizado diversas disoluciones

reguladoras mediante tris(hidroximetil)aminometano, fosfato sódico, borato sódico y acetato sódico, entre otros.

Sistemas derivatizantes: Se han utilizado en esta Memoria: nitrato de terbio(III) pentahidrato (ALDRICH), óxido de tri n-octilfosfina (TOPO) (ALDRICH), ácido etilendiamintetraacético (EDTA) (SIGMA), nitrato de aluminio(III) (SIGMA) y sulfato de cerio(IV) (MERCK).

Otros:

- Surfactantes: Tritón X-100 (SIGMA), sodio dodecil sulfato (SDS) (SIGMA) y Bromuro de hexadeciltrimetilamonio (EGA-CHEMIE).
- Fluoróforos de larga longitud de onda: Violeta de cresilo (SIGMA) y verde de indocianina (SIGMA).
- Enzima laccasa (Trametes versicolor) (SIGMA).
- Ácido tetracloroaurico (SIGMA)

2. Sistemas FIA

Para desarrollar las reacciones derivatizantes de los trabajos realizados mediante cromatografía de líquidos, ha sido necesaria la utilización de sistemas FIA que permitan la incorporación de los reactivos en el sistema. Con este fin se ha hecho uso de todos elementos citados a continuación:

- Válvula de inyección Rheodyne 7725i de seis vías (Rohnert Park, CA, USA).
- Bomba peristáltica de baja presión Gilson (Villiers-le-Bel, France) Minipuls 3.
- Tubos de PTFE de 0,5 mm de diámetro interno para conducir las disoluciones, así como para fabricar los bucles y reactores necesarios.
- Conectores de PTFE (Omnifit) para unir los tubos de conducción de los diferentes componentes del sistema de flujo.

3. Instrumentación

En el desarrollo experimental de esta Memoria se ha utilizado la siguiente instrumentación:

- Cromatógrafo de líquidos Agilent 1200 compuesto por una bomba cuaternaria, una unidad desgasificadora, una unidad automuestreadora, compartimento para la columna termostatado y dos detectores: un fotómetro de diodos en fila (DAD) y un fluorímetro. Se ha utilizado una columna Onyx monolítica de C_{18} (Phenomenex, Torrance, CA), de dimensiones 100mm x 4,6mm y tamaño de poro: 13nm (mesoporos) y 2µm (macroporos).
- Fluorímetros: Se ha utilizado un espectrofluorímetro SLM Aminco (Urbana, IL) AB2 con una lámpara contínua de Xenon de 150W y una lámpara pulsada de xenón de 7W, con una célula de flujo 176-052-QS Hellma (Hellma Hispania, Barcelona, Spain) de volumen interno de 18µL. También se ha empleado un espectrofluorímetro Cary Eclipse Varian (Walnut Creek, CA, USA).
- Espectrofotómetro UV/VIS Lambda 35 (Perkin Elmer).
- Dispositivo SPR Biosuplar 400T, (MIVITEC)
- Microscopio electrónico de transmisión Philips CM-10, resolución 0.5 nm × 0.34 nm y equipado con una cámara digital megaview III. Rejillas de cobre (200C-FC) recubiertas con una película de carbón Formvar® 200mesh, suministrado por Aname (Madrid, Spain)

4. Aparatos

Durante el desarrollo de esta Memoria se han empleado los aparatos que se describen a continuación:

- Baño termostático HAAKE DC1 (ThermoElectron, Karlsruhe, Germany).
- Módulo de flujo detenido de cinética rápida RX-2000 (Applied Photophysics, Leatherhead, UK).
- Microbalanza Explorer (OHAUS)

5. Programas informáticos

Se emplearon diferentes programas informáticos para el calculo estadístico y elaboración de las diferentes representaciones gráficas: Origin 7.0 (ORIGIN LAB), Statgraphics 5.1, SPSS 15.0 y Microsoft Excel

CAPITULO 2

INNOVACIONES EN LA DETERMINACIÓN CROMATOGRÁFICA DE ANTIOXIDANTES EN ALIMENTOS

En este capítulo se describen las investigaciones realizadas para el establecimiento de tres métodos cromatográficos para la determinación de compuestos fenólicos en alimentos y un método quimiométrico para la clasificación de vinos. En concreto, estas investigaciones han dado lugar a los siguientes artículos:

- Analytical innovations in the detection of phenolics in wines. P. Russo, A. Andreu-Navarro, J.M. Fernández-Romero, A. Gómez-Hens, *J. Agric. Food Chem.* 2008, 56, 1858-1865.

- Luminescent determination of flavonoids in orange juices by LC with post-column derivatization with aluminium and terbium. A. Andreu-Navarro, J.M. Fernández-Romero, A. Gómez-Hens, *J. Sep. Sci*. 2010, 33, 509-515.

- Long-wavelength fluorescence detection of flavonoids in orange juices by LC. A. Andreu-Navarro, J.M. Fernández-Romero, A. Gómez-Hens, *Chromatographia* 2010, 72, 1115-1120.

- Usefulness of terbium-sensitised luminescence detection for the chemometric classification of wines by their content in phenolic compounds. A. Andreu-Navarro, P. Russo, M.P. Aguilar-Caballos, J.M. Fernández-Romero, A. Gómez-Hens, *Food. Chem*. 2011, 124, 1753-1759.

El aspecto más novedoso de los métodos cromatográficos propuestos se encuentra en el desarrollo de nuevos sistemas de derivatización postcolumna que permitan la utilización de la luminiscencia sensibilizada de terbio y de la fluorescencia de larga longitud de onda como sistemas de detección, con objeto de mejorar los límites de detección y, en su caso, la selectividad, de los métodos cromatográficos previamente descritos para la determinación de polifenoles en alimentos. En la Introducción de esta Memoria se han descrito algunas de las principales características de estos sistemas de detección, así como su aplicabilidad analítica. También se han discutido aspectos básicos de las técnicas quimiométricas utilizadas.

Un factor común a los tres métodos cromatográficos desarrollados ha sido la utilización de una columna monolítica, como alternativa a las columnas empaquetadas utilizadas convencionalmente en cromatografía de líquidos para la separación de polifenoles. A diferencia de estas

columnas, la columna monolítica está formada por un soporte sólido, normalmente de sílice, con ligandos octadecil silano enlazados a su superficie. Su principal característica es su estructura porosa bimodal, con macroporos, cuyo tamaño puede ser de 0.5 a 8 μm, y mesoporos con tamaño de 2 a 20 nm. Los primeros permiten que la fase móvil pase a velocidad relativamente elevada a través de la columna mientras que los mesoporos permiten que se produzca la separación de los analitos. Se ha demostrado que esta estructura disminuye los tiempos de retención y mejora notablemente la resolución frente a las columnas convencionales [1-3]. Aunque otra característica de estas columnas es la posibilidad de utilizar caudales muy elevados, hasta de 9 mL min^{-1}, los métodos presentados en este capítulo utilizan caudales más bajos ya que ha sido necesario compatibilizar el caudal de fase móvil con el caudal del sistema de derivatización para conseguir los máximos valores de los picos cromatográficos. No obstante, la utilización de la columna monolítica ha permitido disminuir notablemente la duración del proceso cromatográfico, como se discute posteriormente.

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J. Agr. Food Chem. 2008, 56, 1858-1865

Analytical innovations in the detection of phenolics in wines

Pietro Russo, Álvaro Andreu-Navarro, María-Paz Aguilar-Caballos, Juan-Manuel Fernández-Romero, Agustina Gómez-Hens
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A liquid chromatographic method with on-line photometric and luminescent detection for the determination of eighteen phenolic compounds in wines is reported. Photometric detection is performed at four wavelengths, namely 256, 280, 320 and 365 nm, using a diode array detection system. The luminescent detection is achieved by means of a post-column derivatization reaction of ten of these compounds with terbium(III) in the presence of synergistic agents, such as ethylenediaminetetracetic acid (EDTA) and n-octyltriphosphine oxide (TOPO). A micellar medium provided by the surfactants sodium dodecylsulfate and Triton X-100 was used for the determination of the luminescent chelates at λ_{ex} 317, λ_{em} 545 nm. The long wavelength emission of lanthanide chelates can minimize interferences from background sample matrix, which usually emit at shorter wavelengths. The analytical features of the photometric and the fluorimetric methods, such as dynamic ranges of the calibration graphs, detection limits and precision data have been obtained. The practical usefulness of the developed methods is demonstrated by the analysis of Spanish and Italian wine samples (red, rosé, oloroso and white), which were diluted and directly injected into the chromatographic system. The accuracy of both methods was checked out by assaying a recovery study, which was performed at three different analyte levels for each type of sample.

Introduction.

Phenolics are a wide group of compounds constituted by phenolic aldehydes, hydroxybenzoic and hydroxycinnamic acids, catechins, flavonols, and stilbenes, in their monomeric form or conjugated to some species, such as tartaric acid in the case of cinnamic acids (*1*), among others. These compounds are present in wines because they are secondary metabolites of plants. The composition of phenolics and their concentration depend on grape variety, geographical origin, soil type, collection system, and grape processing. These compounds are responsible of the sensory properties of the wines, and, also, they are anti-carcinogenic and have an anti-inflammatory action when they are regularly ingested. A particular example of the importance of monitoring phenolic concentration to control the quality of wines is the presence of aromatic aldehydes, formed by a group of volatile compounds that are extracted from wood lignin during the winemaking process. The presence of these aldehydes in wines is an indicator of fermentation and aging in oak barrels, their absence being indicative of counterfeit aged wines. Vanillin and syringaldehyde are the most abundant aromatic aldehydes in wines.

The occurrence of phenolics has been extensively studied by liquid chromatographic methods (*1–18*). In most of them, conventional reversed-phase columns, constituted by packed microparticulate bonded silica, have been used (*1–5, 7–15, 18*), which generally feature separations of 14–25 compounds in almost 1h or 32 phenolics in 90 min, which makes routine analysis of these compounds very tedious. The use of other materials such as mesoporous silica has given rise to monolithic columns, which operate at higher flow rates with lower back- pressures than conventional columns (*16, 17*). Thus, they allow the analysis of samples using direct injection or low sample dilutions, because the cleaning and regeneration of the column can be done more quickly than in the conventional ones due to the high flow rates afforded. Monolithic columns have been used for the determination of 15 phenolics in red and white wine samples (*16*) with separation times around 30 min and, also, for the direct analysis of cider samples (*19*).

Diode array detection (*1, 3–17*) has been extensively used for the development of liquid chromatography methods, whereas fluorometric (*8,*

12) and mass spectrometry (*2*) detection systems have been used to a lesser extent. Most fluorometric methods proposed are based on measurements of the intrinsic fluorescence of some phenolics. Although these methods feature generally lower detection limits than photometric methods, the number of compounds determined by measuring their native fluorescence is low (*8, 12*). An approach that has been described recently is the use of a postcolumn derivatization reaction with terbium(III) for the determination of eight compounds, namely, hydroxybenzoic acids and catechins (*18*). The method involves the formation of luminescent chelates between phenolics and the lanthanide ion in an alkaline medium in the presence of ethylenediaminetetraacetic acid (EDTA) to prevent terbium precipitation. The detection limits achieved were lower than or comparable to those reported by other methods (*1, 3–11, 13–17*).

The work presented here reports the determination of 18 phenolics, which include hydroxybenzoic (gallic, protocatechuic, *p*-hydroxybenzoic, salicylic, vanillic, and syringic) and hydroxycinnamic (caffeic, ferulic, *p*-coumaric) acids, phenolic aldehydes (syringaldehyde, vanillin), catechins (catechin, epicatechin), flavonols (rutin, quercetin, kaempferol), and stilbenes (*cis*- and *trans*-resveratrol). The separation is achieved in <25 min using a monolithic column. Diode array (LC-DAD) and luminescence (LC-FL) detection systems are used simultaneously to detect and quantify these phenolics. The luminescent detection is based on the reaction of terbium(III) with 10 of these phenolics to give rise to luminescent chelates at a slightly basic medium, using tri *n*-octylphosphine oxide (TOPO) and EDTA as synergistic agents. Hydroxybenzoic acids, catechins, and aldehydes are detected by means of this derivatization reaction. Terbium-sensitized luminescence is used for the first time to determine aromatic aldehydes. The luminescence of the chelates is protected from non radiative processes by a micellar medium provided by Triton X-100 and sodium dodecyl sulfate. The photometric detection is accomplished at four wavelengths: 256, 280, 324, and 365 nm, whereas the luminescent detection is performed using 317 and 545 nm as excitation and emission wavelengths, respectively. Both detection systems are complementary tools to identify and quantify phenolic compounds in different kinds of wine samples, such as red, rosé, oloroso, and white. The only treatment needed is sample dilution prior to the injection onto the chromatographic system. This treatment is simpler than those described elsewhere (*1, 3, 8,*

10, 12, 14), which involve the extraction and/or fractionation of phenolics. The improved selectivity of sensitized luminescence can facilitate the identification of some compounds that cannot be easily identified using UV detection in the presence of complex wine samples. The analytical features of both methods as well as their performance in the analysis of wine samples are compared.

1. Materials and methods

1.1. Apparatus and Instruments.

An Agilent 1200 series liquid chromatography system composed of a quaternary pump, a degasser unit, a vial autosampler, a thermostated column compartment, and a diode array detector was used. An SLM Aminco (Urbana, IL) AB2 luminescence spectrometer provided with a 150 W continuous xenon lamp and a 7 W pulsed xenon lamp, furnished with a 176-052-QS Hellma (Hellma Hispania, Barcelona, Spain) flow cell with an inner volume of 18 μL, was used to monitor fluorescence measurements. A Gilson (Villiers-le-Bel, France) Minipuls 3 low-pressure peristaltic pump and Omnifit (Cambridge, U.K.) Teflon tubing of 0.5 mm i.d. were also used for constructing the postcolumn derivatization manifold. Chromatographic separation was performed using an Onyx monolithic C18 column (Phenomenex, Torrance, CA), 100 mm × 4.6 mm i.d.; pore sizes were mesopores (13 nm) and macropores (2 μm).

1.2. Reagents.

All chemicals used were of analytical reagent grade. Stock solutions (5000 mg L^{-1}) of phenolics were prepared as follows: phenolic acids, such as vanillic, gallic, protocatechuic, *p*-hydroxybenzoic (Sigma), syringic (Fluka), and salicylic (Aldrich) acids, were prepared by dissolving them in a minimum volume of ethanol (10-25 mL, depending upon the phenolic considered) and bringing them up to the final volume (50 mL) with distilled water. The same procedure was used for hydroxycinnamic acids, such as ferulic, caffeic, and *p*-coumaric (Sigma) acids; the aromatic aldehydes vanillin (Aldrich) and syringaldehyde (Fluka); the catechins

catechin and epicatechin, and, also, resveratrol (Sigma), all of which were degassed using nitrogen to prevent their oxidation by dissolved oxygen. Quercetin (Aldrich), rutin (Sigma), and kaempferol (Sigma) were dissolved in absolute ethanol. *cis*-Resveratrol was obtained by irradiating an aliquot of *trans*- resveratrol stock solution with a UV lamp at 360 nm for 2 h at room temperature. The observed yield for this change was calculated by taking into account the decrease in the area of the peak of *trans*-resveratrol at 324 nm. Fifty percent of *trans*-resveratrol was converted under the mentioned irradiation conditions. Intermediate solutions of 100 mg L^{-1} were prepared by diluting the stock solutions in distilled water, except for quercetin, rutin, resveratrol, and kaempferol, which required absolute ethanol to be stable. Stock and intermediate solutions were stored at 4 °C in the dark and were stable for at least 2 weeks. Working standard solutions were prepared from intermediate solutions by their dilution in distilled water.

The mobile phase used for the separation was constituted by solvent A (acetic acid 0.02%, pH adjusted to 3.85 with sodium hydroxide, Panreac Quimica, S.A., Barcelona, Spain) and solvent B [HPLC-grade pure acetonitrile (ACN), Panreac Quimica, S.A.], which were mixed by operating in the gradient mode during the chromatographic separation. The derivatization reagent was formed in situ by mixing two streams: the first one contained a solution of terbium(III) (7.5×10^{-3} M), prepared from terbium(III) nitrate pentahydrate (Aldrich), and the second one, a continuously stirred mixture integrated by TOPO (Sigma) (7×10^{-4} M), Triton X-100 (Fluka) (0.8%), EDTA (Fluka) (1.2×10^{-3} M), sodiumdodecylsulfate (Merck) (10^{-3} M), and tris(hydroxymethyl)aminomethane (Merck) buffer (Tris) (0.2 M, pH 9.5).

1.3. Manifold and Procedure.

Figure 1 shows the three-step integrated LC separation/derivatization/detection approach. Standards or diluted samples (50 µL), containing the analytes at concentrations within their corresponding dynamic ranges, were injected into the column. The mobile phase was pumped at 2 mL min-1, and the system operated under the gradient conditions included in **Table 1**. The variation of absorbance with

time was monitored at 256, 280, 320, and 365 nm. The time necessary to achieve a LC-DAD chromatogram was 25 min. Then, a cleanup and conditioning step were applied to have the chromatographic system ready for the next injection after 5 min.

The eluate of the column after passing through the diode array detection system was merged at point α with the derivatizing solution, which was pumped at a flow rate of 0.8 mL min^{-1}. The mixed solution passed through the reactor L1, in which the derivatization reaction took place. The fluorescence intensity was monitored at λex 317 and λem 545 nm for 20 min, and the corresponding blank solutions were subtracted. Chromatograms were taken using the original software of the luminescence spectrometer, and the raw data of luminescence intensity and time were exported and treated using adequate software packages for the estimation of the main chromatographic parameters.

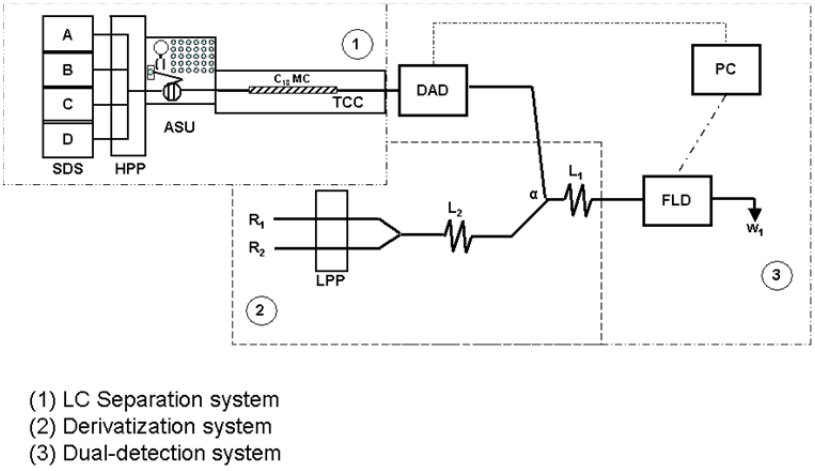


Figure 1. Integrated separation-derivatization and detection approach. 1, 2 and 3 depict the chromatographic, derivatizing and detection subsystems, respectively. A, and B, denotes 0.02% acetic acid and acetonitrile solutions, respectively; SDS, solvent delivery system; HPP, High-pressure quaternary gradient pump; ASU, autosampler unit; C_{18} MC, C_{18} monolithic column; TCC, thermostated column compartment; DAD diode array detection system; R_1 and R_2, reagent streams 1 and 2; LPP, low-pressure pump; L_1, mixing reactor; FLD, fluorescence detector; PC, personal computer; w_1, waste.

Table 1. Gradient elution conditions

Time (min)	%A	%B	Flow (ml min^{-1})
0	2.0	98.0	2.0
7.00	2.0	98.0	2.0
9.00	6.0	94.0	2.0
14.00	6.0	94.0	2.0
19.00	16.0	84.0	2.0
20.00	16.0	84.0	2.0
22.00	30.0	70.0	2.0
24.00	30.0	70.0	2.0
25.00	2.0	98.0	2.0
Conditioning step			
25.50	80.0	20.0	2.5
27.00	80.0	20.0	2.5
27.10	2.0	98.0	2.0
30.0	2.0	98.0	2.0

A: acetonitrile, B: acetic acid (0.02 %, pH 3.85)

1.4. Assessment of Precision.

Precision was evaluated at two different concentration levels, 200 and 600 ng mL^{-1} (except for *cis*- and *trans*- resveratrol, which were 100 and 300 ng mL^{-1} and 300 and 900 ng mL^{-1} , respectively). Seven solutions were subjected to the chromatographic separation on the same day and 10 solutions made in duplicate on five different days to calculate in-day and interday, respectively, precision for retention times and areas by obtaining the percentage of relative standard deviation of these parameters in each case.

1.5. Estimation of LODs.

The estimation of LODs was done following IUPAC recommendations (*20*), which involve the use of a signal-to-noise ratio of 3. The signal considered as blank signal was the standard deviation of the *y*-intercept of the calibration curve made using 10 mixtures of aqueous standards of the phenolics determined.

1.6. Analysis of Wine Samples.

Wine samples were diluted with distilled water to match the linear ranges of calibration for each analyte and were directly injected onto the chromatographic system following the procedure above indicated. Each determination was the mean of three measurements.

2. Results and discussion

2.1. Study of Diode Array and Luminescent Detection Systems.

Absorbance spectra were recorded in the range of 200–400 nm for selecting the wavelengths of the LC-DAD method. Four wavelengths (256, 280, 324, and 365 nm), which are close to the maximum absorption wavelength of each phenolic, were chosen.

Fluorometric detection was achieved using terbium(III) as derivatizing reagent. The relatively long emission wavelength of terbium(III) chelates can improve the selectivity and sensitivity of the measurements because some fluorescent signals from sample matrix, which could overlap the chromatographic peaks of the phenolics, are minimized or avoided. Postcolumn derivatization with terbium(III), using an alkaline medium and in the presence of EDTA, has been previously reported for the determination of some hydroxybenzoic acids and catechins in white wine samples (*18*). However, aromatic aldehydes do not give any luminescent signal under these experimental conditions. It has been necessary to modify this derivatization reaction to obtain luminescent terbium(III) chelates of aromatic aldehydes. These chelates are formed in a slightly basic medium, provided by a Tris buffer solution, and using TOPO and EDTA as synergistic agents. Hydroxybenzoic acids and catechins also give luminescent signals under these conditions. **Table 2** shows the maximum absorption wavelengths of phenolics, which were used to establish the LC-DAD method. Some of these compounds also present other less intense absorption wavelengths, which appear in this table as confirmation wavelengths. These wavelengths can be chosen as an alternative when an improvement of the selectivity is required due to chromatographic overlapping of other peaks at the maximum absorption

wavelength, although the signal obtained is lower. **Table 2** also shows the excitation and emission wavelengths corresponding to 10 of the phenolics assayed, which form luminescent chelates with terbium(III). Although they show different maximum excitation wavelengths, their broad excitation bands allow the selection of 317 nm as the working wavelength for the simultaneous excitation of these chelates. This wavelength was chosen to get the maximum sensitivity for catechin, vanillin, syringaldehyde, and epicatechin, which exhibited lower luminescence intensities than hydroxybenzoic acids under the experimental conditions assayed. The luminescence emission is measured at 545 nm, which is the most intense emission wavelength of terbium(III).

Table 2. Characteristic wavelengths of the studied compounds

Compound	LC-DAD		LC-FL Phenolic-terbium chelate	
	Maximum absorption wavelength (nm)	Confirmation wavelength (nm)	Maximum excitation wavelength (nm)	Emission wavelength (nm)
Gallic acid	280	256	317	545
Protocatechuic acid	256	280	315	
p-Hydroxy benzoic acid	256	280	245, 299	
Salicylic acid	280	---	295	
Vanillic acid	256	280	298	
Caffeic acid	324	280	---	
Syringic acid	280	256	293	
Catechin	280	---	302	
Vanillin	280	256, 324	324	
p-Coumaric acid	280	324	---	
Syringaldehyde	324	280	335	
Epicatechin	280	256, 324	299	
Ferulic acid	324	280	---	
Rutin	256	365	---	
trans-Resveratrol	324	---	---	
Quercetin	365	---	---	
cis-Resveratrol	280	---	---	
Kaempferol	365	---	---	

2.2. Optimization of Variables.

The hydrodynamic and chemical variables involved in LC-DAD and LC-FL methods were optimized using the univariate methodology. Values chosen were those yielding the maximum absorbance and luminescence signals with a minimum standard deviation.

2.3. Chromatographic Variables.

The luminescence of lanthanide chelates can be quenched by the vibration of hydroxyl groups of water molecules (*21*), which is really notable when RP-LC is used due to the relatively high content of water in the mobile phases used. The addition of a synergistic agent or the introduction of an acid in the mobile phase can help to achieve the required chromatographic sensitivity. It has been previously described that an adequate luminescent signal can be obtained in the presence of acetate ions (*22, 23*), which can be ascribed to their capability to displace water from the first coordination sphere of terbium(III) (*24*).

Chromatographic variables were optimized to separate the analytes in the shortest separation time. The study of the effect of the apparent pH of the mobile phase showed that it is a critical variable to achieve the required chromatographic selectivity. The shape of peaks was more definite at pH 3.85, using 0.02% acetic acid. The pH of the mobile phase was critical for the separation of the peaks of caffeic and syringic acids and the peaks of syringaldehyde and epicatechin. Binary mixtures of acetic acid (0.02%, pH 3.85) and ACN using different gradient profiles were tried at the optimum flow rate (2 mL min-1), finding that the use of increasing percentages of ACN in two subsequent steps (**Table 1**), from 14 to 19 min and from 20 to 22 min, was useful for the resolution of *p*-coumaric, syringaldehyde, epicatechin, and ferulic acid. Initial percentages of ACN >2% allowed lower retention times for all of the compounds, but they were overlaped.

The optimum conditions found were those appearing in **Table 1**, which were used for the chromatographic separation. As can be also seen from this table, cleaning and conditioning steps, which take only 5 min, are

included in the gradient routine to ensure reproducibility of retention times between two repeated injections.

2.4. Postcolumn Derivatization Variables.

The study of the flow rate of the derivatizing reagent solution showed that 0.8 mL min^{-1} provided the optimum derivatizing solution column effluent ratio when 2 mL min^{-1} was used for the chromatographic separation. The length of the reactor L1 used was 50 cm, which is enough for the development of the derivatization reactions due to the relatively fast rate for the terbium chelate formation. A Tris buffer solution was chosen to increase the pH of the chromatographic eluent, which is required to achieve the optimum luminescence signal without terbium hydroxide precipitation. The concentration of this buffer was studied in the range of 0.05–0.2 M, the pH being adjusted to 8.7 using the highest concentration. The influence of terbium(III) concentration was evaluated for each phenolic-terbium chelate in the range of 3×10^{-4} - 1.0×10^{-2} M (**Figure 2**).

The concentration of TOPO was studied in the range of 1.5×10^{-4} - 1.6×10^{-3} M. This synergistic agent notably enhances the sensitized luminescence of salicylic acid and catechin when it is used at (2–7.5) × 10^{-4} M. This behavior was less significant for the other phenolics assayed. EDTA was mainly used to prevent the terbium precipitation, and its influence was studied in the range of 2×10^{-4} - 1.0×10^{-2} M. It was found that this compound improves the signal of gallic acid, but decreases the signal from the other compounds. Thus, a compromise solution was taken by using 1.2×10^{-3} M EDTA. The surfactants Triton X-100 and sodium dodecyl sulfate were used to protect the luminescence of the chelates from nonradiative processes. Triton X-100 had a positive influence on the signal of gallic acid, although the luminescence from other phenolic chelates was unaltered until 0.8%, from which a decrease was observed, this concentration being chosen as optimum. The other surfactant increases the signal of syringaldehyde, which was practically negligible in its absence. **Figures 3** and **4** depict the chromatograms obtained for a standard mixture of phenolics, carried out under optimum conditions using LC-DAD and LC-FL methods, respectively. As can be seen from **Figure 3**, the highest number of analytes appears in the chromatogram obtained at 280 nm.

which was practically negligible in its absence. **Figures 3** and **4** depict the chromatograms obtained for a standard mixture of phenolics, carried out under optimum conditions using LC-DAD and LC-FL methods, respectively. As can be seen from **Figure 3**, the highest number of analytes appears in the chromatogram obtained at 280 nm.

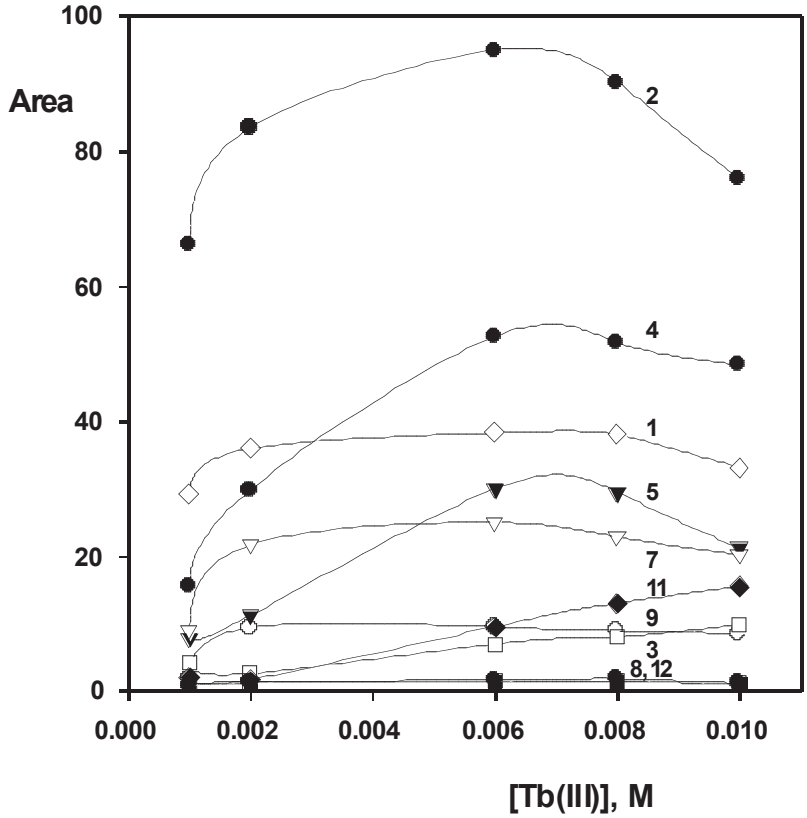


Figure 2. Influence of the terbium (III) concentration for (600 ng mL^{-1}): 1 gallic acid; 2, protocatechuic acid; 3, p-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 7, syringic acid; 8, catechin; 9, vanillin; 11, syringaldehyde; 12, epicatechin, under the optimum experimental conditions.

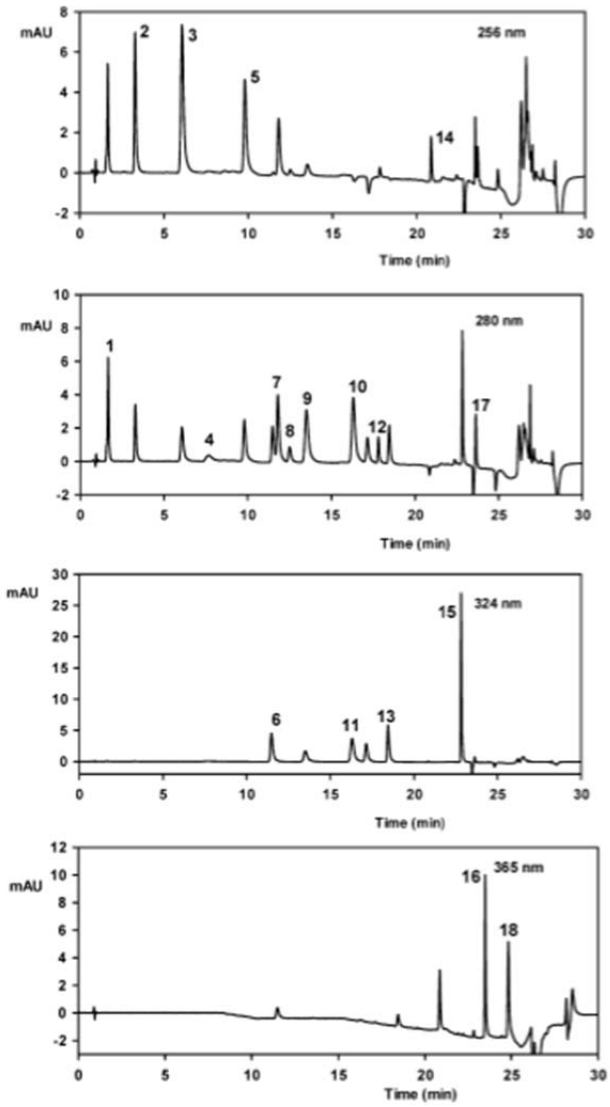


Figure 3. Typical chromatograms achieved using the LC-DAD method at 256, 280, 324 and 365 nm, by injection of a mixture of a aqueous standard solution containing 600 ng mL^{-1} of each analyte (excepting cis- and trans-resveratrol, which were 900 and 300 ng mL^{-1})and processed under the optimum conditions described in the Manifold and Procedure section. 1 gallic acid; 2, protocatechuic acid; 3, p-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, catechin; 9, vanillin; 10, p-coumaric acid; 11, syringaldehyde; 12, epicatechin; 13, ferulic acid; 14, rutin; 15, trans-resveratrol; 16, cis-resveratrol; 17, quercetin; 18, kaempferol.

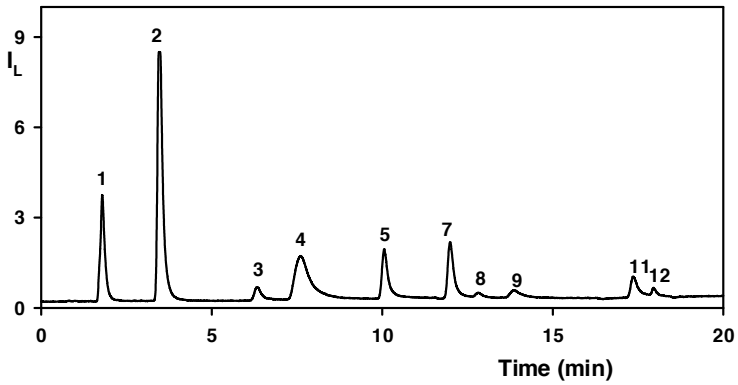


Figure 4. Chromatogram achieved using the LC-FL method by injection of a mixture of aqueous standards at 600 ng mL^{-1} of each analyte and processed under the optimum experimental conditions. 1 gallic acid; 2, protocatechuic acid; 3, p-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 7, syringic acid; 8, catechin; 9, vanillin; 11, syringaldehyde; 12, epicatechin.

2.5. Analytical Features.

Calibration graphs were run under the optimum experimental conditions by using an external calibration method. **Table 3** shows the retention time for each phenolic, the calibration parameters, and the detection limits (LODs) obtained for both methods, which were calculated according IUPAC recommendations (*20*). These detection limits are lower than or comparable to those of other methods previously reported (*1–17, 19*). For LC-DAD, this fact can be explained by two reasons: (1) the use of a monolithic column, which provides narrower peaks than the particulate columns, enhancing the peak height and improving the signal-to-noise ratio; (2) a higher injection volume (50 μL) than those used in the other methods, which are typically 10–20 μL. This injection volume does not provide notable peak broadening due to the relatively high flow rate used in the present method. The $r2$ values obtained indicate a very good correlation of experimental data to calibration curves. The precision of both methods has been studied for retention times and areas at two different analyte concentration levels, and the results obtained at 600 ng mL^{-1} for most analytes, expressed as the percentage of relative standard deviation, are summarized in **Table 4**. It can be seen that the repeatability of peak areas for both methods was equal to or lower than 6%, the results for the LC-FL method being slightly higher than those obtained by applying

the LC-DAD method. The precision was evaluated also at 200 ng mL^{-1}, finding that the results obtained ranged from 0.1 to 1.3% and from 0.3 to 4.3% for the in-day and interday, respectively, precisions of retention times and from 1.2 to 7.4% and from 3.2 to 9.7% for the in-day and interday, respectively, precisions of areas obtained using the FL method. For the DAD method, the results ranged from 0.07 to 1.5% and from 0.15 to 3% for the in-day and interday, respectively, precisions of retention times and from 0.5 to 7.5% and from 1.3 to 10.7% for the in- day and interday, respectively, precisions of areas. This difference could be explained by bearing in mind that LC-FL can be affected by more sources of variability of the results, such as the use of a second pump and the derivatization reaction.

Table 3. Analytical features of LC-DAD and LC-FL methods

Compound	Retention time (min)	Dynamic range* (ng mL^{-1})	Slope ± SD	y-intercept ± SD	r^2	LOD (ng mL^{-1})
Gallic acid	1.66	10-900	0.0642 ± 0.0001	-0.19 ± 0.07	0.9999	3
	1.79	*10-900*	*0.0645 ± 0.0003*	*0.5 ± 0.1*	*0.9999*	*5*
Protocatechuic acid	3.26	100-10000	0.0942 ± 0.0004	-3 ± 1	0.9997	36
	3.43	*10-900*	*0.1608 ± 0.0003*	*0.6 ± 0.1*	*0.9999*	*2*
p-Hydroxybenzoic acid	6.06	100-15000	0.1652 ± 0.0003	-4 ± 1	0.9999	22
	6.26	*50-2000*	*0.0176 ± 0.0002*	*0.30 ± 0.09*	*0.9990*	*15*
Salicylic acid	7.36	50-1000	0.0127 ± 0.0001	0.25 ± 0.06	0.9995	14
	7.51	*50-1000*	*0.0862 ± 0.0009*	*1.5 ± 0.4*	*0.9991*	*14*
Vanillic acid	9.80	100-20000	0.0975 ± 0.0002	-3 ± 1	0.9999	30
	10.0	*50-2000*	*0.0538 ± 0.0004*	*0.7 ± 0.3*	*0.9994*	*17*
Caffeic acid	11.47	10-900	0.0901 ± 0.0002	0.02 ± 0.08	0.9999	3
Syringic acid	11.78	30-15000	0.06116 ± 0.00003	-0.3 ± 0.2	0.9999	10
	11.95	*10-900*	*0.0454 ± 0.0001*	*0.45 ± 0.04*	*0.9999*	*3*
Catechin	12.64	20-2000	0.02096 ± 0.00008	-0.22 ± 0.06	0.9999	8
	12.77	*300-5000*	*0.00269 ± 0.00005*	*-0.1 ± 0.1*	*0.9976*	*114*
Vanillin	13.64	50-15000	0.0931 ± 0.0001	-1.9 ± 0.6	0.9999	19
	13.82	*100-2000*	*0.0140 ± 0.0002*	*0.4 ± 0.2*	*0.9989*	*40*
p-Coumaric acid	16.27	50-15000	0.1141 ± 0.0001	-0.2 ± 0.5	0.9999	14
Syringaldehyde	17.22	10-1000	0.0616 ± 0.0002	0.07 ± 0.07	0.9999	3
	17.35	*50-2000*	*0.0221 ± 0.0002*	*0.4 ± 0.1*	*0.9996*	*15*
Epicatechin	17.85	20-5000	0.02061 ± 0.00004	-0.25 ± 0.05	0.9999	8
	17.95	*100-2000*	*0.00361 ± 0.00004*	*0.03 ± 0.03*	*0.9986*	*29*
Ferulic acid	18.42	20-15000	0.10498 ± 0.00004	-0.4 ± 0.2	0.9999	5
Rutin	20.86	100-20000	0.02364 ± 0.00003	-0.7 ± 0.2	0.9999	24

Table 3. Continuation

Compound	Retention time (min)	Dynamic range* (ng mL^{-1})	Slope ± SD	y-intercept ± SD	r^2	LOD (ng mL^{-1})
trans-Resveratrol	22.86	10-7500	0.13782 ± 0.00003	0.2 ± 0.08	0.9999	2
Quercetin	23.52	200-3500	0.097 ± 0.001	-5 ± 2	0.9995	60
cis-Resveratrol	23.67	100-5000	0.0595 ± 0.0003	-1.7 ± 0.7	0.9999	35
Kaempferol	24.84	50-2000	0.0892 ± 0.0005	3.1 ± 0.5	0.9999	18

(*the lower limit of dynamic range for each phenolic is its LOQ). Italics: fluorimetric method

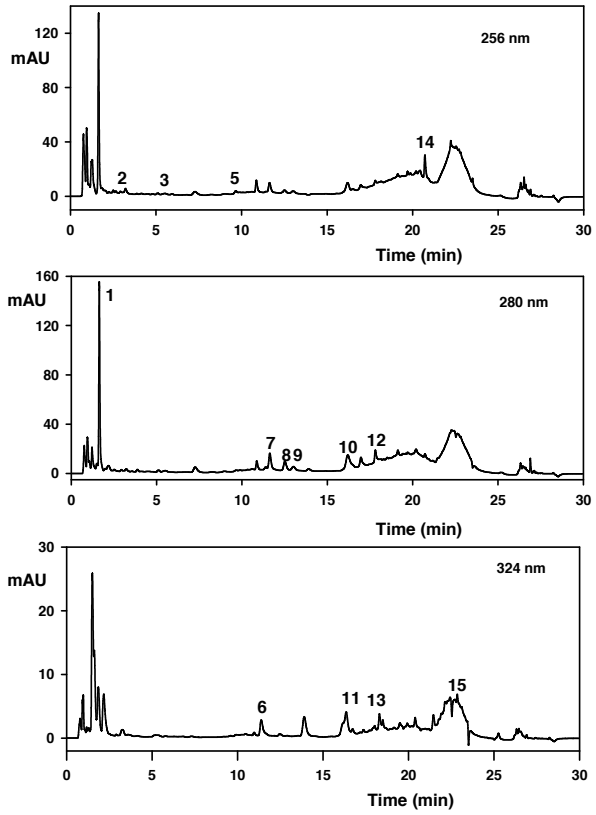
Table 4. Precision (%RSD) of LC-DAD and LC-FL methods

	LC-DAD method				LC-FL method			
	Retention time[a]		Area[a]		Retention time[a]		Area[a]	
Compound	A	B	A	B	A	B	A	B
Gallic acid	2.0	1.8	1.2	3.2	0.5	1.3	3.0	4.7
Protocatechuic acid	0.3	3.0	1.3	2.4	0.7	0.6	1.2	2.7
p-Hydroxybenzoic acid	0.5	1.4	1.3	2.1	1.3	1.9	4.7	5.3
Salicylic acid	1.5	2.3	4.2	5.2	0.8	1.5	3.9	1.5
Vanillic acid	0.4	0.7	1.3	3.4	0.4	1.0	2.6	6.4
Caffeic acid	0.3	0.8	1.5	3.0				
Syringic acid	0.3	0.7	0.8	3.1	0.6	0.4	3.4	5.5
Catechin	0.3	0.5	1.6	3.7	0.1	0.6	4.9	5.0
Vanillin	0.3	0.6	1.4	3.5	0.5	0.2	5.4	2.0
p-Coumaric acid	0.2	0.6	1.3	2.5				
Syringaldehyde	0.1	0.3	0.7	3.0	0.2	0.1	4.1	4.7
Epicatechin	0.1	0.2	1.7	3.2	0.5	0.08	6.0	2.8
Ferulic acid	0.1	0.1	1.2	2.5				
Rutin	0.05	0.1	2.0	4.0				
trans-Resveratrol	0.01	0.1	1.1	2.4				
Quercetin	0.2	0.2	3.3	3.5				
cis-Resveratrol	0.1	0.1	1.5	2.3				
Kaempferol	0.3	0.09	2.5	9.1				

[a]Retention times and areas obtained from standard mixtures of 600 ng mL^{-1} of each analyte, excepting for trans-resveratrol (900 ng mL^{-1}) and cis-resveratrol (300 ng mL^{-1}).
A: Experiments carried out in the same day (n = 7). B: Experiments carried out in different days (n= 5).

2.6. Applications.

LC-DAD and LC-FL methods were applied to the analysis of 15 wine samples belonging to different wine varieties (red, oloroso, rosé, and white). Samples W1-W5 were red wines, W6 was rosé wine, W7–W10 included oloroso wines, and W11–W15 were white wine samples. These wine samples belonged to Córdoba (Spain) (samples W1, W3, W4, W6, W10, W11, W14, and W15) and Sicily (Italy) (samples W2, W5, W7–W9, W12, and W13) geographical areas. Wine samples were diluted with distilled water to match the linear range of the calibration graph for each analyte. **Figures 5** and **6** correspond to the typical chromatograms achieved for a red wine sample using LC-DAD and LC-FL methods, respectively.



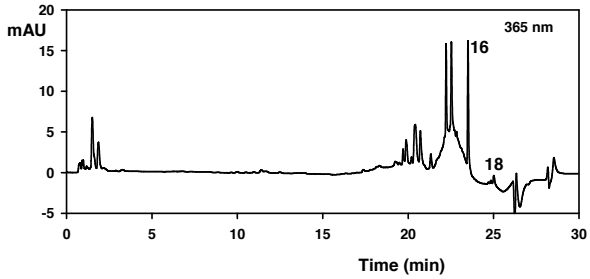


Figure 5. Chromatograms of a red wine sample (Sample W2, dilution 1/10) using the LC-DAD method obtained by direct injection of 50 µL of diluted sample. 1 gallic acid; 2, protocatechuic acid; 3, p-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, catechin; 9, vanillin; 10, p-coumaric acid; 11, syringaldehyde; 12, epicatechin; 13, ferulic acid; 14, rutin; 15, trans-resveratrol; 16, quercetin; 17, cis-resveratrol; 18, kaempferol.

As can be seen from **Figure 5**, there is an increase in the baseline of the chromatograms in the range of 22–24 min. It has been reported that this baseline drift can be ascribed to the presence of polymeric compounds, which have not been separated in the experimental conditions (*1*). However, the chromatogram corresponding to the LC-FL method (**Figure 6**) is cleaner than that obtained for the LC-DAD method. This demonstrates the higher selectivity level obtained using terbium-sensitized luminescence, which is less prone to baseline drifts and to the interference from other sample components. An example of the complementarity of the information supplied by both LC-DAD and LC-FL methods in the analysis of real samples is the determination of salicylic acid, which cannot be determined with accuracy in some wine samples using photometric measurements, due to the presence of an overlapping peak at the same retention time. However, this interference is not observed for the LC-FL method, which also gives satisfactory results when standards of salicylic acid are added. Thus, the LC-FL method is suitable for the identification and confirmation of some of the peaks found by LC-DAD.

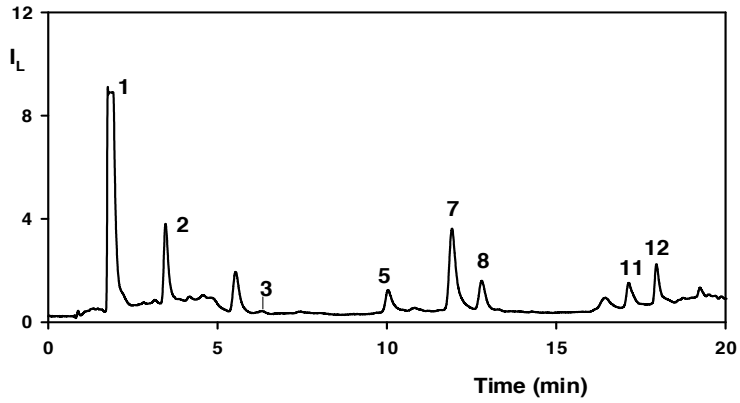


Figure 6. Chromatogram of a red wine sample (Sample W2, dilution 1/10) using the LC-FL method obtained by direct injection of 50 µL of the sample. 1 gallic acid; 2, protocatechuic acid; 3, p-hydroxybenzoic acid; 4, salicylic acid; 5, vanillic acid; 7, syringic acid; 8, catechin; 9, vanillin; 11, syringaldehyde; 12, epicatechin.

Tables 5 and **6** summarize the phenolic content found for the Spanish and Italian wines analyzed, respectively; it can be seen that the highest values were found in red wine samples. The phenolics that form luminescent chelates with terbium(III) were determined using both LC-DAD and LC-FL methods, and the results obtained by both methods were statistically compared using a regression test. The regression study carried out with the concentration values obtained by both methods showed that they did not differ significantly taking into account the correlation parameters obtained ($Y = 0.185 + 0.979X$, $r = 0.954$, Y and X being the results for LC-FL and LC-DAD, respectively). *cis*-Resveratrol was not found in any of the samples. Sicilian red wines contain high concentrations of gallic acid, which agree with the values found in the literature (*2*). Protocatechuic and syringic acids are the most abundant phenolics found in white wine samples.

Table 5. Phenolic content of the Spanish wines analyzed

	Content[a] (mg L^{-1})							
Compound	W1	W3	W4	W6	W10	W11	W14	W15
Gallic acid*	2.8 ± 0.1	30.5 ± 0.8	33± 1	3.60 ± 0.3	1.70 ± 0.05	1.9 ± 0.1	2.16 ± 0.08	1.84 ± 0.08
Protocatechuic acid*	1.268 ± 0.002	1.09 ± 0.01	1.269 ± 0.001	0.58 ± 0.01	0.77 ± 0.02	1.14 ± 0.06	1.524 ± 0.004	0.868 ± 0.002
p-Hydroxybenzoic acid*	0.8 ± 0.01	0.265 ± 0.005	n.d.	0.63 ± 0.02	n.q.	n.q.	0.34± 0.04	0.79 ± 0.02
Salicylic acid*	n.q.	n.q.	0.32± 0.02	n.d.	0.33 ± 0.02	0.215 ± 0.001	0.21 ± 0.01	0.240 ± 0.008
Vanillic acid*	1.21 ± 0.02	1.020 ± 0.003	0.610 ± 0.003	0.33 ± 0.06	0.30 ± 0.03	1.26 ± 0.04	1.16 ± 0.08	0.208 ± 0.008
Caffeic acid	2.90 ± 0.04	9.7 ± 0.1	10.7 ± 0.3	1.71 ± 0.02	0.674 ± 0.003	0.163 ± 0.003	n.q.	1.68 ± 0.08
Syringic acid*	4.18 ± 0.02	3.46 ± 0.03	0.37 ± 0.05	1.4 ± 0.1	0.30 ± 0.02	0.36 ± 0.03	0.80 ± 0.03	0.090 ± 0.003
Catechin*	26.3 ± 0.2	30 ± 2	8.1 ± 0.5	n.d.	n.d.	0.95 ± 0.03	1.2 ± 0.1	n.d.
Vanillin*	n.q.	n.d.	n.d.	n.q.	n.d.	n.d.	0.56 ± 0.04	n.d.
p-Coumaric acid	6.4 ± 0.1	11.8 ± 0.2	11.6 ± 0.3	0.11 ± 0.01	n.d.	0.51 ± 0.01	n.d.	0.91 ± 0.05
Syringaldehyde*	1.3 ± 0.1	1.6 ± 0.1	n.d.	n.d.	0.45 ± 0.05	n.d.	0.43 ± 0.02	n.d.
Epicatechin*	14.0 ± 0.1	13.3 ± 0.3	29.1 ± 0.1	1.94 ± 0.80	n.d.	n.q.	2.9 ± 0.3	n.d.
Ferulic acid	0.399 ± 0.004	0.445 ± 0.003	0.56 ± 0.02	0.14 ± 0.02	0.42 ± 0.04	n.q.	0.21 ± 0.02	0.47 ± 0.02
Rutin	n.d.	5.53 ± 0.02	0.75 ± 0.03	0.81 ± 0.02	n.q.	n.d.	0.212 ± 0.004	n.q.
trans-Resveratrol	0.42 ± 0.02	0.460 ±0.004	0.094 ± 0.002	0.83 ± 0.03	n.q.	0.015 ± 0.007	n.q.	n.q.
Quercetin	5.3 ± 0.2	6.5 ± 0.2	7.0 ± 0.5	0.47 ± 0.02	0.39 ± 0.01	n.d.	n.d.	0.325 ± 0.003
Kaempferol	0.32 ± 0.01	0.560 ± 0.009	0.490 ± 0.005	n.q.	n.q.	n.d.	n.d.	n.d.

*measured using LC-FL method

[a] Mean ± SD (n = 3)

(n.d.: below detection limit; n.q.: not quantified, below quantification limit)

Table 6. Phenolic content of the Italian wines analysed

	Content[a] (mg L^{-1})						
Compound	W2	W5	W7	W8	W9	W12	W13
Gallic acid*	98.0 ± 4.0	87.2± 0.8	9.45 ± 0.07	10.9 ± 0.2	15.3 ± 0.9	2.0 ± 0.1	2.6 ± 0.1
Protocatechuic acid*	1.6 ± 0.1	2.2 ± 0.2	1.615 ± 0.001	1.50 ± 0.080	1.642 ± 0.003	0.65 ± 0.01	0.41 ± 0.04
p-Hydroxy benzoic acid*	n.q.	n.q..	0.344 ± 0.004	0.48 ± 0.04	0.175 ± 0.005	0.252 ± 0.004	0.11 ± 0.04
Salicylic acid*	n.d.	n.d.	0.20 ± 0.01	0.886 ± 0.002	0.32 ± 0.02	0.090 ± 0.004	n.q.
Vanillic acid*	1.25 ± 0.05	2.2 ± 0.1	0.27 ± 0.01	0.34 ± 0.02	0.29 ± 0.02	0.114 ± 0.008	0.106 ± 0.008
Caffeic acid		7.27 ± 0.07	1.81 ± 0.01	1.574 ± 0.006	3.20 ± 0.01	1.312 ± 0.006	1.43 ± 0.01
Syringic acid*	6.3± 0.4	5.1 ± 0.3	0.200 ± 0.008	0.46 ± 0.01	0.139 ± 0.002	0.30 ± 0.01	0.128 ± 0.004
Catechin*	3.8± 0.2	33 ± 2	n.d.	1.00 ± 0.02	n.d.	3.2 ± 0.1	1.30 ± 0.06
Vanillin*	n.d.	n.d.	n.d.	n.q.	n.q.	n.d.	0.20 ± 0.02
p-Coumaric acid	16.3 ± 0.2	24.6 ± 0.2	2.04 ± 0.03	1.296 ± 0.006	1.68 ± 0.05	0.590 ± 0.004	0.60 ± 0.04
Syringaldehyde*	3.0 ± 0.2	2.12 ± 0.03	n.q.	n.d.	n.d.	n.q.	0.14 ± 0.01
Epicatechin*	32.0 ± 0.3	26.5 ± 0.5	0.332 ± 0.006	0.52 ± 0.02	0.50 ± 0.04	1.32 ± 0.1	0.43 ± 0.02
Ferulic acid	0.46 ± 0.02	0.36 ± 0.01	1.00 ± 0.05	0.34 ± 0.02	0.540 ± 0.005	0.486 ± 0.006	0.344 ± 0.002
Rutin	19.0 ± 1.0	3.5 ± 0.2	0.961 ± 0.001	0.46 ± 0.02	0.65 ± 0.03	0.73 ± 0.02	0.20 ± 0.02
trans-Resveratrol	0.34 ± 0.01	0.72 ± 0.03	n.d.	0.050 ± 0.002	n.q.	0.040 ± 0.004	0.008.4 ± 0.0002
Quercetin	11.24 ± 0.05	13.0 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d
Kaempferol	0.216 ± 0.002	0.47 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.

*measured using LC-FL method

[a] Mean ± SD (n = 3)

(n.d.: below detection limit; n.q.: not quantified, below quantification limit)

The recovery study was carried out by adding three different amounts of each analyte to four of the samples analyzed at concentrations in the range of 0.5–20 mg L^{-1} and subtracting the results obtained from similarly unspiked samples. **Table 7** shows the mean recovery values obtained, which were in the range of 68.0–116.9%, most of them being >85%, except those for rutin in sample W1 and syringaldehyde in sample W11, which gave values of 68%, although the values obtained for the other analyzed samples were closer to 100%. Also, the LC- DAD method gave results similar to those of the LC-FL method. The recoveries obtained for the rest of compounds were in agreement with those provided by other methods (*3, 6, 13, 14, 16*). In some of these methods, the accuracy was determined using standards or synthetic wine samples (*6, 13, 14*) and, therefore, the results cannot be directly compared with those obtained with the proposed method, which were obtained in the presence of commercial wine samples. Despite this fact, the results obtained were closer to 100% than those provided by other methods involving several extraction steps (*3*).

This study shows the usefulness of terbium-sensitized luminescence as a complementary tool for the identification and quantification of phenolic compounds in complex wine samples. This is the first time that aromatic aldehydes have been determined using terbium-sensitized luminescence. The spectral selectivity achieved using this approach can be profited to avoid or minimize the potential interferences from the sample matrix. The sensitivity levels achieved with both LC-DAD and LC-FL methods allow higher dilution of samples to be done, which is useful to improve the selectivity. The practical application of both methods has been shown by the analysis of 15 wine samples with satisfactory results.

Table 7. Mean recoveries obtained by the analysis of different wine samples

Compound	Recovery (%)			
	W1	W2	W7	W11
Gallic acid*	102.3	95.8	102.9	94
Protocatechuic acid*	92.6	90.6	94.0	81.3
p-hydroxy benzoic acid*	99.8	92.7	98.4	92.1
Salicylic acid*	108.9	88.6	104.8	94.2
Vanillic acid*	87.8	94.2	99.7	88.5
Caffeic acid	108.9	112.3	103.3	115.0
Syringic acid*	99.3	101.9	89.3	95.6
Catechin*	93.6	110.7	115.0	103.5
Vanillin*	97.8	103.4	101.6	92.7
p-coumaric acid	96.8	105.8	101.1	85
Syringaldehyde*	83.8	103.5	94.9	68.0
Epicatechin*	98.0	108.4	76.0	89.2
Ferulic acid	90.5	93.6	86.0	102.9
Rutin	68.0	114.2	99.2	100.5
trans-resveratrol	93.6	105.7	95.7	96.0
Quercetin	116.9	108.8	109.7	115.1
Kaempferol	88.0	100.2	88.1	88.6

*measured using LC-FL method

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Usefulness of terbium-sensitized luminescence detection for the chemometric classification of wines by their content in phenolic compounds

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A method for wine classification based on the phenolic compound content, wine variety and geographical area is described. The method involves the use of the results obtained from the analysis of fifteen samples of Italian and Spanish wines from different geographical origins [Sicilia (Italy) and Córdoba (Spain)] using liquid chromatography (LC) with photometric and fluorimetric detection, in which eighteen phenolics were determined: gallic acid, protocatechuic acid, p-hydroxybenzoic acid, salicylic acid, vanillic acid, caffeic acid, syringic acid, catechin, vanillin, p-coumaric acid, syringaldehyde, epicatechin, ferulic acid, rutin, trans- and cis-resveratrol, quercetin and kaempferol. Photometric measurements were performed selecting four wavelengths (256, 280, 320 and 365 nm), using a diode array detection system. The fluorimetric detection was achieved by measuring the sensitized luminescence provided by the chelates formed between each analyte and terbium (III). All samples were commercial wines bought in local markets and analyzed immediately after they were opened. The pattern data matrix was constructed by the concentration of each analyte present in wine, which was determined by the most adequate method, namely LC-photometric or LC-fluorimetric method. This data matrix was subjected to different algorithms in order to classify and characterize the wine samples adequately. Supervised (LDA)

and un-supervised (FA) pattern recognition methods were used. The wine pattern generation with LC separation and dual detection approach to determine eighteen phenolic compounds and the chemometric treatment provide an appropriate way with recognition and prediction rates. The values obtained for these rates were 100 % when fluorimetric detection was used. These results can be considered satisfactory, which proves the usefulness of the selected variables.

Introduction

In recent years, there has been an increasing interest in the development of chemometric applications in order to establish the variety, geographical origin, manipulation and other technological features, which define the taste of foods and beverages from agricultural origin [1-4]. The reasons for this interest are: 1) laws enforce labeling of the geographical origin of the foodstuff and beverages in many countries due to demands of more information from consumers and improvement of their domestic production; 2) producers have begun to advertise their brands of high-quality products by including geographical origin and other features in the label for economic reasons; 3) prevention of frauds and adulteration on the label, production and commercialization. Thus, the development of methods giving acceptable information is highly desirable for consumers, producers and administrative authorities.

Wine industry and the market sector are particular examples in which the development of sophisticated chemometric techniques are essential for the improvement of the analytical information about wine composition and for assessing wine authenticity [5,6]. Several chemometric procedures have been used as the basis for discrimination of wines according to vinification technology and classification according to region, type and variety. Different pattern recognition techniques (PRT), such as Principal Component Analysis (PCA) [7,8], Linear and Canonical Discriminant Analysis (LDA and CDA) [9-11], Probabilistic Neural Network (PNN) [12], K-Nearest Neighbors (KNN)[13], Cluster Analysis (CA) [11], Multiregression Analysis (MRA), Partial Least Squares (PLS) [14-16], and CAIMAN [17] have been used for this purpose. There is a great number of combined techniques employing group classification to evaluate wine authenticity using different analytes such as volatile compounds [17], minerals and trace elements [14,18] aminoacids [14] and phenolics [7,16].

Phenolics are important constituents of wines which contribute, either directly or indirectly, to wine sensory properties (colour, astringency, bitterness and aroma). Many of these compounds have shown potent antioxidant activity, acting as bioactive agents that may contribute to prevent degenerative processes in human health [19] such as cancers, cardiovascular disorders, and osteoporosis, which are associated with

oxidative damage of DNA, proteins and lipids. The antioxidants existing in wines can help to prevent these damages by reacting directly or by stimulating endogenous protection systems [20].

Phenolic compounds have been successfully used for assessing wine authenticity and classification procedures since these compounds are characteristic of wine type and can provide information about the geographical origin. It is well known that during fermentation the must in contact with oxygen can undergo oxidation of phenolics, which causes wine browning. When the maturation is finished, phenolic oxidation decreases and the phenolic content is stabilized. Liquid chromatography (LC) with diode-array detection (DAD) has been applied to determine the phenolic fraction [13], which has been used for an effective wine, vinegar and liquor classification and prediction of the sample membership according to employed treatment methods, varieties or geographical origin.

The aim of this paper has been the development of a method for wine classification using the information provided by an LC method with photometric and fluorimetric detection for the determination of eighteen phenolic compounds [21]. These compounds are gallic acid (GAL), protocatechuic acid (PTC), p-hydroxybenzoic acid (PHB), salicylic acid (SAL), vanillic acid (VAI), caffeic acid (CAF), syringic acid (SIR), catechin (CAT), vanillin (VAI), p-coumaric acid (PCU), syringaldehyde (SIA), epicatechin (EPI), ferulic acid (FER), rutin (RUT), trans- and cis-resveratrol (t-RSV and c-RSV), quercetin (QUE) and kaempferol (KAE). The information about phenolic content of fifteen wine samples together with the variety (red, dry sherry, rosé and white), and geographical areas of these wine samples, have been used for classification purposes in this study. The photometric detection was accomplished at four wavelengths, whereas the fluorimetric detection involved the post-column derivatization of ten of these compounds with terbium(III) to form the corresponding luminescent chelates. The geographical areas have been also separated in four types of wines according to the local origin: Córdoba and Montilla-Moriles (Spain) and Sicilian and Marsala (Italy) wines. An additional objective of this paper has been to elucidate which method (LC-photometric or LC-fluorimetric) provides the best pathway to classify wines according to their variety and geographical origin. Although only fifteen wine samples have been used to develop the proposed method, a similar number of samples, in the range of

9-22 samples, have been used in other chemometric studies with satisfactory results [7,16].

1. Experimental

1.1. Wine samples

The fifteen samples of different Italian and Spanish wines were bought from local markets in sealed 750-ml bottles. The samples analysed were red (WR, five samples), rosé (WS, one sample), "dry sherry" (WO, four samples) and white (WW, five samples) wine samples. Samples WR001, WR003, WR004 and WS001 were from Córdoba, samples WR002, WR005 WW002 and WW003 were from Sicilia, samples WO001, WO002 and WO003 were from Marsala , and samples WO004, WW001, WW004 and WW005 were from Montilla-Moriles. Each sample was diluted with distilled water to match dynamic calibration ranges and then, directly injected onto the chromatographic system and treated following the procedure below indicated. Each determination was assayed in triplicate.

1.2. LC-separation and photometric/fluorimetric detection

An Agilent 1200 series liquid chromatography system (Agilent Technologies España, Las Rozas, Spain) composed by a quaternary pump, a degasser unit, a vial autosampler, a thermostated column compartment and a diode array detector, was used. An SLM Aminco (SLM Instruments, Urbana, IL, USA) AB2 luminescence spectrometer provided with a 150 W continuous xenon lamp and a 7 W pulsed xenon lamp, furnished with a 176-052-QS Hellma (Hellma Hispania, Barcelona, Spain) flow cell with an inner volume of 18 ml was used for fluorescence measurements. A Gilson (Gilson Inc., Villiers-le-Bel, France) Minipuls 3 low-pressure peristaltic pump and Omnifit (Diba Industries Ltd., Cambridge,UK) Teflon tubing of 0.5 mm i.d. were also used for constructing the post-column derivatization manifold. Chromatographic separation was performed using an Onyx monolithic C_{18} column (Phenomenex,Torrance, USA) 100 mm x 4.6 mm i.d., pore sizes: mesopores (13 nm), macropores (2 nm). Standards or diluted samples (50 ml), containing the analytes at concentrations within their corresponding dynamic ranges, were injected into the column. Details concerning reagent and solutions, elution programme, photometric

detection, on-line derivatization and fluorimetric detection are described elsewhere [21]. The time necessary to achieve LC-photometric and LC-fluorimetric chromatograms was 25 min. Photometric detection was accomplished at 256 nm (for PTC, PHB, VAI, RUT), 280 nm (for GAL, SAL, SIR, CAT, VAA, PCU, EPI, c-RSV), 324 nm (for CAF, SIA, FER, t-RSV) and 365 nm (for QUE, KAE), which correspond to the maximum absorption wavelength of each phenolic compound. After each injection, a column cleaning-up and conditioning step was introduced to have the chromatographic system ready for the next operation after 5 min. The fluorimetric derivatization reagent was terbium(III), which forms fluorescent chelates with the following analytes: GAL, PTC, PHB, SAL, VAI, SIR, CAT, VAA, SIA and EPI. The fluorescence was monitored at λ_{ex} 317, λ_{em} 545 nm for 20 min, and the corresponding blank solutions were subtracted. Chromatograms were taken using the original software of the luminescence spectrometer and the raw data of luminescence intensity and time were exported and treated using adequate software packages for the estimation of the main chromatographic parameters.

2. Results and discussion

2.1. Analytical features

Calibration graphs were run under the optimum experimental conditions by using an external calibration method. **Table 1** shows the retention time for each phenolic, the calibration parameters and the detection limits (LODs) obtained for both methods, which were calculated according IUPAC recommendations [22].

Table 1. Analytical features of LC-DAD and LC-FL methods

Compound	Retention time (min)	Dynamic range* (ng mL^{-1})	Slope ± SD	y-intercept ± SD	r^2	LOD (ng mL^{-1})
Gallic acid	1.66	10-900	0.0642 ± 0.0001	-0.19 ± 0.07	0.9999	3
	1.79	*10-900*	*0.0645 ± 0.0003*	*0.5 ± 0.1*	*0.9999*	*5*
Protocatechuic acid	3.26	100-10000	0.0942 ± 0.0004	-3 ± 1	0.9997	36
	3.43	*10-900*	*0.1608 ± 0.0003*	*0.6 ± 0.1*	*0.9999*	*2*
p-Hydroxybenzoic acid	6.06	100-15000	0.1652 ± 0.0003	-4 ± 1	0.9999	22
	6.26	*50-2000*	*0.0176 ± 0.0002*	*0.30 ± 0.09*	*0.9990*	*15*
Salicylic acid	7.36	50-1000	0.0127 ± 0.0001	0.25 ± 0.06	0.9995	14
	7.51	*50-1000*	*0.0862 ± 0.0009*	*1.5 ± 0.4*	*0.9991*	*14*
Vanillic acid	9.80	100-20000	0.0975 ± 0.0002	-3 ± 1	0.9999	30
	10.0	*50-2000*	*0.0538 ± 0.0004*	*0.7 ± 0.3*	*0.9994*	*17*
Caffeic acid	11.47	10-900	0.0901 ± 0.0002	0.02 ± 0.08	0.9999	3
Syringic acid	11.78	30-15000	0.06116 ± 0.00003	-0.3 ± 0.2	0.9999	10
	11.95	*10-900*	*0.0454 ± 0.0001*	*0.45 ± 0.04*	*0.9999*	*3*
Catechin	12.64	20-2000	0.02096 ± 0.00008	-0.22 ± 0.06	0.9999	8
	12.77	*300-5000*	*0.00269 ± 0.00005*	*-0.1 ± 0.1*	*0.9976*	*114*
Vanillin	13.64	50-15000	0.0931 ± 0.0001	-1.9 ± 0.6	0.9999	19
	13.82	*100-2000*	*0.0140 ± 0.0002*	*0.4 ± 0.2*	*0.9989*	*40*
p-Coumaric acid	16.27	50-15000	0.1141 ± 0.0001	-0.2 ± 0.5	0.9999	14
Syringaldehyde	17.22	10-1000	0.0616 ± 0.0002	0.07 ± 0.07	0.9999	3
	17.35	*50-2000*	*0.0221 ± 0.0002*	*0.4 ± 0.1*	*0.9996*	*15*
Epicatechin	17.85	20-5000	0.02061 ± 0.00004	-0.25 ± 0.05	0.9999	8
	17.95	*100-2000*	*0.00361 ± 0.00004*	*0.03 ± 0.03*	*0.9986*	*29*
Ferulic acid	18.42	20-15000	0.10498 ± 0.00004	-0.4 ± 0.2	0.9999	5
Rutin	20.86	100-20000	0.02364 ± 0.00003	-0.7 ± 0.2	0.9999	24

Table 1. Continuation

Compound	Retention time (min)	Dynamic range* (ng mL^{-1})	Slope ± SD	y-intercept ± SD	r^2	LOD (ng mL^{-1})
trans-Resveratrol	22.86	10-7500	0.13782 ± 0.00003	0.2 ± 0.08	0.9999	2
Quercetin	23.52	200-3500	0.097 ± 0.001	-5 ± 2	0.9995	60
cis-Resveratrol	23.67	100-5000	0.0595 ± 0.0003	-1.7 ± 0.7	0.9999	35
Kaempferol	24.84	50-2000	0.0892 ± 0.0005	3.1 ± 0.5	0.9999	18

(*the lower limit of dynamic range for each phenolic is its LOQ). Italics: fluorimetric method

2.2. Statistical and Preliminary data exploration

All the statistical analyses were performed by means of the statistical software package STATGRAPHICS Plus (Statgraphics Consulting, Software Científico S.L., Madrid, Spain) for Windows 5.1 and SPSS 15.0 (SPSS Inc., Chicago, IL, USA) for Windows 5.1 using a Hewlett Packard HP4180C (Hewlett Packard España, Las Rozas, Spain) as graphic output. LC analysis was performed in triplicate and the data are given as individual values ± standard deviation (S.D.). Each analyte was processed according with the selected separation/derivatization and detection mode. **Table 2** summarizes the phenolic content of each wine sample, which was obtained from the chromatographic methods depicted in **Table 1.** This information was added to a matrix of data, which also included the data corresponding to wine variety (WV) and geographic origin (GO). The mean values obtained in the different groups were compared by One-way ANOVA and the t-test, assuming that there were significant differences between mean values when statistical comparison gave $p<0.05$. In multivariate analysis each sample was considered to be a data vector represented by the concentration of the selected phenolic compounds. Missing observations can be problematic in multivariate analysis, and sometimes measurements cannot be computed if there are missing values in the series. Usually, these missing values appeared when a particular observation was un-known or when the concentration value was lower than the detection limit. Hence, three different alternatives for replacing missing values were assayed: the replacement by the series mean (MEA), by half of the value of LOD (LOD/2) and by half of the value of LOQ (LOQ/2).

Table 2. Phenolic content in the analyzed samples

Compound [a]	Sample code							
	WR001	WR002	WR003	WR004	WR005	WS001	WO001	WO002
Gallic acid*	2.8 ± 0.1	98.0 ± 4.0	30.5 ± 0.8	33± 1	87.2± 0.8	3.60 ± 0.3	9.45 ± 0.07	10.9 ± 0.2
Protocatechuic acid*	1.268 ± 0.002	1.6 ± 0.1	1.09 ± 0.01	1.269 ± 0.001	2.2 ± 0.2	0.58 ± 0.01	1.615 ± 0.001	1.50 ± 0.080
p-hydroxy benzoic acid*	0.8 ± 0.01	n.q.	0.265 ± 0.005	n.d.	n.q..	0.63 ± 0.02	0.344 ± 0.004	0.48 ± 0.04
Salicylic acid*	n.q.	n.d.	n.q.	0.32± 0.02	n.d.	n.d.	0.20 ± 0.01	0.886 ± 0.002
Vanillic acid*	1.210 ± 0.02	1.25 ± 0.05	1.020 ± 0.003	0.610 ± 0.003	2.2 ± 0.1	0.33 ± 0.06	0.27 ± 0.01	0.34 ± 0.02
Caffeic acid	2.90 ± 0.04	n.q.	9.7 ± 0.1	10.7 ± 0.3	7.27 ± 0.07	1.71 ± 0.02	1.81 ± 0.01	1.574 ± 0.006
Syringic acid*	4.18 ± 0.02	6.3± 0.4	3.46 ± 0.03	0.37 ± 0.05	5.1 ± 0.3	1.4 ± 0.1	0.200 ± 0.008	0.46 ± 0.01
Catechin*	26.3 ± 0.2	3.8± 0.2	30 ± 2	8.1 ± 0.5	33 ± 2	n.d.	n.d.	1.00 ± 0.02
Vanillin*	n.q.	n.d.	n.d.	n.d.	n.d.	n.q.	n.d.	n.q.
p-coumaric acid	6.4 ± 0.1	16.3 ± 0.2	11.8 ± 0.2	11.6 ± 0.3	24.6 ± 0.2	0.11 ± 0.01	2.04 ± 0.03	1.296 ± 0.006
Syringaldehide*	1.3 ± 0.1	3.0 ± 0.2	1.6 ± 0.1	n.d.	2.12 ± 0.03	n.d.	n.q.	n.d.
Epicatechin*	14.0 ± 0.1	32.0 ± 0.3	13.3 ± 0.3	29.1 ± 0.1	26.5 ± 0.5	1.94 ± 0.80	0.332 ± 0.006	0.52 ± 0.02
Ferulic acid	0.399 ± 0.004	0.46 ± 0.02	0.445 ± 0.003	0.56 ± 0.02	0.36 ± 0.01	0.14 ± 0.02	1.00 ± 0.05	0.34 ± 0.02
Rutin	n.d.	19.0 ± 1.0	5.53 ± 0.02	0.75 ± 0.03	3.5 ± 0.2	0.81 ± 0.02	0.961 ± 0.001	0.46 ± 0.02
trans-resveratrol	0.42 ± 0.02	0.34 ± 0.01.	0.460 ±0.004	0.094 ± 0.002	0.72 ± 0.03	0.83 ± 0.03	n.d.	0.050 ± 0.002
Quercetin	5.3 ± 0.2	11.24 ± 0.05	6.5 ± 0.2	7.0 ± 0.5	13.0 ± 0.3	0.47 ± 0.02	n.d.	n.d.
Kaempferol	0.32 ± 0.01	0.216 ± 0.002	0.560 ± 0.009	0.490 ± 0.005	0.47 ± 0.01	n.q.	n.d.	n.d.

*measured using LC-FL method, [a] Content in mg L^{-1}, Mean ± SD (n = 3), (n.d.: below detection limit; n.q.: not quantified, below quantification limit

Table 2. Continuation.

Compound [a]	Sample code						
	WO003	WO004	WW001	WW002	WW003	WW004	WW005
Gallic acid*	15.3 ± 0.9	1.70 ± 0.05	1.9 ± 0.1	2.0 ± 0.1	2.6 ± 0.1	2.16 ± 0.08	1.84 ± 0.08
Protocatechuic acid*	1.642 ± 0.003	0.77 ± 0.02	1.14 ± 0.06	0.65 ± 0.01	0.41 ± 0.04	1.524 ± 0.004	0.868 ± 0.002
p-hydroxy benzoic acid*	0.175 ± 0.005	n.q.	n.q.	0.252 ± 0.004	0.11 ± 0.04	0.34± 0.04	0.79 ± 0.02
Salicylic acid*	0.32 ± 0.02	0.33 ± 0.02	0.215 ± 0.001	0.090 ± 0.004	n.q.	0.21 ± 0.01	0.240 ± 0.008
Vanillic acid*	0.29 ± 0.02	0.30 ± 0.03	1.26 ± 0.04	0.114 ± 0.008	0.106 ± 0.008	1.16 ± 0.08	0.208 ± 0.008
Caffeic acid	3.20 ± 0.01	0.674 ± 0.003	0.163 ± 0.003	1.312 ± 0.006	1.43 ± 0.01	n.q.	1.68 ± 0.08
Syringic acid*	0.139 ± 0.002	0.30 ± 0.02	0.36 ± 0.03	0.30 ± 0.01	0.128 ± 0.004	0.80 ± 0.03	0.090 ± 0.003
Catechin*	n.d.	n.d.	0.95 ± 0.03	3.2 ± 0.1	1.30 ± 0.06	1.2 ± 0.1	n.d.
Vanillin*	n.q.	n.d.	n.d.	n.d.	0.20 ± 0.02	0.56 ± 0.04	n.d.
p-coumaric acid	1.68 ± 0.05	n.d.	0.51 ± 0.01	0.590 ± 0.004	0.60 ± 0.04	n.d.	0.91 ± 0.05
Syringaldehide*	n.d.	0.45 ± 0.05	n.d.	n.q.	0.14 ± 0.01	0.43 ± 0.02	n.d.
Epicatechin*	0.50 ± 0.04	n.d.	n.q.	1.32 ± 0.1	0.43 ± 0.02	2.9 ± 0.3	n.d.
Ferulic acid	0.540 ± 0.005	0.42 ± 0.04	n.q.	0.486 ± 0.006	0.344 ± 0.002	0.21 ± 0.02	0.47 ± 0.02
Rutin	0.65 ± 0.03	n.q.	n.d.	0.73 ± 0.02	0.20 ± 0.02	0.212 ± 0.004	n.q.
trans-resveratrol	n.q.	n.q.	0.015 ± 0.007	0.040 ± 0.004	0.084 ± 0.002	n.q.	n.q.
Quercetin	n.d.	0.39 ± 0.01	n.d.	n.d.	n.d	n.d.	0.325 ± 0.003
Kaempferol	n.d.	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.

*measured using LC-FL method, [a] Content in mg L^{-1}, Mean ± SD (n = 3), (n.d.: below detection limit; n.q.: not quantified, below quantification limit

A second treatment, consisting on the normalization of the data in order to avoid initial weighing influences on the concentration values prior to the classification analysis, was also assayed. Two normalization procedures were tested: interval scaling (IS) and autoscaling (AS). These different strategies lead to the selection of the method, providing the best sample classification. Finally, the selected data matrices were constructed using IS and LOQ/2 pre-processing methods as normalized and hole replacing procedures [15,23]. Three data matrices were constructed using the following structure: a first column including wine variety, a second column including the geographical region and different columns including the concentration of the selected analytes. The matrix type A corresponds to the concentration of seventeen analytes (GAL, PTC, PHB, SAL, VAI, PCU, SIR, CAF, VAA, SIA, CAT, EPI, FER, RUT, QUE, KAE and t-RSV) using LC-photometric and LC-fluorimetric approaches. The analyte c-RSV provided incomplete information for the selected fifteen wines and, thus, it was removed. The second matrix, namely type B, was generated using the information provided by ten analytes quantified by LC-fluorimetric approach (GAL, PTC, PHB, SAL, VAI, SIR, CAT, VAA, SIA and EPI). Finally, the matrix type C was constructed using the information provided by sixteen analytes quantified by the LC-photometric method (GAL, PTC, PHB, VAI, CAF, SIR, CAT, VAA, PCU, SIA, EPI, FER, RUT, t-RSV, QUE and KAE). Partial information provided by SAL and c-RSV were not used for developing this matrix.

2.3. Factor Analysis using Principal Components

Factor Analysis (FA), using principal components as the method for factor extraction, was employed to summarize the information in a reduced factor number. This procedure allows reducing data dimensionality, a data exploration that investigates how many components (a linear combination of original variables) are necessary to explain the more significant parts of the variance in the method with a minimum loss of information. **Table 3** summarizes the features of the FA achieved using the three data sets, namely A, B and C, which included the initial Eigenvalues for the principal component found, their percentage of Variance and the Cummulative percentage of Variance. This Table also depicts the Variable influence obtained for each PCA after Varimax Rotation including their Communality values. The first type of FA (type A data) was performed using the analyte

concentrations obtained using both LC-photometric and LC-fluorimetric information. The objective of this FA is to obtain a reduced number of factors which explain the variability of the selected analytes. When this data set was used, the first five factors were chosen (87.65 % of the total variance) because the eigenvalues were higher than 1, and therefore, they explain more variance than each original variable. All variables present a communality equal to or higher than 0.649, and therefore, they are well represented by the five factors. A Varimax rotation was carried out to minimize the number of variables that influence each factor, and then, to facilitate the interpretation of the results. The first factor that explains the higher percentage of variance (52.04 %) is mainly associated with SIA, SIR, RUT, GAL and QUE. The second factor that explains the 11.00 % of the total variance is related to CAF and KAE and CAT. The remaining factors selected, with 24.60 % of the total variance explained, are related to FER (third factor) and PHB (fifth factor). On the basis of the first FA, these 10 variables can be used to repeat the FA obtaining similar results with respect of the original FA and, thus, would be selected in further chemometric analysis without appreciable variation.

The second FA study including Varimax rotation was performed using the information of the ten analytes determined by the LC-fluorimetric method (type B). Four factors were extracted from this FA with eigenvalues higher than 1, which explains the 87.30 % of the variability from the original variables (see **Table 3**). The first factor that explains the higher percentage of variance (52.37 %) is mainly associated with SIR, SIA, VAI, GAL and EPI. The other three factors explain the 33.17 % of the whole variability and were associated with PHB (second factor), SAL (third factor) and VAA (fourth factor).

Table 3. Features of Factor Analysis [a]

Data set	Initial Eigenvalues				Variables after Varimax Rotation (Communality values) [b]
	Factor Number	Total	% of Variance	Cumulative %	
Type A	1	8.847	52.038	52.038	SIA (0.932), SIR (0.907), RUT (0.905), GAL (0.897)
17 analytes	2	1.871	11.008	63.046	CAF (0.932), KAE (0.897), CAT (0.816)
(10 reduced	3	1.802	10.599	73.645	FER (0.769)
variables)	4	1.342	7.893	81.538	-
(photometric and fluorimetric)	5	1.039	6.119	87.647	PHB (0.894)
Type B	1	5.237	52.366	52.366	SIR (0.937), SIA (0.898), VAI (0.879), GAL (0.808), EPI (0.804)
10 analytes	2	1.341	13.410	65.776	PHB (-0.944)
(8 reduced	3	1.119	11.188	76.964	SAL (0.860)
variables)	4	1.033	10.333	87.296	VAA (0.970)
(fluorimetric)					
Type C	1	7.117	44.483	44.483	SIA (0.945), VAI (0.904), RUT (0.896), PTC (0.862), EPI (0.816)
16 analytes	2	3.636	22.725	67.208	KAE (0.911), PCU (0.892), CAF (0.861)
(10 reduced	3	1.952	12.200	79.408	SIR (0.742)
variables)	4	1.039	6.495	85.903	FER (-0.900)
(photometric)					

a) Extraction method using Principal Component Analysis with Kaiser Normalization, b) using Principal Component Analysis

The last FA study was performed using information from the sixteen analytes quantified by the LC-photometric method (type C). As can be seen in **Table 3**, four factors were extracted (85.90 %), in which the first factor explains the 44.48 % of variance with associated variables of SIA, VAI, RUT, PTC and EPI. The second factor was associated to the variables KAE, PCU and CAF with a variance percent of 22.73 %. Finally, SIR (third factor) and FER (fourth factor) represent the last 18.69 % of the variability. The data sets A and B shows high coincidence in the results with at least four of the variables at the same significance level (SIR, SIA, GAL and PHB) and exhibits variance percentages quite similar. The FA type C also provides a reduction of the variables in an acceptable concordance level with the FA type A (also four variables). However, other six variables were not concordant at the significance level, which hinders their applicability in further chemometric analysis. As a result of Factor Analysis, the new latent variables, which are a linear combination of the initial experimental variables, represent a similar significance degree in the variability of the initial experimental variables. These variables were also used in further chemometric treatments.

2.4. Discriminant analysis

Linear discriminant analysis (LDA) is a supervised classification procedure in which the number of categories and the samples that belong to each category are previously defined. This statistical technique is based on the extraction of linear discriminant functions of the independent variable by means of a quantitative dependent variable (category) and the quantitative independent variables. Currently, two different processes can be applied in LDA: 1) Stepwise LDA that selects the quantitative variables which enhanced discrimination of the groups established by the dependent variable. For this purpose, the criteria for this selection is the Wilks' lambda, which is a measurement of how well each function separates objects (samples) into groups, and it is equal to the ratio of the total variance in the discriminant scores not explained by differences among the groups. Smaller values of Wilks' lambda indicate greater discriminatory ability of the function. The associated chi-square statistic test hypothesizes that the means of the function listed are equal across groups and the small significance values indicate that the discriminant function does better than chance at separating the groups; 2) Introduction of all independent

variables. The objective of this process is to keep all the original information, although the system obtained is more complex. This approach is applied when the stepwise method cannot produce a good classification of the samples as a function of the quantitative variable.

According to the data set, a LDA was developed, using new matrices of data integrated by the reduced original variables, which were selected from to the previous FA. Each of the reduced data set of types A, B and C was also subjected to the LDA, considering two types of classification criteria: according to the wine variety (red, dry sherry, rosé and white) or the geographical origin (Córdoba, Montilla-Moriles, Sicilia and Marsala). The results of the LDA were validated using the leave-on-out cross-validation. In this procedure, a defined number of samples were sequentially removed from the original data set and the classification model was rebuilt. All the samples of the data set are sequentially removed and classified. Finally, after a significant number of iterations, the objects were classified and the percentage of correct classification is given in all instances. The classification results according to the wine variety and geographical origin are shown in **Table 4.** As can be seen, classification parameters such as number of eigenvalues, linear correlation, Wilks' lambda and Chi-square coefficients reveal acceptable aggregation for at least three of the discriminant factors defined, in which the quantitative values of the variables correlate well with the previous FA. The predicted results provided a percentage of predicted membership values according to the wine variety of 73.3 % using the LC-photometric method and 97.92 % for the combination of LC with fluorimetric and photometric detection. The second attempt to classify the objects according to the geographical origin provided very similar results, in which the discriminant parameters and the classification results were achieved using the reduced data sets A, B and C. The prediction membership exhibited prediction percentages between 91.10 and 97.80 %. However, when the cross-validation procedure is used, these values decrease to 86.7 % and 95.6 %.

Table 4. Results of the initial Linear Discriminant Analysis

(I) data from Wine Variety

Set of type A data (10 analytes with photometric and fluorimetric detection)

	Initial Eigenvalues					Wilks' lambda			
Function	Eigenvalues [(a)]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	354.956	98.90	98.90	0.999	1 through 3	0.000	297.025	30	0.000
2	2.667	0.70	99.60	0.853	2 through 3	0.116	79.657	18	0.000
3	1.348	0.40	100.00	0.758	3	0.426	31.578	8	0.000

Variable passing tolerance test: SIR, CAT, SIA, FER, KAE, PHB, QUE, GAL, CAF and RUT

Set of type B data (8 analytes with fluorimetric detection)

	Initial Eigenvalues					Wilks' lambda			
Function	Eigenvalues [(a)]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	22.953	91.10	91.10	0.979	1 through 3	0.010	176.917	24	0.000
2	1.458	5.80	96.90	0.770	2 through 3	0.228	56.226	14	0.000
3	0.787	3.10	100.00	0.664	3	0.560	22.055	6	0.001

Variable passing tolerance test: EPI, SIR, SIA, GAL, SAL, PHB, VAA and VAI

Set of type C data (10 analytes with photometric detection)

	Initial Eigenvalues					Wilks' lambda			
Function	Eigenvalues [(a)]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	82.840	97.90	97.90	0.994	1 through 3	0.003	209.363	30	0.000
2	1.164	1.40	99.30	0.733	2 through 3	0.292	45.493	18	0.000
3	0.580	0.70	100.00	0.606	3	0.633	16.928	8	0.031

Variable passing tolerance test: KAE, PCU, CAF, RUT, SIA, FER, SIR, PTC, VAI and EPI

Table 4. Continuation

(II) data from Geographical Origin									
Set of type A data (10 analytes with photometric and fluorimetric detection)									
	Initial Eigenvalues					Wilks' lambda			
Function	Eigenvalues [a]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	50.052	90.50	90.50	0.990	1 through 3	0.002	238.403	30	0.000
2	3.498	6.30	96.90	0.882	2 through 3	0.081	92.887	18	0.000
3	1.737	3.10	100.00	0.797	3	0.365	37.257	8	0.000
Variable passing tolerance test: FER, QUE, GAL, SIR, SIA, KAE, RUT CAT, PHB and CAF									
Set of type B data (8 analytes with fluorimetric detection)									
	Initial Eigenvalues					Wilks' lambda			
Function	Eigenvalues [a]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	5.539	68.60	68.60	0.920	1 through 3	0.031	132.406	24	0.000
2	1.660	20.60	89.20	0.790	2 through 3	0.201	61.052	14	0.000
3	0.874	10.80	100.00	0.683	3	0.534	23.871	6	0.001
Variable passing tolerance test: EPI, GAL, SAL, SIA, PHB, VAI, VAA and SIR									
Set of type C data (10 analytes with photometric detection)									
	Initial Eigenvalues					Wilks' lambda			
Function	Eigenvalues [a]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	19.028	80.30	80.30	0.975	1 through 3	0.005	195.514	30	0.000
2	3.469	14.60	94.90	0.881	2 through 3	0.102	84.620	18	0.000
3	1.203	5.10	100.00	0.739	3	0.454	29.222	8	0.000
Variable passing tolerance test: VAI, SIA, RUT, PTC, KAE, CAF, PCU, FER, EPI and SIR [a] the first 3 linear discriminant functions were used in the analysis									

Table 5. Results of LDA and classification according to the wine variety and geographical origin using LC-fluorimetric parameters

Set of data type B for wine variety (10 analytes with fluorimetric detection)									
Initial Eigenvalues					Wilks' lambda				
Function	Eigenvalues [a]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	102.323	83.50	83.50	0.995	1 through 3	0.000	305.816	30	0.000
2	19.311	15.80	99.30	0.975	2 through 3	0.027	134.215	18	0.000
3	0.852	0.70	100.00	0.678	3	0.540	22.803	8	0.004

Variable passing tolerance test: GAL, PTC, PHB, SAL, VAI, SIR, CAT, VAA, SIA and EPI, [a] the first 3 linear discriminant functions were used in the analysis

Data type B		Wine variety	Predicted Group Membership				Total
			1	2	3	4	
Original	Count	1	15	0	0	0	15
		2	0	12	0	0	12
		3	0	0	3	0	3
		4	0	0	0	15	15
	Percentage		100.00	100.00	100.00	100.00	100.00
Cross-validated(a)	Count	1	15	0	0	0	15
		2	0	12	0	0	12
		3	0	0	3	0	3
		4	0	0	0	15	15
	Percentage		100.00	100.00	100.00	100.00	100.00

Table 5. Continuation

Set of data type B for geographical origin (10 analytes with fluorimetric detection)

Initial Eigenvalues					Wilks' lambda				
Function	Eigenvalues [a]	% of Variance	Cummulative %	Linear Correlation	Test Function	Wilks' lambda	chi-square	Differences	Significance
1	109.933	90.10	90.10	0.995	1 through 3	0.000	297.225	30	0.000
2	10.663	8.70	98.90	0.956	2 through 3	0.036	122.995	18	0.000
3	1.382	1.10	100.00	0.762	3	0.420	32.107	8	0.000

Variable passing tolerance test: VAA, PTC, GAL, PHB, SIA, SAL, SIR, EPI, VAI, and CAT, [a] the first 3 linear discriminant functions were used in the analysis

Data type B		Wine variety	Predicted Group Membership				Total
			1	2	3	4	
Original	Count	1	12	0	3	0	12
		2	0	12	0	0	12
		3	0	0	12	0	12
		4	0	0	0	9	9
	Percentage		100.00	100.00	100.00	100.00	100.00
Cross-validated(a)	Count	1	12	0	5	0	12
		2	0	12	0	0	12
		3	0	0	12	0	12
		4	0	0	0	9	9
	Percentage		100.00	100.00	100.00	100.00	100.00

From the results obtained by the classification studies, according to the variety of wines or to their geographical origin, it can be concluded that the best separation and, thus, classification is obtained using the information provided by the LC-fluorimetric methodology (data set B). As can be seen, this data matrix only provides a partial reduction in two variables when compared to the original type B matrix (prediction of 93.30 %). If the Linear Discriminant Analysis using the original data set (10 variables achieved using the LC-fluorimetric detection procedure) is developed, a significant improvement of the percentage of predicted sample membership using wine variety or geographical origin as classification factor was achieved. **Table 5** shows the classification parameters and the percentage of the predicted membership obtained. As can be seen, the percentage of prediction was complete in all instances (100 % of the predicted membership). **Figure 1** depicts the classification of wines according to the variety (A) and geographical origin (B). As can be seen, the similarities between wines of the same variety or the same geographical origin are well established in all instances, leading to a correct discrimination among the different groups.

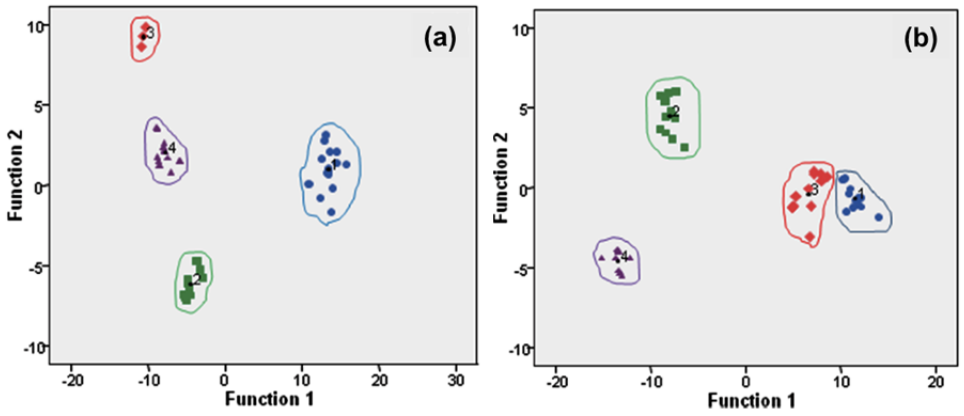

Figure 1. Prediction examples using LDA for varieties (a) and geographical origins (b) of the wines using reduced data matrix type B (n=10) for LC-fluorimetric method. Symbol for wine classification according variety, (●) denotes red, (■) dry sherry, (◆) rosé and (▲) white, respectively, and for geographical origin, (●) denotes Córdoba (Spain), (■) Montilla-Moriles, (◆) Sicilia (Italy) and (▲) Marsala.

3. Conclusions

According to the results obtained, it should be emphasized that: a) It has been established that the LC-fluorimetric detection method using a combined technique of FA/LDA provides an adequate pathway to classify the wines according to their variety or geographical origin, b) The proposed method constitutes an original procedure to classify wines using LC and optical detection, c) The fluorescence of the derivatized analytes provides a quantitative and selective method, using a reduced number of analytes, to develop an adequate chemometric classification procedure, and d) A correct classification pathway with 100 % of the correct predicted membership is achieved by selecting only a reduced number of phenolic compounds, such as hydroxybenzoic acids (GAL, PTC, PHB, SAL, VAI and SIR), phenolic aldehydes (SIA and VAA) and flavonoids (RUT and KAE).

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Luminescent determination of flavonoids in orange juices by liquid chromatography with post-column derivatization with aluminium and terbium

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A new post-column derivatization system is described and applied to the determination of flavonoids in citric beverages after their separation by liquid chromatography using a monolithic column. The derivatization involves the formation of the chelates of the analytes with aluminium (III) and terbium (III) in the presence of the surfactant sodium dodecylsulfate and the measurement of the terbium sensitized luminescence at λ_{ex}360 and λ_{em} 545 nm. Naringin, hesperidin, quercetin, naringenin, and kaempferol have been chosen as analyte models. The large Stokes shift and the relatively long wavelength emission of terbium(III) can minimize interferences from background sample matrix, which usually emit at shorter wavelengths. Calibration graphs were constructed in the intervals 6.0 – 1700 ng/mL naringin, 9.8 – 1700 ng/mL hesperidin, 2.1 - 2000 ng/mL quercetin, 5.2 – 1500 ng/mL naringenin and 2.5 – 2000 ng/mLkaempferol, with regression coefficients higher than 0.9935 in all instances. The precision of the method, expressed as RSD%, was established at two concentration levels, with values of 1.3 and 4.7 %, which corresponded to the minimal and maximal error zones of the calibration graphs. The practical usefulness of the method is demonstrated by the analysis of orange juices, which were diluted and directly injected into the chromatographic system, obtaining recoveries between 86.9 and 108.2 %.

Introduction

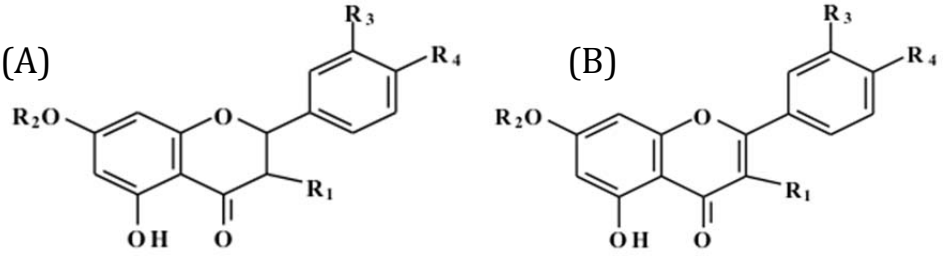
Flavonoids are a group of polyphenolic compounds widely distributed in vegetables and fruits [1]. The determination of these compounds is of great interest owing to their multiple biological effects, including antioxidant activity, antitumor, antimutagenic, antibacterial and angioprotective properties [2, 3]. They also contribute to different plant properties such as colour, flavour, fragrance, nutrition, stability and therapeutic properties. Flavonoids as 2-phenyl-benzo-α-pyrones, are classified according to the multitude of substitution patterns in the two bencene rings of their basic structure. Variation in their heterocyclic rings gives rise to flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavones [4]. The flavonoid content in orange juices has special interest as it contributes to the quality of these samples [5].

The main separation technique applied to the determination of flavonoids in orange juices is liquid chromatography (LC) [6-11], using reversed-phase columns, although gas chromatography [12], involving the formation of trimethylsilyl derivatives, has been also described for this purpose. The usefulness of a monolithic column, which can operate at higher flow-rates with lower back-pressures than conventional columns, has been previously described for the LC separation of some flavonoids in wine samples [13]. However, to the best of our knowledge, this type of column has not been used up to date for the determination of these compounds in orange juices.

Photometric [6-10], coulometric [10] and mass spectrometric (MS) [6, 11] detection systems have been reported in the LC methods described for the analysis of these samples. Some flavonoids, such as quercetin and kaempferol, have been fluorimetrically determined in body fluids using aluminium(III) as post-column derivatization reagent to obtain fluorescent chelates [14]. Another post-column derivatization system previously described for the determination of these compounds in wine samples involves the formation of the corresponding terbium(III) chelates and the measurement of the sensitized luminescence [13]. This phenomenon is an intermolecular energy transfer process, in which the ligand acts as donor and terbium(III) acts as acceptor and emits luminescence, that allows very

sensitive and selective determinations, although it use as detection system in LC has been relatively limited [15]

This article reports for the first time the use of a monolithic column for the direct separation of flavonoids in orange juice samples and a new post-column derivatization system for the luminescent detection of these compounds. The derivatization system is based on the capability of flavonoids to form stable chelates with aluminium(III) and terbium(III) and the measurement of the terbium sensitized luminescence at λ_{ex}360 and λ_{em} 545 nm, in the presence of a sub-micellar medium provided by sodium dodecylsulfate (SDS). The large Stokes shift and the relatively long wavelength emission minimize potential interferences from background sample matrix, which usually emit at shorter wavelengths. Two flavone aglycones (quercetin and kaempferol), a flavanoneaglycone (naringenin) and two flavanone-O-glycosides (hesperidin and naringin) (**Figure 1**) have been chosen as model analytes, since they are the most representative flavonoids in orange juices. The flow rate used was lower than that usually reached using a monolithic column, since it was necessary to make compatible the separation process with the post-column system. However, the use of this column improves the chromatographic resolution and the separation of the analytes requires a shorter time than that using a conventional column (7-9).



Type of polyphenols	Name	Abbreviation	Substituents			
			R_1	R_2	R_3	R_4
Flavanone O-glycoside (A)	Naringin	NAR	H	Glycoside	H	OH
Flavanone O-glycoside (A)	Hesperidin	HES	H	Glycoside	OH	OCH_3
Flavanoneaglycone (A)	Naringenin	NAN	H	H	H	OH
Flavone aglycone (B)	Quercetin	QUE	OH	H	OH	OH
Flavone aglycone (B)	Kaempferol	KAE	OH	H	H	OH

Figure 1. Chemical structures and nomenclature of the analytes selected in this research.

1. Experimental

1.1. Instrumentation

An Agilent 1200 Series Liquid Chromatography System composed by a quaternary pump, a degasser unit, a vial autosampler, a thermostated column compartment has been used. An SLM Aminco (Urbana, IL) AB2 luminescence spectrometer provide with a 150 W continuous xenon lamp and a 7 W pulsed xenon lamp, furnished with a 176-052-QS Hellma (Hellma Hispania, Barcelona, Spain) flow cell with an inner volume of 18 μL, was used to monitor fluorescence detection. A Gilson (Villiers-le-Bel, France) Minipuls 3 low-pressure peristaltic pump and Omnifit (Cambridge, UK) Teflon tubing of 0.5 mm i.d. were also used for constructing the post-column derivatisation manifold. Chromatographic separation was performed using an Onyx monolithic C18 column (Phenomenex, Torrance, USA), 100 mm x 4.6 mm i.d., pore sizes: mesopores (13 nm), macropores (2 μm).

1.2. Reagents

All chemicals used were of analytical reagent grade. Stock solutions (5000 mg/mL) of the flavonoids quercetin (QUE) (Aldrich), kaempferol (KAE) (Sigma), naringin (NAR) (Fluka), naringenin (NAN) (Aldrich) and hesperidin (HES) (Fluka) were prepared in absolute ethanol. Intermediate solutions of 100 mg/mL were also prepared by diluting the stock solutions in absolute ethanol. These solutions were stored at 4 ºC in the dark and were stable for at least two weeks. Working standard solutions were prepared from the intermediate solutions by their dilution in distilled water.

The mobile phase for the chromatographic separation was constituted by a ternary mixture of 0.15 M acetic acid, pH 4.0 (solvent A), acetonitrile (ACN) (solvent B), and methanol (solvent C), which were mixed operating in gradient mode during the chromatographic separation. All solvents were of analytical or HPLC grade (PanreacQuimica, S.A., Barcelona, Spain). The derivatization reagent solution contained terbium(III) (5.0 mM), from terbium(III) nitrate pentahydrate (Aldrich), aluminium (III) (15.0 mM), from aluminium (III) nitrate (Merck) and 0.1 mM sodium

dodecylsulfate (SDS) (Merck) dissolved in 0.15 M HAc/NaAc buffer of pH = 4.0. Ethylenediaminetetraacetic acid (EDTA) (Fluka), n-trioctylphosphine oxide (TOPO) (Sigma), cetyltrimethyl ammonium bromide (CTAB) (Sigma) and Triton X-100 (Fluka) were also used.

1.3. Manifold and Procedure

Figure 2 shows the integrated LC separation/derivatization/detection approach. Standards or diluted samples (200 μL), containing a mixture of the analytes at concentrations within their corresponding dynamic ranges, were injected in triplicate into the column. The mobile phase was pumped at a flow-rate of 0.7 mL/min and the system operated in an optimized gradient mode in order to obtain the adequate separation of the analytes.

After the chromatographic separation, the elution solution was merged with the derivatizing solution, which was pumped at a flow-rate of 0.5 mL/min. The mixture passed through the reaction coil, in which the derivatizing reactions took place, and the luminiscence intensity of each analyte was monitored at λ_{ex} 360 and λ_{em} 545 nm for 25 min. Chromatograms were taken using the original software of the luminescence spectrometer and the raw data of luminescence intensity and time were exported and treated using adequate software packages for the estimation of the main chromatographic parameters.

1.4. Analysis of orange juice samples

Commercial or natural orange juice samples were diluted, about 500-times, with distilled water to match the linear ranges of calibration for each analyte and were directly injected onto the chromatographic system following the procedure above indicated. Each determination was the mean of three measurements.

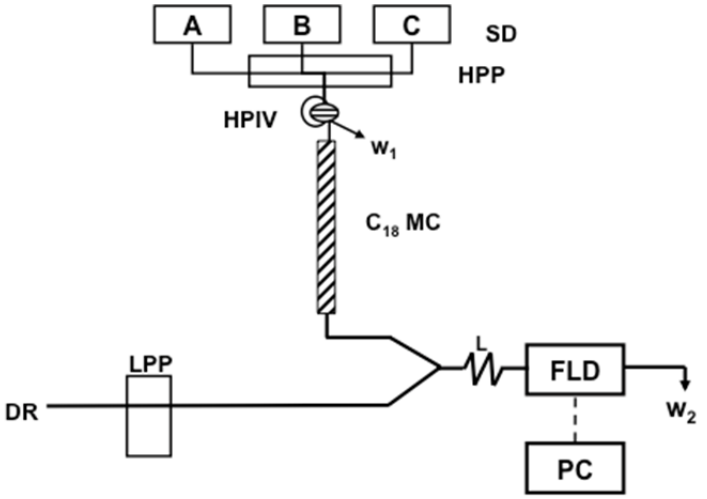


Figure 2. Integrated separation-derivatization and detection approach. A, B and C denote 15.0 mM acetic acid, acetonitrile and methanol solutions, respectively; SD, solvent delivery system; HPP, High-pressure quaternary gradient pump; HPIV, High Pressure injection valve; C_{18} MC, C_{18} monolithic column; DR, derivatizing reagent solution; LPP, low-pressure pump; L, mixing reactor; FLD, fluorescence detector; PC, personal computer; w, waste.

2. Results and discussion

2.1. Study of the luminescence reaction

Flavonoids have strong coordination ability toward a wide range of cations such as iron(II) [16], chromium(III) [17], aluminium(III) [14, 18-21], and lanthanide [13, 22, 23] ions, which is ascribed to the presence of multiple hydroxyl groups and the carbonyl group on the heterocyclic ring of their chemical structure. Experimental studies reveal that the preferred complexation sites for the flavonoids involve the hydroxyl group on carbon 3 or 5 and the adjacent 4-carbonyl group [18-21]. Aluminium(III) and lanthanide ions such as terbium(III) form fluorescent chelates with these compounds, which have different fluorescent features. While aluminium chelates show the wide emission band corresponding to molecular fluorescence, terbium chelates show the typical narrow emission bands of

sensitized luminescence, which involves an intramolecular energy transfer process from the ligand to the terbium(III) ion and the emission of the lanthanide ion [15].

Several assays were carried out to study the fluorescent behavior of the analytes using both terbium(III) and aluminium(III) ions, in order to obtain a potential increase on the luminescence signals. **Figure 3** shows the excitation and emission spectra obtained for quercetin in the presence of SDS as sub-micellar medium. The excitation spectra (**Figure 3.A**) show a band with maximum excitation wavelength at 360 nm, which corresponds to the terbium quelate, and another more intense band at 450 nm, corresponding to the aluminium chelate. However, in the presence of both terbium(III) and aluminium(III), the intensity at 360 nm increases, whereas that at 450 nm decreases. The emission spectra obtained by exciting at 360 nm (**Figure 3.B**) show the narrow emission bands at 490, 545, 585 and 620 nm, corresponding to the terbium sensitized luminescence. As can be seen, the intensity of these bands increases when both terbium(III) and aluminium(III) are together, obtaining the highest signal at 545 nm. However, using 450 nm as the excitation wavelength, the emission spectra (**Figure 3.C**) show a wide band corresponding to the emission of the aluminium(III) chelate, which decreases in the presence of terbium(III). A potential explanation of the increase on the intensity of the terbium(III) emission in the presence of aluminium(III) could be ascribed to the co-luminescence effect, in which the aluminium chelate contributes to improve the terbium(III) luminescence [15].

The special luminescent behavior of the analytes in the presence of both aluminium(III) and terbium(III) ions has been exploited to develop a new post-column derivatization system, using an adequate mixture of these ions in a sub-micellar medium integrated by SDS, monitoring the luminescence intensity of each chromatographic band at λ_{ex} 360 and λ_{em} 545 nm.

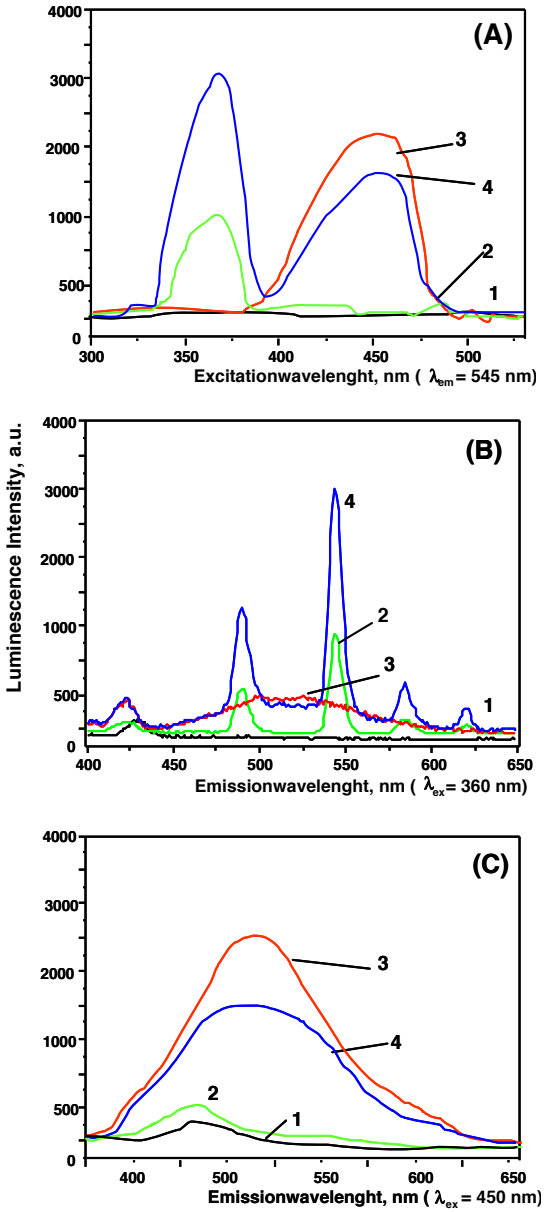


Figure 3. Excitation and emission spectra of 100 ng/mL quercetin dissolved in a mixture containing the initial mobile phase composition and 0.1 M SDS. (A) excitation (B) emission spectra of the following solutions: (1) blank solution, (2) 5 mM terbium (III), (3) 15 mMaluminium (III) and (4) a mixture containing 15 mM of aluminium and 5 mM of terbium solutions (for detail see the text).

2.2. Optimization of Variables

The variables affecting the method were optimized using the univariate methodology. **Table 1** shows the range studied for each variable and the values chosen, which were those yielding maximum luminescence signals with minimum standard deviations.

Table 1. Optimization of variables

Variables	Variables	Range studied	Optimum value
Instrumental variables	Excitation wavelength, nm	200 – 600	360
	Emission wavelength, nm	400 – 600	545
	Ex/em slits	2.5 – 15	10/10
	PMT gain, V	400 - 800	500
Chromatographic variables	HPP flow-rate, mL/min	0.5 – 3.0	0.7
	Injection volume, µL	20 – 500	200
	Elution mode	Gradient	-
	Temperature, ºC	15 – 35	25º C
	[HAc], M	0.05 – 0.25	0.15
	pH	2.5 – 6.0	4.0
Derivatizing variables	LPP flow-rate, mL/min	0.2 – 1.5	0.5
	L_1 reactor length, cm	50 – 300	100
	[Al(III)], mM	0.2 -20.0	15.0
	[Tb(III)], mM	0.1 – 10.0	5.0
	[HAc], M	0.05 – 0.25	0.15
	pH	1.5 – 6.0	4.0
	[SDS], mM	0.01 – 0.5	0.1

2.2.1. *Chromatographic variables*

Chromatographic variables were optimized in order to obtain acceptable resolution of the analytes in the lowest separation time. The composition and influence of the mobile phase was studied using ternary mixtures integrate by 0.15 M acetic acid, pH 4.0 (A), ACN (B) and methanol (C). In all cases, the methanol amount remained constant, in a percentage of 20 %. Binary mixtures of acetic acid (0.15 M, pH 4.0) and ACN were assayed, using different gradient and isocratic profiles, at the optimum flow-rate of 0.7 mL/min. A linear gradient between 10 to 20 % of ACN in 12 min provides the separation of the flavanone-O-glycosides naringin and hesperidin. The second part is a gradient zone in which the ACN percentage is rapidly reduced to 0 %, followed of an increase to 40 %, which allows the elution of quercetin before 17 min. Finally, an isocratic segment is used to separate naringenin and kaempferol.

The influence of the chromatographic flow-rate was studied in the range 0.5-3 mL/min with acceptable separation in all instances. It was necessary to make compatible the flow rate with the mobile-phase composition, in order to obtain a good peak resolution, and the separation process with the post-column derivatization system. A flow-rate of 0.7 mL/min was acceptable as it generates pressures lower than the maximum pressure tolerated in the luminescence detector.

2.2.2. *Post-column derivatization variables*

The study of the flow-rate of the derivatizing reagent solution showed that 0.5 mL/min provided an adequate flow-rate to the optimum derivatizing reactions when 0,7 mL/min was used for the chromatographic separation. The length of the mixture reactor L was 100 cm, which was enough for the development of these reactions, due to the relatively fast rate formation of the aluminum(III) and terbium(III) chelates.

The luminescence of lanthanide chelates can be quenched by the vibrational energy of the hydroxyl groups of water molecules [15], which is really notable when reverse-phase LC is used due to the relative high content of water in the mobile phase. It has been previously described that an adequate luminescent signal can be obtained in the presence of acetate

ions [24, 25], which can be ascribed to their capability to displace water from the first coordination sphere of terbium(III). A sodium acetate/acetic acid buffer solution was chosen to keep the adequate pH in the final solution and reach the highest luminescence signal by the positive effect of acetate ions. The best acetic acid concentration was 0.15 M, being the pH of the derivatization solution adjusted to 4.0.

Figure 4 depicts the effect of three chemical variables on the luminescence of the flavanoids chelates. The influence of terbium(III) concentration was evaluated in the range 0.1 to 10.0 mM (**Figure 4.A**), choosing a concentration of 5.0 mM as optimum. According to the study of the influence of aluminium(III) concentration (**Figure 4.B**), a value of 15.0 mM was chosen. The potential positive effect of a surfactant such as Triton X-100, CTAB and SDS was also studied. The luminescence of the flavanoids chelates was not modified by the presence of the two firsts, but it increased in the presence of SDS, as **Figure 4.C** shows. A 0.1 mM SDS concentration was selected. In order to improve the luminescence signal, trioctylphosphine oxide and EDTA, as effective luminescence synergetic agents, were tested, but not changes in the luminescence signal were obtained.

2.3. Analytical features

Calibration graphs were run under the optimal experimental condition depicted in Table 1. Seven standard solutions of mixtures of the five analytes, covering the concentration range between 1 and 3000 ng/mL, were assayed in triplicate. **Table 2** summarizes the figures of merits of the method, including average retention times, calibration equations, regression coefficients, residual standard deviations, linear ranges, detection limits (LODs) and precision, expressed as relative standard deviation (RSD %) values. The LODs were calculated according IUPAC recommendations [26]. As can be seen, the method was enough sensitive for the determination of the analytes at the ng/mL level, giving wide dynamic ranges, between 2.1 and 2000 ng/mL, depending on the analyte selected. The regression values ($r^2 \geq 0.9936$, n = 7) and residual standard deviation ($\sigma_{y/x} \leq 0.93$) coefficients were acceptable in all instances.

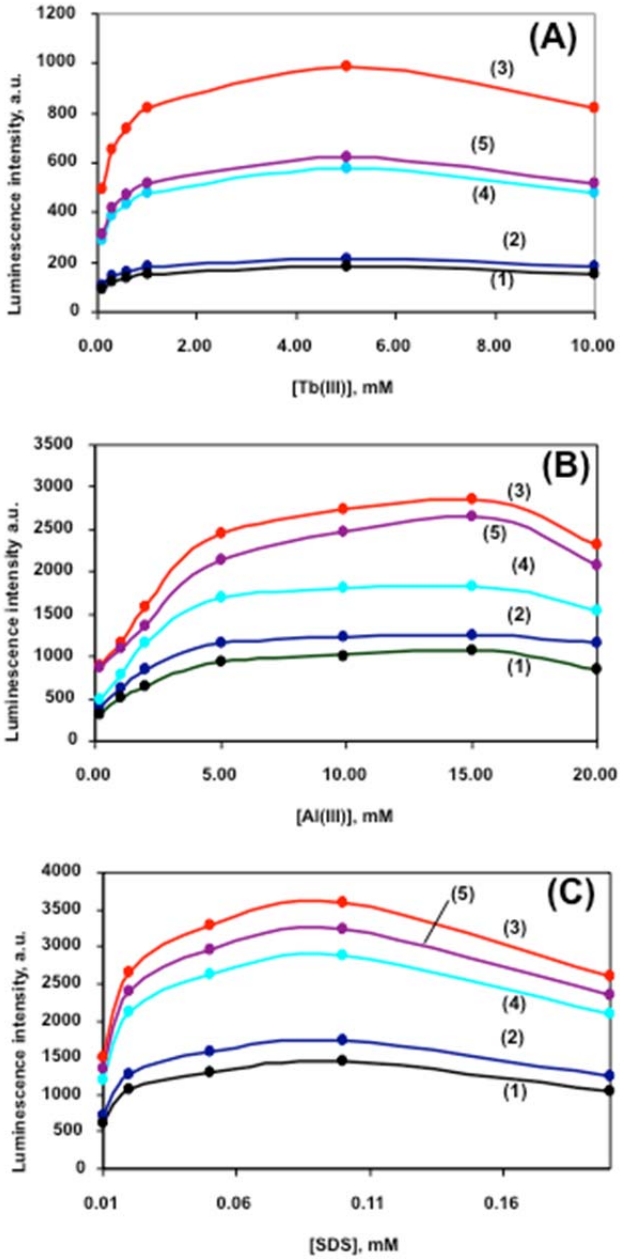


Figure 4. Influence of Tb(III) (A), Al(III) (B) and SDS (C) concentrations on the luminescence of naringin (1), hesperidin (2), quercetin (3), naringenin (4) and kaempferol (5). [Analyte] = 100 ng/ml. [Al(III)] = 2 mM (A) and 15 mM (C). [Tb(III)] = 5 mM (B and C). [SDS] = 0.05 mM (A and B)

The precision, expressed as percentage of the relative standard deviation was established in two characteristic zones corresponding to the minimal (centroid point) and maximal (nearly of the average of the LOQs) errors of the dynamic calibration graphs. The RSD % values, estimated for n = 11 solutions containing 500 and 10 ng/mL of each analyte, respectively, injected in triplicate, ranged between 1.3 and 4.7 %.

2.4. Application of the method

The proposed method was applied to the analysis of orange juice samples from natural and commercial origin. The samples only required the adequate dilution, about 500-times, with distilled water to match the linear range of the calibration graph for each analyte. **Figure 5** shows the chromatograms obtained for a standard solution containing 100 ng/mL of each analyte (**Fig. 5.A**), and for natural (**Fig. 5.B**) and commercial orange juice samples (**Fig. 5.C**). The method was tested in two ways: a) by determining the concentration of the five analytes (naringin, hesperidin, quercetin, naringenin and kaempferol) in the samples, and b) by studying the recovery after addition of two standard aliquots of 10.0 and 500.0 ng/mL of each analyte. The samples were analyzed following the sampling procedure described in the experimental section. The results achieved are presented in **Table 3**, in which can be seen that all the analytes were found in the analyzed samples, at concentration levels similar to those described elsewhere [5], except kaempferol, which was not quantified in samples 1 and 3. The recoveries ranged between 86.9 and 108.2 % in all instances, which was indicative of the low matrix interference.

Table 2. Features of the method

Analyte	Retention time (min)	Equation [1]	r^2	$\sigma_{y/x}$ [2]	Linear range (ng/mL)	Detection limit (ng/mL)	RSD% [3]	
							low level	high level
Naringin	10.20	y= (5.00 ± 0.02) x +5.63 ± 3.54	0.9955	0,24	6.0 -1700	1.0	3.9	3.8
Hesperidin	11.20	y= (0.56 ± 0.01) x + 0.20 ± 0.57	0.9936	0,04	9.8 – 1700	2.7	3.7	4.7
Quercetin	16.80	y= (59.08 ± 0.27) x – 20.66 ±10.5	0.9957	0,40	2.1 – 2000	1.0	1.3	4.6
Naringenin	21.20	y=(21.95 ± 0.14) x + 0.31 ± 11.4	0.9958	0,65	5.2 – 1500	1.5	4.3	4.1
Kaempferol	22.50	y= (25.34 ± 0.07) x - 0.28 ± 6.32	0.9983	0,93	2.5 – 2000	0.8	2.6	3.3

[1] *y* denotes peak area, *x* denotes analyte concentration (ng/mL), [2] Residual standard deviation, [3] RSD values at 10.0 and 500.0 ng/mL of each analyte for maximal and minimal error zones, respectively.

Table 3. Application of the method

Analytes	Natural juice			Commercialjuice 1			Commercialjuice 2		
	Conc. found[1]	Recovery % [2]		Conc. found[1]	Recovery % [2]		Conc. found[1]	Recovery % [2]	
		1st	2nd		1st	2nd		1st	2nd
Naringin	86.5	100.0	92.1	1010.0	95.7	93.7	255.0	105.2	105.0
Hesperidin	200.7	105.0	107.6	820.0	86.9	106.3	161.0	89.7	102.6
Quercetin	360.0	100.0	98.6	220.0	106.5	90.4	97.5	95.4	92.3
Naringenin	240.0	101.2	98.6	550.0	100.0	94.6	52.5	91.3	92.5
Kaempferol	n.d	-	-	30.0	102.6	92.6	n.d.	-	-

[1] Concentracion in µg/mL, [2] Recoveries after addition of 10.0 and 500 ng/mL, first and second addition, respectively.

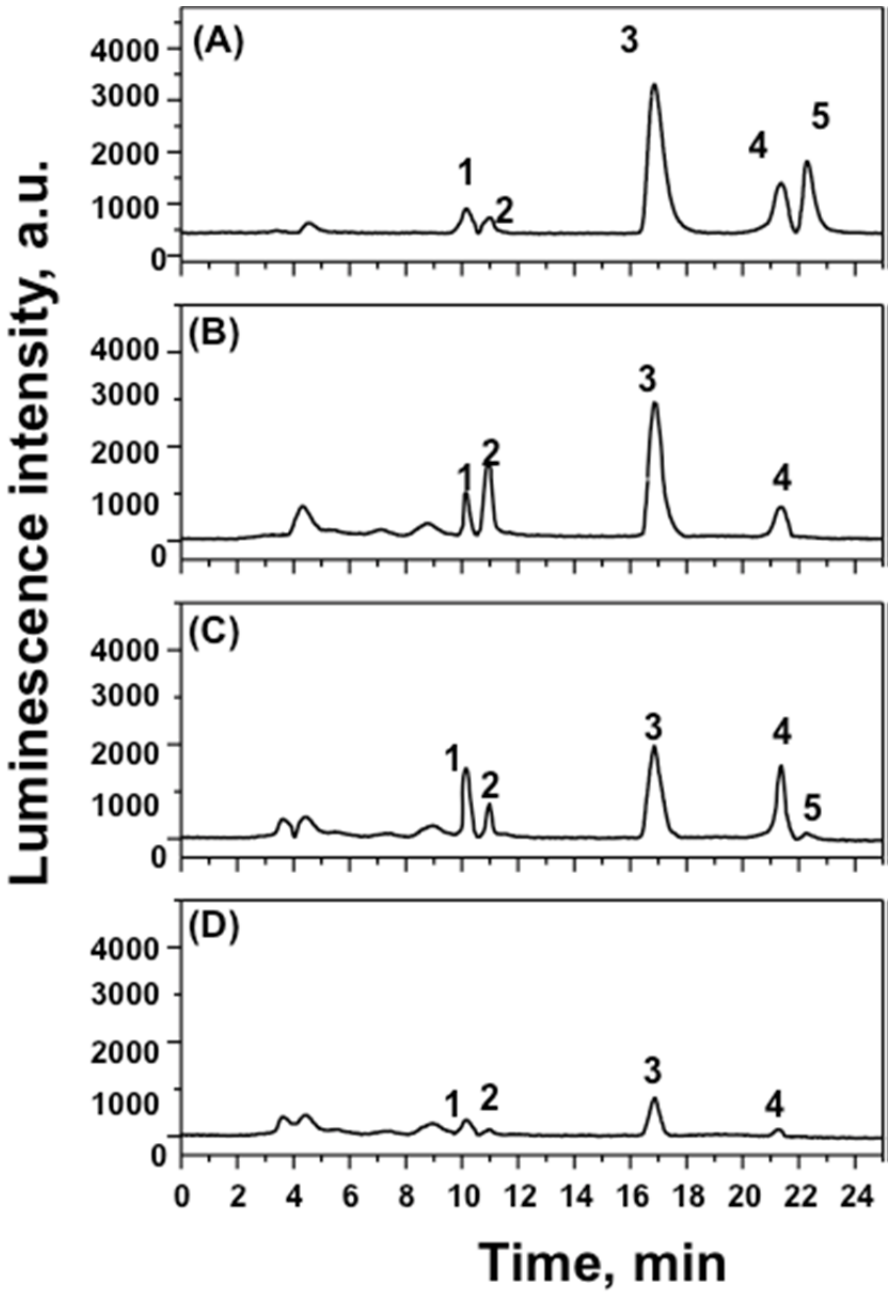


Figure 5. Chromatograms achieved using the proposed approach. (A) An aqueous standard solution containing 100 ng/mL of each analyte, (B) natural and commercial (C-D) juice samples.Analytes: (1) naringin, (2) hesperidin, (3) quercetin, (4) naringenin and (5) kaempferol.

3. Conclusions

This article shows the usefulness of a new post-column derivatization reaction for the LC separation and luminescent detection of flavonoids in orange juices. It is the first time that the simultaneous formation of analyte chelates with two metal ions to obtain sensitized luminescence is described. Unlike other LC methods for the analysis of similar samples, which involve a previous sample treatment [6-10], the proposed method allows the direct injection of the diluted samples. Also, the time required for the chromatographic separation is shorter than that required for the separation of similar mixtures of these compounds [7-9]. Finally, regarding LOD values, the luminescent detection improve the values previously described using photometric, coulometric or MS detection [8, 10].

Acknowledgements

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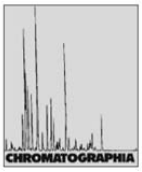

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Long-Wavelength Fluorescence detection of Flavonoids in Orange Juices using Liquid Chromatography

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A new post-column liquid chromatographic reaction system for the determination of flavonoids in orange juices is based on the use of the long wavelength fluorophor cresyl violet and cerium(IV) in a cetyl trimethylammonium bromide micellar medium. Two flavone aglycones (quercetin and kaempferol), a flavonone aglycone (naringenin), one flavone-O-glycoside (rutin) and two flavanone-O-glycosides (hesperidin and naringin) have been used as analyte models. The reaction process involves the interaction between the analyte, cerium(IV) and cresyl violet giving rise to a decrease in the fluorescence, measured at λ_{ex} 585, λ_{em} 625 nm, which is proportional to the analyte concentration. Dynamic ranges of the calibration graphs and detection limits, obtained with standard solutions of the analytes are (ng mL^{-1}): quercetin (12.2 – 4000, 3.7), kaempferol (3.5 – 1000, 1.0), naringenin (6.7 – 1000, 2.0), rutin (5.0 – 800, 1.5), hesperidin (10.1 – 1000, 3.0), and naringin (17.8 – 800, 1.8). The determination coefficients were higher than 0.993 in all instances. The precision of the method, expressed as RSD%, was established at two concentration levels, with values ranging between 2.8 and 6.2 %. The practical usefulness of the developed method is demonstrated by the

analysis of natural and commercial orange juices, which were filtered, diluted and directly injected into the chromatographic system, with apparent recoveries between 86.9 and 107.0%.

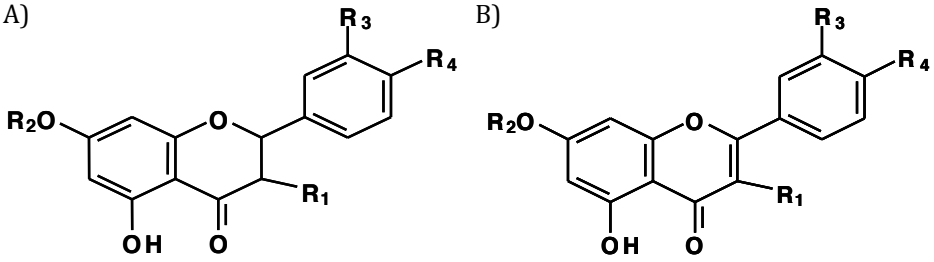
Introduction

The use of long-wavelength fluorophores (LWF) as analytical reagents is an attractive option to improve the selectivity of fluorimetric analysis and avoid or minimize the sample treatment step of the analytical process. These dyes allow measurements are obtained in a region of the electromagnetic spectrum (> 600 nm) in which the potential absorption or emission associated to sample matrix is minimized. Also, Raman interference is greatly diminished and the probability of non-radiative quenching processes is decreased due to the usually short fluorescence lifetime of these fluorophores. LWFs have been described as enzymatic substrates, immunoassay labels, sensing systems and derivatizing reagents in liquid chromatography (LC) and capillary electrophoresis (CE) [1]. Although the lack of sufficient reactive groups for targeting of analytes is a limitation of these fluorophores, their analytical usefulness has been expanded using electrostatic and redox interactions [2-6].

This article describes for the first time the use of the oxazine LWF cresyl violet (CV) in a post-column LC reaction system for the indirect determination of six representative flavonoids in orange juices, involving the use of cerium(IV) and a cetyl trimethylammonium bromide (CTAB) micellar medium and fluorescence detection.

Flavonoids are a large group of natural polyphenols found in fruits and vegetables that have been widely studied by LC with photometric or mass spectrometric detection [7]. Some of these methods have been applied to the analysis of orange juice samples, requiring most of them a treatment step, which involves extraction, hydrolysis, centrifugation and/or filtration [8-13]. A recent direct LC method described for this purpose is based on the use of post-column luminescence derivatization reactions of the analytes with aluminium(III) and terbium(III) [14]. Although the detection limits obtained are similar to the values reached in this manuscript, the time required for the mixture resolution is higher. Also, a direct LC method with photometric detection has been proposed for the determination of a mixture of flavonoids, similar to that proposed here (**figure 1**), in orange extracts [15] but the separation required 35 min.

A) B)



A)	R_1	R_2	R_3	R_4
Naringin (Flavanone O-glycoside)	H	Glycoside	H	OH
Hesperidin (Flavanone O-glycoside)	H	Glycoside	OH	OCH_3
Naringenin Flavanone aglycone)	H	H	H	OH
B)				
Rutin (Flavone O-glycoside)	Glycoside	OH	OH	OH
Quercetin (Flavone O-aglycone)	OH	H	OH	OH
Kaemfpherol (Flavone aglycone)	OH	H	H	OH

Figure 1. Chemical structures and nomenclature of the flavonoids selected in this research.

1. Experimental

1.1. Materials

All chemicals used were of analytical reagent grade. Stock solutions (5000 mg L^{-1}) of the analytes were prepared as follows: quercetin (QUE) (Aldrich), rutin (RUT) (Sigma), kaempferol (KAE) (Sigma), naringin (NAR) (Fluka), naringenin (NAN) (Aldrich) and hesperidin (HES) (Fluka) were dissolved in absolute ethanol. Intermediate solutions of 100 mg L^{-1} were also prepared by diluting the stock solutions in absolute ethanol or water, according to the analyte solubility. Stock and intermediate solutions were stored at 4 ºC in the dark and were stable for at least two weeks. Working standard solutions were prepared from the intermediate solutions by their dilution in distilled water.

1.2. Equipment

HPLC. An Agilent 1200 Series Liquid Chromatography System composed by a quaternary pump, a degasser unit, a vial autosampler, and a thermostated column compartment has been used. Chromatographic separation was performed using an Onyx monolithic C18 column (Phenomenex, Torrance, USA), 100 mm x 4.6 mm i.d., pore sizes: mesopores (13 nm), macropores (2 μm).

Post-column reaction system. An SLM Aminco (Urbana, IL) AB2 luminescence spectrometer provided with a 150 W continuous xenon lamp, furnished with a 176-052-QS Hellma (Hellma Hispania, Barcelona, Spain) flow cell with an inner volume of 18 μL, was used to monitor fluorescence detection. A Gilson (Villiers-le-Bel, France) Minipuls 3 low-pressure peristaltic pump and Omnifit (Cambridge, UK) Teflon tubing of 0.5 mm i.d. were also used for constructing the post-column reaction manifold. The reaction solution was also previously merged from two separated solutions containing the reactants (L_1= 50 cm). The mixture passed through the reaction coil (L_2 = 100 cm), in which the reaction process took place, and the fluorescence intensity was monitored using λ_{ex} 585, λ_{em} 625 nm.

1.3. Method

Figure 2 shows the integrated LC separation/reaction/detection approach. Standards or diluted samples (200 μL), containing a mixture of the analytes at concentrations within their corresponding dynamic ranges, were injected in triplicate into the column. The mobile phase was pumped at a flow-rate of 0.7 mL min^{-1} and the system operated in an optimized gradient mode in order to obtain the adequate separation of the analytes. The mobile phase was constituted by a ternary mixture of 0.15 mol L^{-1} acetic buffer, pH 4.0 (solvent A), acetonitrile (solvent B), and methanol (solvent C), which were mixed operating in gradient mode during the chromatographic separation (**Table 1**). All solvents were of analytical or HPLC grade (Panreac Quimica, S.A., Barcelona, Spain). The reaction solution was merged in a continuous system by mixing two solutions: solution 1, containing cresyl violet (CV) (8.5 μmol L^{-1}) (Sigma) and cetyltrimethyl ammonium bromide (CTAB) (1 mmol L^{-1}) (Sigma) dissolved in water, and

solution 2, containing cerium(IV) sulfate (0.1 mmol L^{-1}) (Merck) dissolved in 15 mmol L^{-1} sulfuric acid.

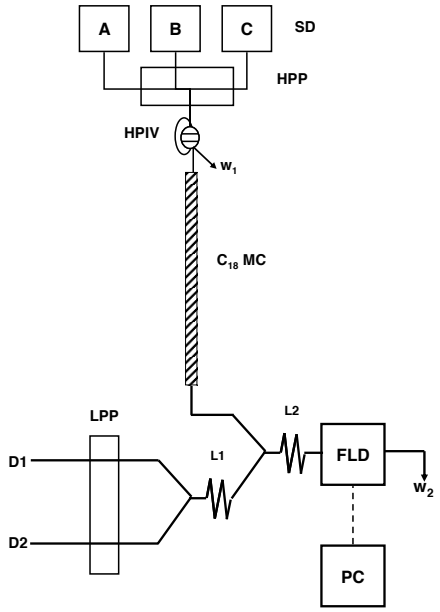


Figure 2. Integrated separation-derivatization and detection approach. A, B and C denote 0.15 mol L^{-1} acetic buffer, acetonitrile and methanol solutions, respectively; SD, solvent delivery system; HPP, High-pressure quaternary gradient pump; HPIV, High pressure injection valve; C_{18} MC, C_{18} monolithic column; D1, CV in CTAB micellar solution; D2, cerium(IV) in sulfuric acid solution; LPP, low-pressure pump; L_1 and L_2, mixing reactors; FLD, fluorescence detector; PC, personal computer, w, waste solution.

Table 1. Elution gradient conditions

Time (min)	*% Acetic buffer (0.15 mol L^{-1}, pH 4.0)*	*% Acetonitrile*	*% Methanol*
0	75	5	20
5	70	10	20
12	50	30	20
13	50	30	20

After the chromatographic separation, the elution solution was merged with the reaction solution, which was pumped at a flow-rate of 0.9 mL min^{-1}. Chromatograms were taken using the original software of the spectrofluorimeter and the raw data of fluorescence intensity and time were exported and treated using suitable software packages for the estimation of the main chromatographic parameters.

1.4. Analysis of orange juice samples

Commercial and natural orange juice samples were filtered and diluted 500-times with distilled water, to match the linear range of the calibration graph for each analyte and were directly injected onto the chromatographic system following the procedure above indicated. Each determination was the mean of three measurements.

2. Results and discussion

2.1. Study of the fluorescent system

Figure 3 shows the emission spectra (λ_{ex} 585 nm) of CV alone and in the presence of the different components of the system, using quercetin as a flavonoid model. The spectra were obtained in a solution containing the initial mobile phase composition (75% acetic buffer, 5% acetonitrile and 20% methanol) used for the chromatographic separation. As can be seen, the fluorescence of CV (curve 1) shows a slight increase in the presence of CTAB micelles (curve 2), in a similar way to the behaviour previously described for its absorbance spectrum [16]. The oxidation of CV by cerium(IV), which is made evident by the drastic fluorescence quenching (curve 3), is practically hindered in the presence of CTAB (curve 4) because the dye is protected by the micelles and only a partial decrease of the fluorescence occurs. However, the presence of quercetin causes a quenching in the CV fluorescence (curves 5 and 6), which is proportional to the flavonoid concentration.

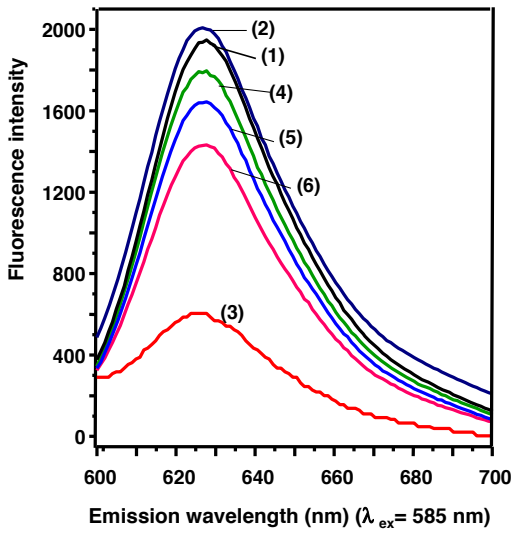


Figure 3. Emission spectra (λ_{ex} 585 nm) of (1) CV alone and (2) in the presence of CTAB, (3) cerium(IV), (4) CTAB + cerium(IV), (5) CTAB + cerium(IV) + quercetin (20 ng mL^{-1}), (6) and CTAB + cerium(IV) + quercetin (100 ng mL^{-1}). All spectra were obtained in the presence of a solution containing 75% acetic buffer (0.15 mol L^{-1}), 5% acetonitrile and 20% methanol, which corresponds to the initial mobile phase composition. [CV] = 8.5 µmol L^{-1}, [CTAB] = 1.0 mmol L^{-1}, cerium(IV) = 0.1 mmol L^{-1}.

This fluorescence quenching is probably the result of a combination of redox, acid-base and hydrophobic processes. It has been described that quercetin has a strong interaction with CTAB micelles, which modifies their structure [17]. Thus, the decrease in the fluorescence of CV could be ascribed to the interaction of the flavonoid with the micelles, which facilitates that the dye leaves the micelles and is oxidized by cerium(IV). It is also logical to assume that the flavonoid can be oxidized by cerium(IV) before its interaction with CTAB micelles. In fact, it has been described that CTAB micelle facilitates the oxidation of quercetin [17]. On the other hand, although the dissociation constants of quercetin are relatively high, and the apparent pH of the medium is about 3.8, the CTAB micelles can provide a relative alkaline microenvironment [18]. This medium can promote the formation of quercetin anions, giving rise to an additional electrostatic attraction with positively charged CTAB micelle, besides the hydrophobic interaction.

2.2. Method Performance

The hydrodynamic and chemical variables affecting the system were optimized using the univariate method. **Table 2** shows the range studied for each variable and the values chosen, which were those yielding the best differences between the blank solution and the solution containing the analytes, and with minimum standard deviation.

Table 2. Optimization of variables

Type of Variables	*Variables*	*Range studied*	*Optimum value*
Instrumental	Excitation wavelength, nm	300 – 620	585
	Emission wavelength, nm	610 - 800	625
	Ex/em slits	2.5 – 15	8/8
	PMT gain, V	400 - 800	600
Chromatographic	HPP flow-rate, mL min^{-1}	0.5 – 3.0	0.7
	Injection volume, µL	20 – 500	200
	Elution mode	Gradient	-
	Temperature, ºC	25.0 – 45.0	35.0
	[HAc] mol L^{-1}	0.05 – 0.25	0,15
	pH	2.5 – 6.0	4.0
Derivatizing	LPP flow-rate, mL min^{-1}	0.2 – 1.5	0.9
	L_1 reactor length, cm	50 - 300	50
	L_2 reactor length, cm	50 – 300	100
	[Cresyl violet] µmol L^{-1}	1.0 -20.0	8.5
	[Ce (IV)] mmol L^{-1}	0.05 – 0.2	0.10
	[H_2SO_4] mmol L^{-1}	5.0 – 50.0	15.0
	[CTAB] mmol L^{-1}	0.01 – 2.0	1.0

2.3. Chromatographic separation

Chromatographic variables were optimized in order to obtain acceptable resolution of the analytes in the lowest separation time. The composition and influence of the mobile phase was studied using ternary mixtures integrated by 0.15 mol L^{-1} acetic buffer, pH 4.0 (A), acetonitrile (B) and methanol (C). Different gradient profiles were assayed at the optimum flow rate, finding that the use of a constant methanol percentage

(20%) and increasing percentages of acetonitrile until 30 %, with the simultaneous decreasing of the acetic buffer, up to 12 min (Table 1), was useful for the resolution of the analytes.

The influence of the chromatographic flow-rate was studied in the range 0.5 – 3 mL min^{-1} with acceptable separation in all instances. Although monolithic columns allow the use of high flow-rates, it was necessary to choose a relatively low flow-rate, 0.7 mL min^{-1}, to make compatible the separation process with the post-column reaction system and the pressure tolerated by the fluorescence detector.

2.4. Post-column reaction

The study of the flow-rate of the reaction reagent solution showed that 0.9 mL min-1 was a suitable value for the process when a 0.7 mL min-1 flow-rate was used for the chromatographic separation. The length of the mixture reactor L2 was 100 cm, which was enough for the development of the different reactions involved in the process. Now that a sensitive response has been demonstrated, future optimization of the design and connections in the post-column reactor may be possible to reduce the peak widths.

Figure 4 shows the influence of CV, cerium(IV) and CTAB concentration on the fluorescence of the system in the presence of rutin (curve 1), naringin (curve 2) and quercetin (curve 3). The values of the y-axis correspond to the net area of the negative peaks achieved in each chromatogram. The effect of the CV concentration (**Figure 4a**), tested in the range of 1.0 – 20 μmol L-1, and in the presence of cerium(IV) 0.05 mmol L-1 and CTAB 1.0 mmol L-1, was similar for the three analytes. A concentration of 8.5 μmol L-1 was selected as optimum. As it was necessary to dissolve cerium(IV) in sulfuric acid to avoid its precipitation, the effect of the concentration of this acid was evaluated in the range 5.0 – 50 mmol L-1, finding that a 15 mmol L-1 concentration provided enough acid medium to dissolve cerium(IV). The influence of cerium(IV) concentration was studied in the range 0.05 – 0.2 mmol L-1 (**Figure 4b**), in the presence of CV 8.5 μmol L-1 and CTAB 1.0 mmol L-1 . As can be seen, the effect of cerium(IV) in the presence of the glycoside flavonoid derivatives rutin and naringin is slightly different to that obtained in the

presence of the aglycone derivative quercetin. A 0.10 mmol L-1 cerium(IV) concentration was selected as optimum. The CTAB concentration is a critical variable of the system. **Figure 4c**, obtained in the presence of cerium(IV) 0.1 mmol L-1 and CV 8.5 μmol L-1, shows that the maximum signal in the presence of rutin and naringin is obtained at a 0.5 mmol L-1 CTAB concentration, which corresponds to the critical micellar concentration described for this surfactant [19]. In the presence of quercetin, the maximum signal is obtained at 1 mmol L-1, choosing this concentration as the optimum value.

Figure 5a shows the chromatogram obtained under the optimum experimental conditions for a standard solution containing 100 ng mL-1 of each analyte. Although the separation does not reach the baseline in some instances, it is enough to allow analyte determination. The slight increase of the baseline is ascribed to the variation of the fluorescence of CV with the solvent gradient. As can be seen, the mixture of the six flavonoids can be resolved in about 13 min. Several phenolic and hydroxycinnamic acids, such as gallic, caffeic and coumaric acids, were tested, but they were co-eluted with the front of the solvent.

2.5. Analytical features

Calibration graphs were run under the optimal experimental conditions depicted in **Table 2**, and the linearity of the method was established using the residual analysis test Eight standard solutions of mixtures of the six analytes, covering the concentration range between 1 and 4000 ng mL^{-1}, were assayed in triplicate. **Table 3** summarizes the figures of merits of the method, including average retention time, calibration equations, determination coefficients, residual standard deviations, lack-of-fit p-values, linear ranges, detection limits (LODs) and precision. The determination coefficient values ($R2 \geq 0.993$, $N = 8$) and residual standard deviation ($\sigma y/x \leq 1.15$) were acceptable in all instances.

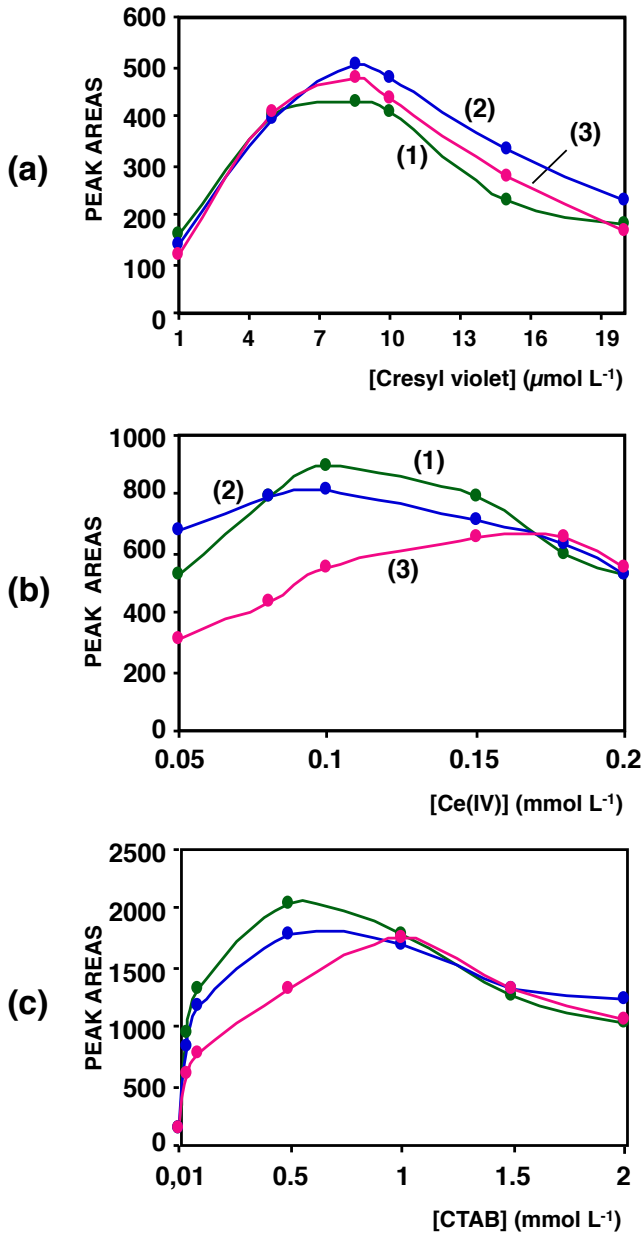


Figure 4. Influence of CV (a), cerium(IV) (b) and CTAB (c) concentrations on the fluorescence system containing rutin (1), naringin (2) and quercetin (3). [Analyte] = 100 ng mL^{-1}. [cerium(IV)] = 0.05 mmol L^{-1} (a) and 0.1 mmol L^{-1} (c), [CV] = 8.5 µmol L^{-1} (b and c), [CTAB] = 1.0 mmol L^{-1} (a and b). (The y-axis values correspond to the absolute peak areas achieved for the selected analytes after the analytical signal correction from the blank).

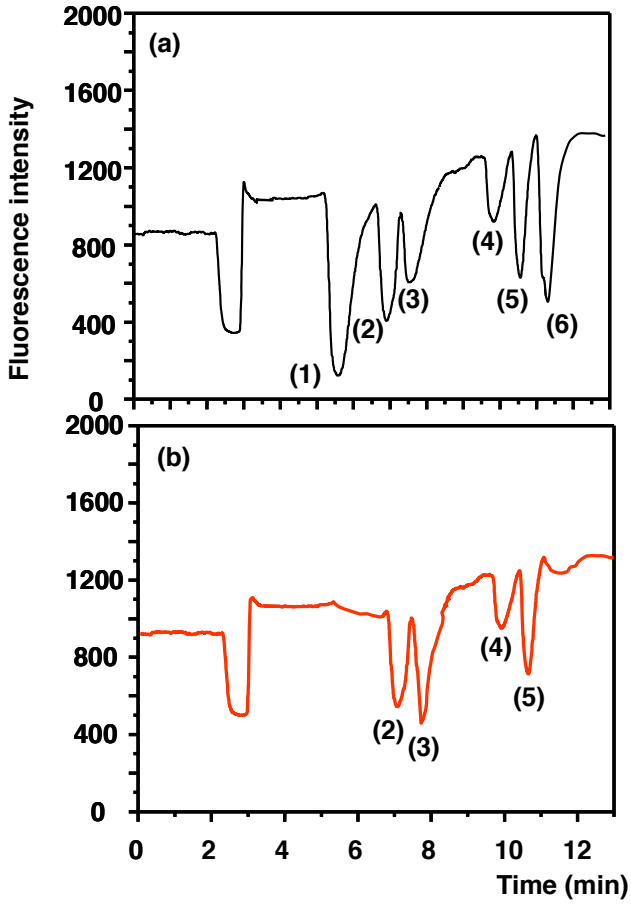


Figure 5. Chromatograms achieved using the proposed approach. (a) Aqueous standard solution containing 100 ng mL^{-1} of each analyte: rutin, (1) naringin, (2) hesperidin, (3) quercetin, (4) naringenin (5) and kaempferol (6), and (b) natural orange juice 2 (Navel) containing: naringin, (2) hesperidin, (3) quercetin, (4) and naringenin (5).

The linearity of the calibration curves was also estimated using the lack-of-fit test, which revealed a good statistical correlation between the variables in the selected concentration intervals, with p-values higher than 0.1 in all instances. The interval of the calibration graphs ranged between 5 - 800 ng mL^{-1} for rutin and 12.2 – 4000 ng mL^{-1} for quercetin. The LODs, calculated according IUPAC recommendations [20], ranged between 1.0 ng mL^{-1} for kaempferol and 3.7 ng mL^{-1} for quercetin. The precision of the method, expressed as percentage of relative standard deviation (%RSD), was studied at two concentrations of each analyte, 20 and 100 ng mL^{-1} (N=11), injecting each sample in triplicate. The values obtained ranged between 2.8 and 6.2 %.

2.6. Application of the method

The proposed method was applied to the analysis of five orange juice samples from natural and commercial origin. The samples only required the filtration and adequate dilution, 500-times, with distilled water to match the linear range of the calibration graph of each analyte. The method was tested in two ways: a) by determining the concentration of the six analytes in the samples, and b) by studying the recovery after addition of two standard aliquots of 20.0 and 100.0 ng mL-1 of each analyte. The samples were analyzed following the procedure described in the experimental section (N= 6). The results achieved are presented in **Table 4,** in which can be seen that naringin, hesperidin, quercetin and naringenin were found in all the analyzed samples. Rutin was found in two commercial samples and kaempferol only in one of them. **Figure 5b** shows the chromatogram obtained for the natural orange juice 2. The concentration levels found are similar to those described elsewhere [8-12]. The apparent recoveries ranged between 86.9 and 107.0 %, which was indicative of the low matrix interference.

Table 3. Features of the method

Analyte	*Retention time ±SD (min)*[1]	*Equation* [2]	R^2	$\sigma_{y/x}$ *(p-value)* [3]	*Linear range (ng mL^{-1})*	*Detection limit (ng mL^{-1})*	*RSD%* [4]	
							low level	high level
Rutin	5.6 ± 0.6	y= (0.756 ± 0.009) x + 9.211 ± 0.038	0.9974	1.15 (0.38)	5.0 - 800	1.5	3.9	6.2
Naringin	7.0 ± 0.2	y= (0.306 ± 0.005) x + 4.002 ± 0.182	0.9959	0.31 (0.97)	17.8 - 800	1.8	4.5	4.5
Hesperidin	7.5 ± 0.2	y= (0.278 ± 0.005) x + 16.891 ± 0.281	0.9931	0.62 (0.81)	10.1 - 1000	3.0	3.8	4.7
Quercetin	9.9 ± 0.6	y= (0.304 ± 0.002) x + 11.075 ±0.371	0.9981	0.61 (0.69)	12.2 - 4000	3.7	2.8	3.2
Naringenin	10.6 ± 0.4	y=(0.333 ± 0.004) x + 11.853 ± 0.222	0.9969	0.36 (0.92)	6.7 - 1000	2.0	3.3	4.7
Kaempferol	11.6 ± 0.3	y= (0.779 ± 0.007) x + 9.937 ± 0.269	0.9988	0.34 (0.71)	3.5 - 1000	1.0	3.9	2.8

[1] SD denotes standard deviation, [2] y denotes peak area, x denotes analyte concentration (ng mL^{-1}), [3] residual standard deviation and p-values achieved using the lack-of-fit test and [4] relative standard deviation (N=11) values at 20.0 and 100.0 ng mL^{-1} of each analyte.

Table 4. Application of the method

Natural Juice 1	*Natural Juice 1 (Navel)*			*Natural Juice 2 (Navel)*			*Natural Juice 3 (Clementina)*			*Commercial Juice 1*			*Commercial Juice 2*		
Analytes	*Conc.Found (± SD)* [1]	*Recovery (%)*		*Conc. Found (± SD)* [1]	*Recovery (%)*		*Conc. Found (± SD)* [1]	*Recovery (%)*		*Conc. Found (± SD)* [1]	*Recovery (%)*		*Conc. Found (± SD)* [1]	*Recovery (%)*	
		1st	2nd		1st	2nd		1st	2nd		1st	2nd		1st	2nd
Rutin	n.d.	-	-	n.d.	-	-	n.d.	-	-	1.4 (± 0.3)	99.9	91.8	3.5 (± 0.6)	95	103
Naringin	85.8 (± 0.3)	99.3	91.6	68.8 (± 0.4)	97.9	103	103 (± 1)	102	94.5	870 (± 2)	97.9	103	244 (± 1)	95.7	93.7
Hesperidin	194.8 (± 0.2)	101	93.6	163.3 (± 0.8)	89.9	101	188.9 (± 0.8)	96.9	105	760 (± 2)	89.9	101	172 (± 1)	86.9	106
Quercetin	360 (± 1)	98.5	92.8	90 (± 1)	91.6	94.4	116.8 (± 0.1)	104	89.9	245.0 (± 0.8)	91.6	94.4	96 (± 2)	107	90.4
Naringenin	248 (± 2)	101	96.8	65 (± 1)	103	97.8	186.5 (± 0.2)	103	98.9	550 (± 3)	103	97.8	49 (± 1)	104	94.6
Kaempferol	n.d.	-	-	n.d.	-	-	n.d.	-	-	26 (± 2)	107	106	n.d.	-	-

[1] Concentracion in μg mL^{-1} (SD= standard deviation). [2] Recoveries after addition of 20.0 and 100.0 ng mL^{-1}, first and second addition, respectively.

3. Conclusions

This article shows the usefulness of a new LC post-column fluorescent system for the direct determination of flavonoids in orange juices. The use of long-wavelength measurements to obtain analytical signals avoids the interference of potential signals from the sample matrix. Also, the low detection limits reached allow the use of a high dilution factor, avoiding sample treatment and preconcentration processes. These detection limits are, in general, lower than those described using photometric detection. For instance, the detection limits reported in some of these methods [10, 12, 15] for naringin, quercetin, hesperidin and rutin are in the range 0.03-1.25 μg mL-1, 0.1-2.5 μg mL-1, 0.3-1.0 μg mL-1 and 0.02-0.04 μg mL-1, respectively. A potential limitation of the method is the relative low flow-rate selected using a monolithic column, which was required to make compatible the separation, post-column derivatization and fluorescent detection processes.

Acknowledgements

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CAPITULO 3

INNOVACIONES EN LA DETERMINACIÓN NO CROMATOGRÁFICA DE ANTIOXIDANTES EN ALIMENTOS

Este capítulo presenta las investigaciones que han dado lugar al desarrollo de dos nuevos métodos para la determinación de antioxidantes alimentarios mediante medidas dinámicas. Los resultados de estos estudios se recogen en los siguientes artículos:

- Determination of antioxidant additives in foodstuffs by direct measurement of gold nanoparticle formation using resonalce light scattering detection. A. Andreu-Navarro, J.M. Férnández-Romero, A. Gómez-Hens, *Anal. Chim. Acta*, 2011, 695, 11-17.

- Determination of polyphenolic content in beverages using laccase, gold nanoparticles and long wavelength fluorimetry. A. Andreu-Navarro, J.M. Férnández-Romero, A. Gómez-Hens, *Anal. Chim. Acta*, enviado.

En el primero de ellos se utiliza la capacidad de los antioxidantes para reducir al oro(III) en disolución, dando lugar a la formación de nanopartículas. Este comportamiento ha permitido la cuantificación de estos compuestos mediante medidas de la variación de la dispersión de la radiación de resonancia con el tiempo y utilización de la velocidad inicial del proceso como parámetro analítico. En la introducción de este trabajo se comentan los escasos antecedentes descritos sobre la monitorización de la formación de nanopartículas de oro (AuNPs) con fines analíticos. No obstante, además de su aplicación analítica, cabe destacar que este estudio pone de manifiesto por primera vez la posibilidad de obtener AuNPs en sólo unos segundos mediante un procedimiento muy simple.

El segundo método utiliza la fluorimetría de larga longitud de onda para determinar polifenoles mediante el efecto inhibidor que causan estos compuestos en la oxidación enzimática del fluoróforo verde de indocianina, en presencia de AuNPs cargadas positivamente. Este sistema competitivo origina un periodo de inducción que es proporcional a la concentración de los analitos, en el que éstos impiden la oxidación del fluoróforo, con la correspondiente pérdida de su fluorescencia. Para realizar este estudio se eligió la enzima laccasa que, a diferencia de otras oxidasas, no requiere el uso de reactivos como el peróxido de hidrógeno para actuar como catalizador ya que reduce al oxígeno disuelto, transformándolo en agua, mientras se oxida el sustrato. Aunque se han descrito diversos sustratos coloreados de la laccasa [1], es la primera vez que se utiliza la fluorimetría de larga longitud de onda para la determinación de polifenoles. Otro aspecto novedoso del método propuesto es el uso de AuNPs para mejorar

los límites de detección obtenidos, debido a la interacción de las NPs con la enzima.

Entre los objetivos planteados para el desarrollo de estos métodos se encuentra la búsqueda de nuevas aplicaciones analíticas de las AuNPs, cuyo uso ha tenido un gran impacto en los últimos años en la Química Analítica. Las especiales propiedades ópticas y eléctricas que presentan estas NPs han sido utilizadas en numerosos métodos determinativos, tales como los basados en el desarrollo de biosensores o de nuevos marcadores en inmunoensayo, y en estudios de hibridación de ácidos nucleicos [2]. También se han descrito diversas aplicaciones de las AuNPs en procesos de separación y preconcentración utilizando técnicas cromatográficas y electroforéticas [3]. Sin embargo, su uso con fines determinativos para la detección directa de los analitos mediante el seguimiento de la síntesis de las AuNPs, o su utilización como inhibidores de la actividad enzimática, como se describe en este capítulo, ha sido muy restringido.

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Determination of Antioxidant Additives in Foodstuffs by Direct Measurement of Gold Nanoparticle Formation using Resonance Light Scattering Detection

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The capability of antioxidant compounds to reduce gold(III) to gold nanoparticles has been kinetically studied in the presence of cetyltrimethylammonium bromide using stopped-flow mixing technique and resonance light scattering as detection system. This study has given rise to a simple and rapid method for the determination of several synthetic and natural antioxidants used as additives in foodstuff samples. The formation of AuNPs was monitored by measuring the initial reaction-rate of the system in about 5 s, using an integration time of 0.1 s. Dynamic ranges of the calibration graphs and detection limits, obtained with standard solutions of the analytes, were (μmol L^{-1}): gallic acid (0.04 – 0.59, 0.01), propyl gallate (0.04 – 1.41, 0.01), octyl gallate (0.03 – 0.35, 0.08), dodecyl gallate (0.02 – 0.30, 0.007), butylated hydroxyanisol (0.07 – 0.39, 0.009), butylated hydroxytoluene (0.04 – 0.32, 0.01), ascorbic acid (0.11 – 1.72, 0.03) and sodium citrate (0.07 – 1.29, 0.02). The regression coefficients were higher than 0.994 in all instances. The precision of the method, expressed as RSD%, was established at two concentration levels of each analyte, with values ranging between 0.6 and 4.8 %. The practical

usefulness of the developed method was demonstrated by the determination of several antioxidant additives in foodstuff samples, which were extracted, appropriately diluted and assayed, obtaining recoveries between 95.4 and 99.5 %. The results obtained were validated using two reference methods.

Introduction

Natural and synthetic antioxidants are a group of compounds used as additives to prevent or retard oxidation reactions in food products. There is a trend to limit the use of synthetic antioxidants, owing to their potential toxic effects, but they are still found in a relatively wide range of foodstuffs, although their use is subject to very strict safety regulations [1-6]. Phenolic compounds such as propyl (PG), octyl (OG) and dodecyl (DG) esters of gallic acid (GA), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are the main synthetic antioxidants still authorized, although they are being replaced by natural antioxidants, such as ascorbic (AA) and citric (CA) acids. CA is also used as a food additive for other purposes, such as acidifier and flavouring agent.

The determination of synthetic antioxidants in food samples is mainly carried out by using reverse-phase liquid chromatography (LC) with photometry or mass spectrometry (MS) as detection systems [7, 8]. Methods based on gas chromatography (GC), with flame ionization detector (FID) or MS, and capillary electrophoresis (CE) with photometric or electrochemical detection have been also described [7]. The limits of detection (LODs) reported for most of these methods are at the level of μmol L^{-1}. These methods are very useful for the identification of the analytes, but they are time-consuming for screening purposes.

The use of gold nanoparticles (AuNPs) as analytical reagents has allowed the development of very sensitive methods based on their special optical and electrochemical properties. For instance, they have been used as labels in immunoassays and hybridization assays and as nanoscaffolds to develop chemical sensors [9]. These methods require the previous synthesis of the NPs, using generally tetrachloroauric acid and a reducing reagent, usually citrate [10,11]. Other reagents such as cysteine [12], ascorbic acid [10, 13, 14], sodium borohydride [15, 16] and gallic acid [17, 18] have also shown their usefulness for this purpose. The experimental conditions chosen to obtain AuNPs are critical factors that affect the size, shape and potential aggregation of the NPs and, consequently, their properties. Some of these methods [14, 15] involve the use of cetyltrimethylammonium bromide (CTAB) to favour the synthesis of the NPs. The formation of ion pairs between $AuCl_4^-$ and cationic surfactants

prior to the formation of AuNPs was described [19, 20]. Also, the electrochemical formation of AuNPs in the presence of CTAB has been recently reported [21].

An alternative approach to the use of AuNPs as analytical reagents is the direct formation of these NPs by using $AuCl_4^-$ as reagent, which reacts with an analyte that shows reducing properties. The measurement of a property of the AuNPs formed allows the direct quantification of the analyte. An advantage of this approach is that the control of the size and shape of the NPs formed and their stabilization to avoid their aggregation are not required, provided that the experimental variables are suitably controlled. This approach has been applied to the determination of thioglycolic acid in cosmetic samples using a flow-injection system and measuring the absorbance of the resonance plasmon band of the AuNPs obtained [22]. This optical property has been also used to determine hydroquinone in pharmaceutical preparations and catechol and pyrogallol in water samples [23], using a batch format. Resonance light scattering (RLS) measurements have been described for the determination of hydrogen peroxide [24] using AuNPs seeds, which are enlarged in the presence of $AuCl_4^-$, CTAB and the analyte, increasing the RLS signal. This approach has been extended to the determination of glucose using glucose oxidase to obtain hydrogen peroxide, but the method has not been applied to the analysis of real samples and it is necessary to wait for 15 min to obtain each measurement.

This article describes a method for the fast and automatic determination of several antioxidant additives **(Figure 1)** in foodstuffs based on the capability of these compounds to reduce $AuCl_4^-$ to AuNPs in the presence of CTAB (**Figure 1**). The formation of AuNPs is followed by monitoring the variation of the RLS signal with time, using stopped-flow mixing technique, which allows the measurement of the initial rate of the system. The LODs obtained, at the level of nmol L^{-1}, are lower than those described using absorbance measurements [22, 23]. Although the proposed approach does not distinguish among the different antioxidants, it can be used as a very sensitive and fast screening method to detect the presence of these compounds in foodstuffs.

1. Experimental

1.1. Apparatus and Instruments

A Cary Eclipse Varian spectrofluorimeter (Walnut Creek, CA, USA) was used. The instrument was furnished with an RX-2000 Rapid Kinetic System Stopped-Flow mixing accessory from Applied Photophysics (Leatherhead, UK), which was fitted with an observation cell of 10 x 2 mm length, 60 μL of inner volume and controlled by the associated electronics, the computer and a pneumatic syringe drive system. The temperature of the solution was kept constant at the selected temperature by circulating water from a Haake DC1 thermostatic bath (ThermoElectron, Karlsruhe, Germany).

1.2. Reagents

All chemicals used were of analytical reagent grade. Stock solutions (100 mg mL^{-1}) of the analytes (all supplied by Sigma) were prepared as follows: gallic acid (GA), propyl gallate (PG), octyl gallate (OG), dodecyl gallate (DG), butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT) were dissolved in absolute ethanol. Ascorbic acid (AA) and sodium citrate (SC) were prepared using deionized water purified with a Milli-Q system (Millipore, Bedford, Ma, USA). Stock and intermediate solutions were stored at 4 ºC in the dark and were stable for at least two weeks. Working standard solutions were prepared from intermediate solutions by their dilution in distilled water. Tetrachloroauric acid trihydrate was also purchased from Sigma. Other reagents used were sodium chloride (Sigma), acetic acid (Merck), sodium dodecyl sulphate (SDS, Sigma), Triton X-100 (Sigma) and cetyltrimetylammonium bromide (CTAB, Sigma).

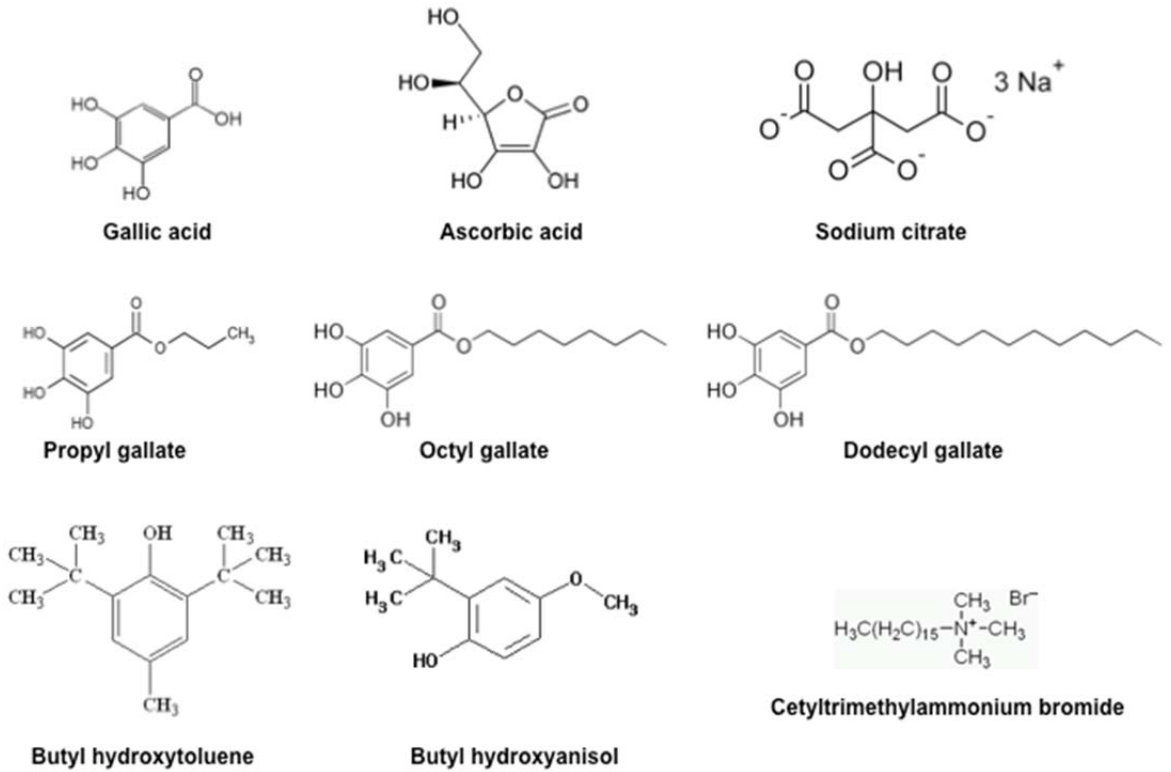

Figure 1. Chemical structures of the antioxidant compounds assayed. The structure of CTAB is also shown.

1.3. Manifold and Procedure

Figure 2 shows the stopped-flow mixing device used for the rapid mixture of the reactants. A solution containing $AuCl_4^-$ (1.0 mmol L^{-1}), prepared in 20 mmol L^{-1} acetic/acetate buffer, adjusted to pH = 4, was used to fill one of the two driving syringes of the stopped-flow module. The other syringe was filled with a pre-mixed solution containing sample or standard and CTAB (0.2 mmol L^{-1}) dilute in the same buffer solution. The pneumatic syringe control device allowed the automation of each injection. In each run, 0.15 mL of each solution was mixed in the mixing chamber of the stopped-flow module and the variation of the RLS signal with time was monitored at $\lambda_{ex} = \lambda_{em}$ = 540 nm ($\Delta\lambda$ = 0 nm) using a spectrofluorimeter. Each standard or sample solution was assayed at least six-times. All measurements were carried out at the selected optimal temperature of 25 ± 0.1 °C.

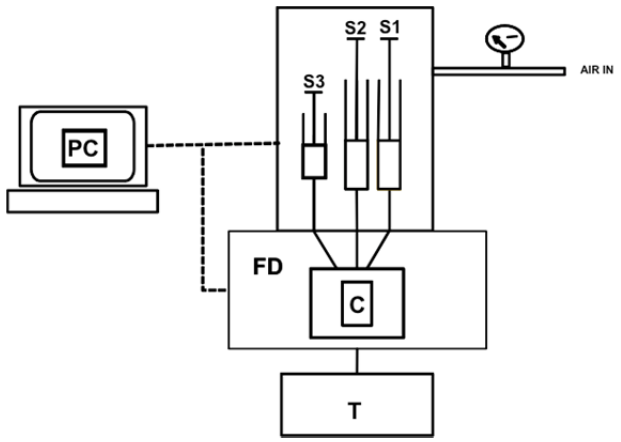

Figure 2. Scheme of the instrumental system: S1 and S2, driving syringes containing $HAuCl_4$ and CTAB + antioxidant sample, respectively; S3, stopping syringe; FD, spectrofluorimeter; C, observation cell; T, thermostat; PC, computer.

Data were acquired and processed using the software incorporated in the spectrofluorimeter for application of the initial-rate kinetic method. The initial rate (v_0) was estimated by measuring the slope of the kinetic curve in the interval 0 – 5 s, being the integration time 0.1 s. The linear calibration graph for each analyte was obtained by plotting the difference obtained for the initial rate in the presence and absence of the analyte versus the analyte concentration. The concentration of the antioxidant compound in the samples was determined by interpolation in the calibration graphs.

1.4. Characterization of the AuNPs

Transmission electron microscopy (TEM) images of different samples were obtained by dropping the resulting AuNPs on a carbon-coated copper grid and setting a completely dried drop by vacuum desiccator. The characterization of the NPs was performed by Transmission Electron Microscopy (TEM), using a CM-10 Philips Microscope with 0.5 x 0.34 nm resolution and equipped with a digital megaview III camera. Copper grids (200C-FC) coated with a Formvar® carbon film 200 mesh, supplied by Aname (Madrid, Spain), were used as support in TEM experiments.

1.5. Analysis of Foodstuff Samples

Four commercial foodstuffs, including biscuits, doughnuts, milkshakes and soup stock cubes, were processed to determine their corresponding antioxidant concentrations. The extraction step was carried out following the official extraction procedure described elsewhere [25]. In summary, 1 g of each sample was exactly weighed in a tube and then extracted three times using 5-mL of a 50% v/v methanol/water solution. The extracts were mixed for 3 min and centrifuged at 3000 rpm for 5 min. The upper solution was collected and diluted with an adequate volume of buffer, in order to fit the linear range of the calibration graph, and was treated as described above. Each determination was carried out in triplicate, using in each instance the mean of the data obtained from at least six kinetic curves.

1.5.1. Reference Methods

Each sample was tested by the AOAC official method 983.15 for the determination of phenolic antioxidants in oils, fats and butter [26]. Briefly, the antioxidants were extracted in acetonitrile and the extract was concentrate and re-dissolved in 2-propanol. A volume (10 μL) of this solution was analyzed by LC with UV detection at 280 nm. This method allows the determination of PG, OG, DG, 2,4,5-trihydroxybutirophenone (THBP), tert-butylhydroquinone (TBHQ), nordihydroguaiaracetic acid (NDGA), BHA, BHT and 2, 6-di terbutyl-4-hydroxymethylphenol (Ionox 100).

The content of AA in the milkshake sample analyzed was determined by another reference method based on the titration with N-bromosuccinimide (NBS), using sodium iodide and starch as the indicator system and oxalic acid as stabilizer [27]. Briefly, the procedure was as follows: a volume of the sample (10 mL) was centrifuged and 5 mL of the supernatant solution were collected and risen up to a final volume of 250 mL. A volume (20 mL) of this solution was transferred to a standard flask and 1.5 mL 10% KI solution, 2 mL 98% acetic acid solution and 3-4 drops of starch solution were added. The titration was carried out with 0.1 mmol L^{-1} NBS standard solution. All titrations were performed in triplicate.

2. Results and discussion

2.1. Study of the Formation of AuNPs

Several synchronous spectra ($\Delta\lambda$ = 0 nm) were obtained to study the behaviour of the system and choose the optimum wavelengths for RLS measurements, using GA as the antioxidant model. **Figure 3.A** depicts the synchronous spectra obtained for solutions containing $AuCl_4^-$ in the presence of GA, CTAB, and both GA and CTAB. The reactants were previously mixed using stopped-flow technique. As can be seen, the solution containing $AuCl_4^-$ and GA does not give any scattering signal, which shows that AuNPs are not formed under these conditions. Although GA has been described as reagent for the formation of AuNPs in the absence of CTAB, it is necessary to wait for at least 30 min [17, 18]. The solution containing $AuCl_4^-$ and CTAB shows a relatively high scattering band, with a

wide maximum at 400-500 nm, which is ascribed to the formation of insoluble ion pairs between these species [19, 20] and, also, to the emission of the light source in the spectrofluorimeter. The synchronous spectrum obtained for GA in the presence of the mixture CTAB-$AuCl_4^-$ shows also a shoulder at about 520-600 nm that is indicative of the formation of AuNPs. The use of an anionic or no ionic surfactant such as SDS or Triton X-100, respectively, alone or together with GA did not give any scattering signal, obtaining spectra similar to that obtained for the solution containing $AuCl_4^-$ and GA (curve 1 in Figure 3.A). These results show that the formation of the ionic pair between $AuCl_4^-$ and the cationic surfactant is necessary, under the experimental conditions used, to obtain the NPs. **Figure 3.B** shows a TEM image obtained for a solution containing $AuCl_4^-$, CTAB and GA, in which the formation of AuNPs can be seen with a wide size range, although most of them ranged between 10 and 20 nm.

Figure 3.C shows the kinetic curves obtained in the absence and in the presence of different GA concentrations using $\lambda_{ex} = \lambda_{em} = 540$ nm and stopped-flow mixing technique. As can be seen, the initial rate can be measured in about 5 s. The low initial rate of the CTAB- $AuCl_4^-$ system in the absence of GA (curve 1) shows the usefulness of kinetic methodology as it avoids the relatively high background signal (**Figure 3.A**, curve 2) that would be obtained using equilibrium measurements. The reactants were distributed in the syringes by introducing the $AuCl_4^-$ solution in one of the syringes and using a premixed solution containing CTAB and the analyte to fill the other syringe. It was not possible to use a solution containing the mixture of $AuCl_4^-$and CTAB owing to the formation of the insoluble ion pairs. Regarding the solution containing $AuCl_4^-$ and the analyte, it showed a cloudy aspect after several minutes, which could be ascribed to the slow reduction of Au(III).

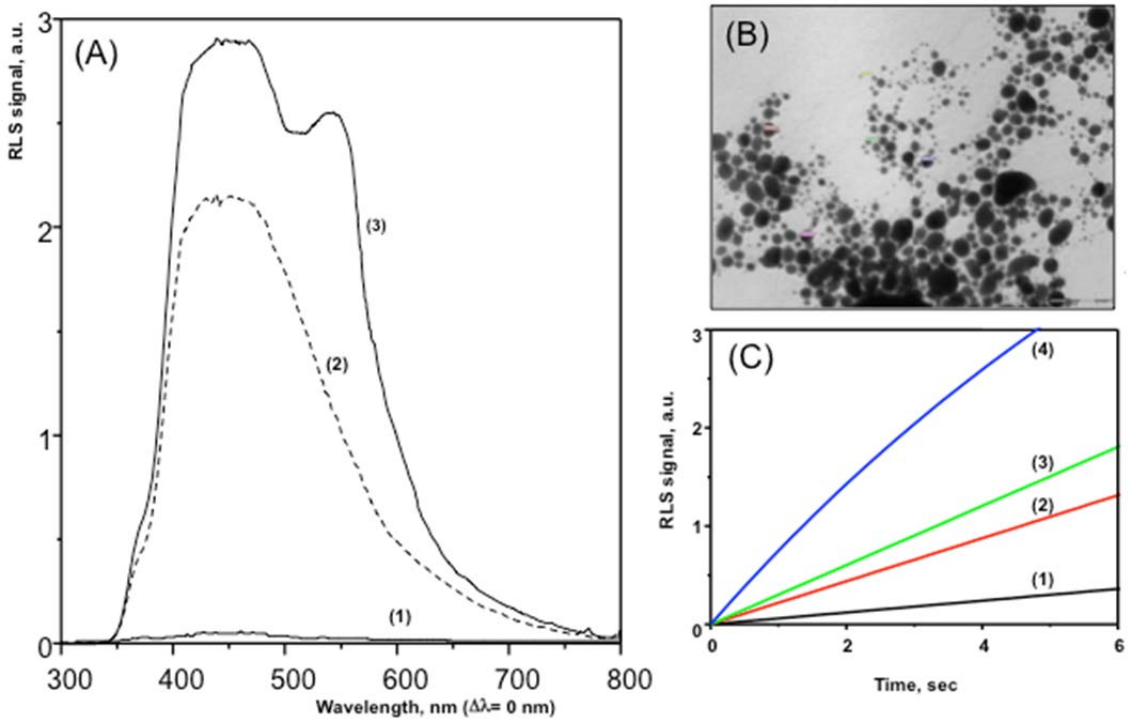

Figure 3. (A) Synchronous spectra obtained after stopped-flow mixing of solutions containing $AuCl_4^-$ in the presence of GA (1), CTAB (2) and CTAB + GA (3) [GA] = 0.1 µmol L^{-1}). (B) TEM image obtained for a solution containing $AuCl_4^-$ + CTAB + GA (0.5 µmol L^{-1}). (C) Kinetic curves obtained for different concentrations of GA (µmol L^{-1}) = (1) 0.0, (2) 0.1, (3) 0.2, (4) 0.5. [$AuCl_4^-$] = 1.0 mmol L^{-1}; [CTAB] = 0.2 mmol L^{-1} (concentrations are referred to the values in the syringes).

2.2. Optimization of Variables

The variables affecting the system were optimized following the univariate method. All reported concentrations are initial concentrations in each syringe, which are twice the final concentrations in the reaction mixture at time zero. The analytical parameter used was the difference obtained for the initial rate of the system in the presence and absence of GA. Each kinetic result was the average of at least six measurements. **Table 1** summarizes the variables studied, including the range assayed and the optimum values chosen.

Table 1. Optimization of variables

Type of variable	Variable	Range studied	Optimum value
Instrumental	Excitationwavelength (nm)	280 - 800	540
	Emission wavelength (nm)	280 - 800	540
	Ex/Em slits (nm)	2 - 20	5/5
	Power energy (V)	300 - 700	430
	Average time (s)	0.012 - 0.15	0.1
Stopped-flow device	Reaction volume (mL)	0.1- 0.5	0.3
	Temperature (°C)	10 - 50	25
Chemical	pH	2 - 6	4.0
	[HAc/Ac$^-$] Buffer (mmol L^{-1})	10 - 200	20
	[$AuCl_4^-$] (mmol L^{-1})	0.0 - 2.5	1.0
	[CTAB] (mmol L^{-1})	0.0 - 0.5	0.2

The initial rate was independent of the mixing cell volume, which was assayed in the range 0.1 - 0.5 mL, choosing a volume of 0.3 mL. The effect of the concentration of $AuCl_4^-$ was studied in the interval 0.0 – 2.5 mmol L^{-1} (**Figure 4.A**). As can be seen, the optimum concentration was close to 1.0 mmol L^{-1}. The influence of the temperature on the AuNPs formation was studied in the range 10 – 50 °C (**Figure 4.B)**, obtaining the maximum initial rate at 25 ºC. **Figure 4.C** shows the pH dependence of the system, which was studied in the range of 2 – 6. A pH of 4.0 was selected as the optimum value, using an acetic acid/sodium acetate buffer solution to adjust this pH. The study of the influence of the concentration of this buffer

on the process, assayed in the range of 10 – 200 mmol L^{-1}, showed that a concentration of 20 mmol L^{-1} concentration was suitable to adjust the pH. Several sodium chloride concentrations, in the range of 0-200 mmol L^{-1}, were assayed to evaluate the influence of the ionic strength on the system, but the results obtained showed that this variable does not modify the kinetic behaviour of the system.

Finally, the study of the influence of the CTAB concentration in the interval 0.0 – 0.5 mmol L^{-1} (**Figure 4.D**) showed that the optimum concentration was 0.2 mmol L^{-1}. Higher concentration values caused an increase of the blank signal, decreasing the net initial rate corresponding to GA.

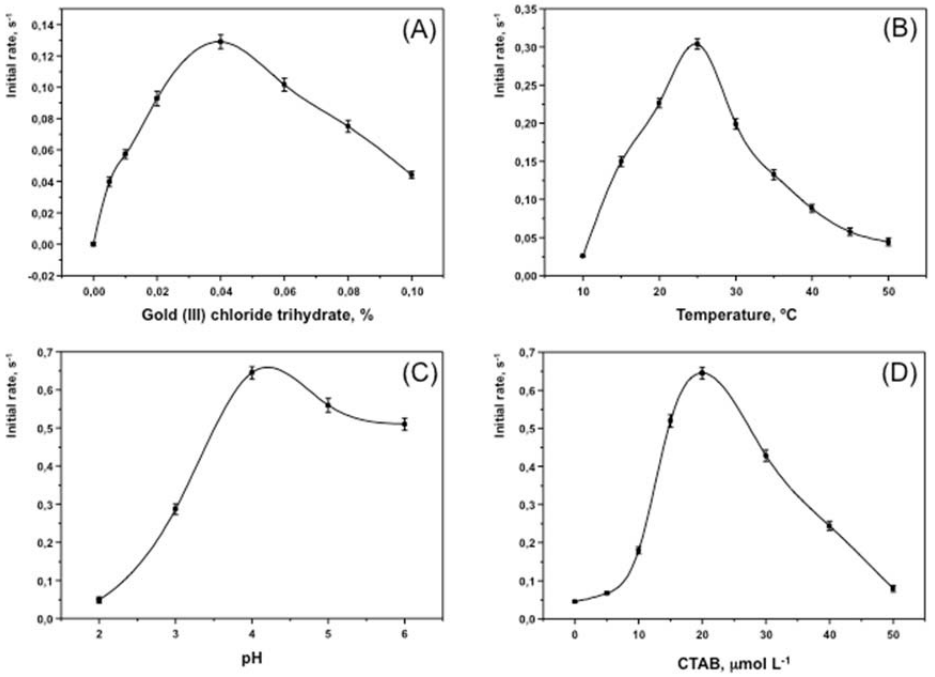

Figure 4. Study of the influence of $AuCl_4^-$ concentration (A), temperature (B), pH (C), and CTAB concentration (D) on the initial rate of the system in the presence of 0.5 μmol L^{-1} GA. [$AuCl_4^-$] = 1.0 mmol L^{-1} on Figures B, C and D; temperature = 25 ºC on Figures A, C and D; pH = 3 on Figures A and B, and 4 on Figure D; [CTAB] = 0.2 mmol L^{-1} on Figures A, B and C.

2.3. Analytical Features

Calibration graphs were run under the optimal experimental conditions summarized in **Table 1**, using the initial rate as the analytical parameter. Seven standard solutions containing different concentrations of each analyte (GA, PG, OG, DG, BHA, BHT, AA and SC), covering the range between 0.01 and 2 μmol L^{-1}, were assayed in triplicate. **Table 2** summarizes the figures of merit of the method, including the calibration equations, linear ranges, LODs and precision data. The LODs obtained, calculated according to IUPAC recommendations [28] ranged between 0.007 μmol L^{-1} for DG and 0.03 μmol L^{-1} for AA. The precision, expressed as relative standard deviation (RSD %) was studied at two concentrations of each analyte, which correspond to the minimal (centroid point) and maximal (close to the limits of quantification) errors of the dynamic calibration graphs. The RSD% values, obtained for n = 11 solutions, ranged between 0.6 and 4.8 %. The estimated sampling frequency under the working conditions was about 8 h^{-1}.

2.4. Application of the method

The proposed method was applied to the analysis of four commercial foodstuffs, including biscuits, doughnut, milkshake and soup stock cubes, to determine their corresponding antioxidant concentrations. The samples were analysed following the sampling procedure described in the experimental section. Each sample was extracted using the above mentioned official extraction process [25] and then adequately diluted with distilled water to match the linear range of the calibration graph for each analyte. The method was tested in two ways: 1) by determining the antioxidant concentration in the samples, and 2) by studying the recovery after addition of two standard aliquots of the corresponding analyte at two concentration levels which covered the linear range of the calibration graph. The results achieved are summarized in **Table 3**, which include the commercial name, the foodstuff type, the dilution factor, the concentrations obtained by both reference and proposed methods and the recovery data obtained at two concentration levels, which are depicted in bracket. The antioxidant content of the sample that contained two analytes (soup stock cubes) is expressed as PG concentration. As can be seen, the results obtained by both the proposed and reference methods are very similar. The

recovery percentages obtained, calculated by subtracting the results obtained from similar unspiked samples, ranged between 95.4 and 99.5 %.

3. Conclusions

This study shows for the first time the usefulness of kinetic methodology for monitoring the formation of AuNPs induced by antioxidant compounds in the presence of CTAB and its analytical usefulness. This approach has allowed the development of a kinetic method for the determination of GA, PG, OG, DG, BHA, BHT, AA and SC by measuring the variation of RLS signal with time. In contrast to equilibrium methods involving the presence of $AuCl_4^-$, the use of stopped-flow mixing technique does not require an incubation step, obtaining the analytical signal in few seconds. The results obtained show that the method can readily be adapted as a fast screening method to automatic quality control of antioxidant additives in foodstuffs.

Acknowledgements

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Table 2. Analytical features of the method

Analyte	Equation [1]	r^2	$\sigma_{y/x}$ [2]	Linear range (μmol L^{-1})	Detection Limit (μmol L^{-1})	RSD% [3]	
						low level	high level
Gallic Acid (GA)	y= (1.17 ± 0.04) x + 0.06 ± 0.005	0.9970	0.015	0.04 – 0.59	0.01	4.8	0.9
Propyl Gallate (PG)	y= (0.48 ± 0.01) x + 0.069 ± 0.002	0.9996	0.005	0.04 – 1.41	0.01	6.8	5.3
Octyl Gallate (OG)	y= (1.46 ± 0.06) x – 0.048 ± 0.006	0.9945	0.015	0.03 – 0.35	0.008	3.5	3.3
Dodecyl Gallate (DG)	y= (2.53 ± 0.03) x + 0.106 ± 0.006	0.9994	0.007	0.02 – 0.30	0.007	4.2	1.7
Butyl Hydroxyanisol (BHA)	y= (1.38 ± 0.03) x + 0.031 ± 0.004	0.9984	0.007	0,07 – 0.39	0.009	1.5	3.6
Butyl Hydroxytoluene (BHT)	y= (2.39 ± 0.13) x + 0.026 ± 0.010	0.9943	0.025	0.04 – 0.32	0.01	1.7	0.6
Ascorbic Acid (AA)	y= (0.82 ± 0.01) x + 0.113 ± 0.009	0.9991	0.016	0.11 – 1.72	0.03	2.6	3.4
Sodium Citrate (SC)	y= (3.67 ± 0.12) x + 0.055 ± 0.025	0.9976	0.028	0.07 – 1.29	0.02	2.9	1.9

(1) y denotes the analytical signal, and x is the analyte concentration (μmol L^{-1}), (2) residual standard deviation (or standard error of estimate), (3) relative standard deviation values achieved at two concentration levels of each analyte, corresponding to the maximal and minimal error zones in the calibration graphs (for more detailed see text).

Table 3. Application of the method

Sample Nº	Commercial Name	Foodstuff Type	Dilution Factor	Analyte	Reference Method		Proposed Method		
					Type(1)	Conc. Found (±SD)(2)	Conc. Found (±SD)(2)	Recovery % (3)	
								1st	2nd
1	Chiquilin	Biscuit	1:500	BHA	LC	100 ± 7	100 ± 7	98.9 (0.12)	98.8 (0.22)
2	Maggi	Soup stock cubes	1:250	PG BHA	LC LC	38 ± 6 72 ± 6	113,7 ± 0.3 (4)	99.2 (0.05)	99.1(0.19)
3	Choleck	Milkshake	1:250	AA	RT	34.68 ± 0.02	32.9± 0.2	99.2 (0.15)	97.9 (1.3)
4	Filipinos	Doughnut	1:500	BHA	LC	192 ± 3	191 ± 8	95.4 (0.12)	97.5 (0.16)

(1) Reference methods: LC, liquid chromatography [a] and RT, redox titration [b].
(2) Concentration in $\mu g\ g^{-1}$.
(3) Recoveries after the first and second addition, respectively [the concentration ($\mu mol\ L^{-1}$) corresponding to the amount added is shown in bracket].
(4) Expressed as PG concentration.

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Determination of polyphenolic content in beverages using laccase, gold nanoparticles and long wavelength fluorimetry

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An enzymatic fluorimetric method for the determination of polyphenol compounds in beverages is described, which is based on the temporal inhibition caused by these compounds on the oxidation of the long wavelength fluorophor indocyanine green (λ_{ex} 764 nm, λ_{em} 806 nm), in the presence of the enzyme laccase and positive charged gold nanoparticles (AuNPs). The oxidation of the dye gives rise to a fast decrease in its fluorescence, but it is delayed by the polyphenol, obtaining a time period directly proportional to its concentration, which has been used as the analytical parameter. The behaviour of several benzenediols and benzenetriols in the system and the modification of the activity of the enzyme by its interaction with AuNPs have been studied.

The system has been optimized using gallic acid as a polyphenol model, but the dynamic ranges of the calibration graphs and the detection limits for several of the polyphenols assayed were obtained (μmol L^{-1}): gallic acid (0.13-5, 0.04), catechol (0.08-5, 0.01), hydroquinone (0.05-2, 0.01), hydroxyhydroquinone (0.09-5, 0.03), pyrogallol (0.17-5, 0.04). Most of the values of the regression coefficients were 0.999 and the precision of the method, expressed as RSD% and checked at two concentration levels of each analyte, ranged between 1.8 and 5.6%. The method has been applied to the determination of polyphenol content in several foodstuff samples and the results compared with those obtained with the standard Folin-Ciocalteu method.

Introduction

The availability of suitable methods for the determination of polyphenolic compounds in food products is still a very active research topic, in spite of the high number of methods described up to date for this purpose [1,2]. This interest is justified by the lack of standardised methods and the widely described connection between the presence of these compounds in food products and their quality properties and health benefits [3].

Methods for the determination of polyphenolic compounds in food samples can be mainly classified in two groups: 1) methods focused to the individual identification and quantification of the constituents of a polyphenol mixture in a sample, which involve the use of a separation technique, such as chromatography [1,4] or capillary electrophoresis [5], and 2) methods aimed to determine collectively polyphenols, obtaining the total polyphenolic content. The choice of each type of methods depends on the objective of the analysis. Usually, the separation and identification of all polyphenols present in a food sample is a complex task owing to the high number of potential analytes and the differences in their concentration levels.

The second type of methods is mainly based on the antioxidant properties of polyphenolic compounds, which are due to the reactivity of hydroxyl groups on the benzene ring. The main limitation of these methods is that only an approximate estimation of the polyphenol content is obtained as the results are referred to a polyphenol model, which is chosen to obtain the calibration plot, but the antioxidant activity of these compounds depends on the position and number of hydroxyl groups on the benzene ring. The most representative method of this group is the photometric Folin-Ciocalteu assay, which involves the use of heteropolyphosphotungstate-molybdate in an alkaline medium and a reference standard polyphenol, such as gallic acid (GA) [6]. This method can lead to an overestimation of total polyphenols due to its poor selectivity as the reagent reacts with other reducing non-phenolic substances, such as ascorbic acid and reducing sugars, which are common food additives or are naturally present in the samples.

A group of methods devoted to the determination of total polyphenol content are based on the use of amperometric biosensors, which involve the immobilization of phenoloxidase enzymes, namely tyrosinase and laccase, on a suitable electrode. Polyphenols are enzymatically oxidized to quinones or radicals and then detected at the electrode by their reduction currents [7]. A limitation of these sensors is that the products can potentially electropolymerize to polyaromatic derivatives damaging the electrode surface and limiting the life time of the biosensor, being necessary to protect the surface of the biosensor [7]. Also, it has been described that the adsorption of oxidized products on the surface of the immobilization support often leads to enzyme inactivation phenomena [8]. Laccase has been described as the most appropriate enzyme for the detection of polyphenolic compounds [7,9], although biosensors based on the immobilization of both laccase and tyrosinase have also shown their usefulness [10,11].

Laccases belong to the so-called blue-copper family of oxidases characterized by containing four catalytic copper atoms. They have found several biotechnological applications in textile, food processing, pharmaceutical and chemical industries [12,13]. A feature of these enzymes is their capability to oxidize different substrates with the concomitant reduction of oxygen to water, avoiding the use of reagents such as hydrogen peroxide for their catalytic action. Several laccase-based amperometric biosensors have been described for the determination of polyphenols in wines [9,10,13], beers [7,11], tea infusions [14] and fruit juices [15] using gallic acid [7, 9-11,13], caffeic acid [14] or guaicol [15] as the polyphenol standard to obtain the calibration plot. The polyphenol content values obtained with these sensors are generally lower than that obtained by the Folin-Ciocalteu method.

In this work, an alternative method for polyphenol determination is described, which is based on the temporal inhibition caused by these compounds on the oxidation of the long wavelength fluorophor indocyanine green (**Figure 1**), also called cardio green, in the presence of laccase and gold nanoparticles (AuNPs). The behaviour of several polyphenols (**Figure 1**) on the system has been studied, choosing gallic acid as the standard for the optimization and development of the method, which has been applied to the determination of polyphenol content in several

beverage samples. The results obtained were compared with the standard Folin-Ciocalteu method.

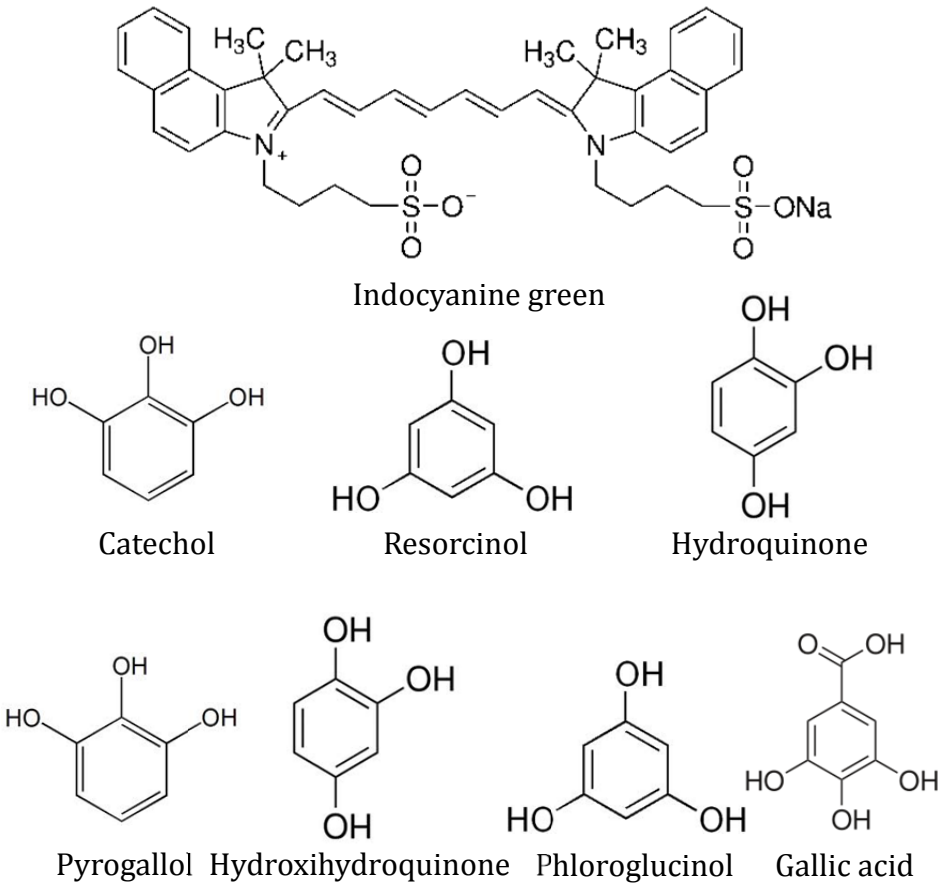

Fig 1. Chemical structures of indocyanine green and the polyphenolic compounds assayed.

1. Experimental

1.1. Apparatus and Instrument

An SLM Aminco (Urbana, IL) AB2 luminescence spectrometer provided with a 150 W continuous xenon lamp and a 7 W pulsed xenon lamp was used. The instrument was furnished with an RX-2000 Rapid Kinetic System Stopped-Flow mixing accessory from Applied Photophysics (Leatherhead, UK), which was fitted with an observation cell of 10 x 2 mm length, 60 μL of inner volume and controlled by the associated electronics, the computer and a pneumatic syringe drive system. The temperature of the solution was kept constant at 20ºC by circulating water from a Haake DC1 thermostatic bath (ThermoElectron, Karlsruhe, Germany).

1.2. Reagents

All chemicals used were of analytical reagent grade. 4-Dimethylaminopyridine, tetraoctylammonium bromide, $HAuCl_4$, $NaBH_4$ and Folin-Ciocalteu reagent were obtained from Sigma. Stock solutions (10 mmol L^{-1}) of the analytes were prepared as follows: gallic acid (GA) (Sigma) was dissolved in the minimum amount of ethanol; catechol (CA) (Sigma), hydroquinone (HY) (Aldrich), hydroxyhydroquinone (HH) (Aldrich), phloroglucinol (PG) (Sigma), pyrogallol (PY) (Sigma-Adrich) and resorcinol (RE) (Sigma-Aldrich) were prepared in water. Stock and intermediate solutions were stored at 4 ºC in the dark and were stable for at least one week, except for PY which had to be prepared daily. Working standard solutions (5 and 100 μmol L^{-1}) were prepared from intermediate solutions by their dilution in water. A stock solution (150 u.a./mL) of laccase (from *Trametes versicolor*) (Sigma) was prepared daily in tris(hydroxymethyl)aminomethane (Tris) buffer solution at pH 7,5. One unit of laccase is defined as the amount of enzyme that transforms 1 μmol of catechol per minute at pH 4.5 and 25ºC. Positive charged AuNPs were synthesized [16] and stored at 4 ºC in the dark and were stable for at least three weeks. A stock solution (130 μmol L^{-1}) of indocyanine green (Sigma) was prepared daily by its dissolution in water. All aqueous solutions were prepared using water purified with a Milli-Q system (Millipore, Bedford, Ma, USA).

1.3. Manifold and procedure

A stopped-flow mixing device was used for the rapid and automatic mixture of the reactants, which is shown in **Figure 2**. A pre-mixed solution containing laccase (0.1 u.a. mL^{-1}), positive charged AuNPs (39,4 µmol L^{-1}) and 25 mmol L^{-1} Tris buffer solution (pH = 7.5), which was let stand for at least two hours, was used to fill one of the two driving syringes of the stopped-flow module. The second syringe was filled with a pre-mixed solution containing sample or standard and indocyanine green (5.2 µmol L^{-1}) solutions, prepared in the same buffer solution. The pneumatic syringe control device allowed the automation of each injection. In each run, 0.15 mL of each solution was mixed in the mixing chamber of the stopped-flow module and the variation of the fluorescence signal of indocyanine green with time was monitored at λ_{ex} = 764 and λ_{em} = 806 nm using a spectrofluorimeter. Each standard or sample solution was assayed at least four-times. All measurements were carried out at the selected optimal temperature of 20 ± 0.1 °C.

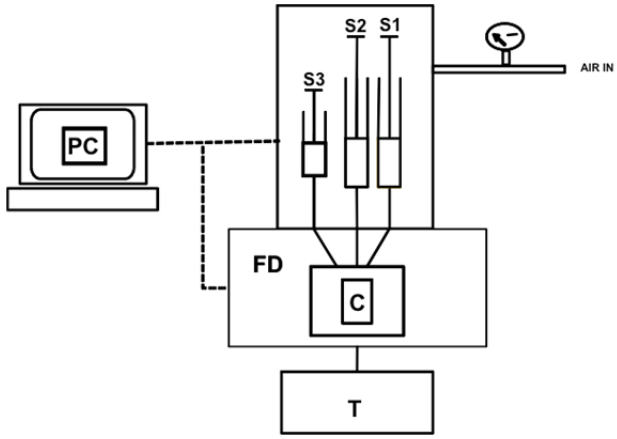

Figure 2. Scheme of the instrumental system: S1 and S2, driving syringes containing laccase + AuNPs and indocyanine green + polyphenolic compound, respectively; S3, stopping syringe; FD, spectrofluorimeter; C, observation cell; T, thermostat; PC, computer.

Data were acquired and processed using the software incorporated in the spectrofluorimeter for treatment of kinetic measurements. The analytical parameter used to quantify the analyte was the difference in the time necessary, expressed in seconds, to observe a decrease on the fluorescence signal in the presence and absence of the analyte, as **Figure 3** shows**.** The concentration of phenolic compounds in the samples was determined by interpolation in the calibration graph obtained for GA, which was chosen as the analyte model.

1.4. Synthesis of negative and positive charged gold nanoparticles

Negative charged AuNPs were synthesized by the method reported by Turkevich [17]. Briefly, 5 mg of tetrachloroauric acid trihydrate was dissolved in 47,5 mL of deionized water in a round-bottom flask and trisodium citrate dehydrate (1.7 mM) was added drop wise in two minutes and then was vigorous stirred under reflux at 100 ºC for 30 min. The final aqueous solution, containing approximately 0.25 mM of AuNps, was kept stable in refrigerator at 4 ºC until use. The size of these AuNPs ranged from 20 to 30 nm.

Positive charged AuNPs were synthesized by the method reported in the literature [16] as follows: a 30 mM aqueous tetrachloroauric acid was added to a 25 mM solution of tetraoctylammonium bromide in toluene (80 mL). Then a 0.4 M solution of freshly prepared $NaBH_4$ was added to the stirred mixture, which caused an immediate reduction to occur. After 30 min, the two phases were separated and the toluene phase was subsequently washed with 0.1 M H_2SO_4, 0.1 M NaOH, and water (three times), and then dried over anhydrous Na_2SO_4. After that, 0.1M 4-dimethylaminopyridine (DMAP) solution was added to the toluene phase. Direct phase transfer across the organic/aqueous boundary was completed within 1h. The size of these AuNPs was from 5 to 15 nm.

1.5. Analysis of foodstuff samples

Commercial juice and tea samples were processed to determine their corresponding polyphenol content. Juice samples only required their direct dilution with distilled water. For tea samples, an infusion of 2,5 g tea bag was prepared in 50 mL of hot distilled water. The extracts were stored

at 4 ºC in the dark. The solutions were diluted in order to fit the linear range of the calibration graph, and were treated as described above. Each determination was carried out in triplicate, using in each instance the mean of the data obtained from at least four measurements.

1.5.1. Reference method

Each sample was tested by the Folin-Ciocalteu method [6] which is the AOAC official method for the determination of total polyphenolic compounds. Briefly, the procedure was as follows: The analyte standard or the sample solution was mixed with 0.25 mL of the Folin Ciocalteu reagent and the mixture was let to react for a few minutes. After, 1 mL of Na_2CO_3 saturated solution was added and the mixture was risen up to a final volume of 5 mL. After 2 h, the absorbance at 765 nm was measured. The linear calibration graph for GA as the analyte model was obtained by plotting the difference obtained for the absorbance values in the presence and absence of the analyte versus the analyte concentration. The polyphenol content in the samples was determined by interpolation in the calibration graph.

2. Results and discussion

2.1. Study of the chemical system

The chemical system used to develop the proposed method is based in the capacity of polyphenolic compounds to delay the oxidation of the long wavelength fluorophor indocyanine green in the presence of laccase. This fluorophor was chosen with the aim of establishing a fluorimetric method in which the potential background signals from the sample matrix are avoided, as they can appear at lower wavelengths. As can be seen in **Figure 3**, when the fluorophor is mixed with laccase, its fluorescence shows a fast decrease, which can be ascribed to the catalytic effect of the enzyme on the oxidation of the dye by dissolved oxygen. However, this effect is delayed in the presence of a polyphenol compound, such as GA, obtaining an apparent induction period that is directly proportional to the concentration of the polyphenol. These results show the sequential catalytic effect of the enzyme, which acts over the fluorophor when the polyphenol has been oxidized. **Figure 3** also shows that the slope of the

kinetic curves obtained in the absence and in the presence of the polyphenol has practically the same value, inferring that the kinetics of the oxidation of the dye are not affected by the oxidation product of the first reaction. It has been described that AuNPs can decrease the activity of lacasse in a homogeneous medium owing to their interaction [18]. With the aim of studying the potential effect of these NPs on the system, several assays were carried out using negative and positive charged AuNPs. The first type of NPs did not modify the kinetics of the system, but an increase on the induction time was found in the presence of positive charged AuNPs, as **Figure 3** shows. This effect can be ascribed to a decrease in the enzymatic activity of laccase due to its electrostatic interaction with the NPs, as the assays were carried out at pH 7.5, in which the enzyme is negatively charged (isoelectric point = 3.5) [19]. The study of the time necessary to attain a suitable laccase-NPs interaction showed that the results were satisfactory and reproducible when the reagent mixture was prepared at least two hours before its use.

The behaviour of several di- (CA, HY, RE) and tri-phenols (HH, PG, PY, GA) on the system was studied with the aim of checking its potential application to the determination of polyphenols. The results obtained showed that the compounds having the hydroxyl groups in position orto or para gave a similar response, as described below, but those in position meta, such as RE and PG, gave a lower response. Also, it was found that the presence of the carboxylic group in GA had only a slight effect on the kinetics of the system as the response obtained was similar to that obtained for PY.

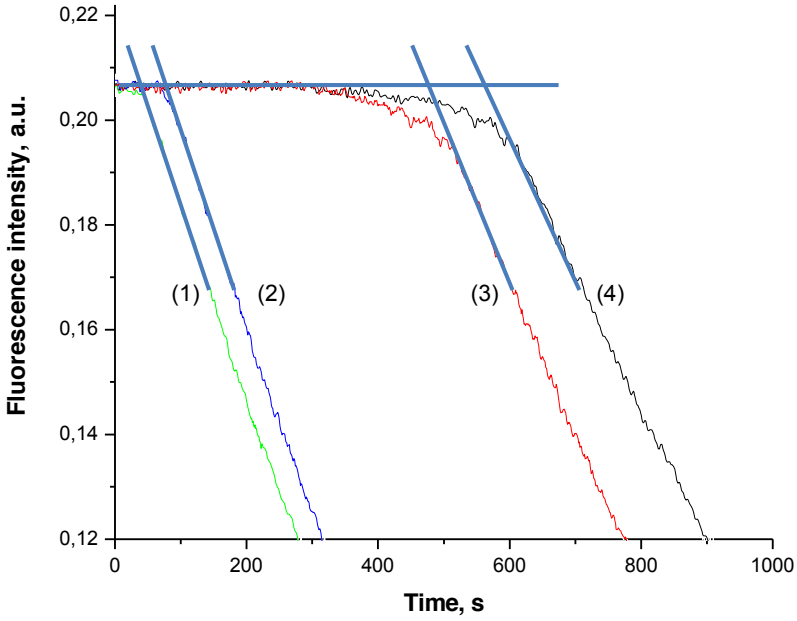

Figure 3. Kinetic curves obtained for the laccase-indocyanine green system alone (1) and in the presence of AuNPs (2), GA (3) and AuNPs + GA (4). [laccase] = 0.1 u.a. mL^{-1}, [indocyanine green] = 5.2 µmol L^{-1}, [GA] = 2 µmol L^{-1}, temperature = 20 ºC, pH = 7.5 and [Tris] = 25 mmol L^{-1}.

2.2. Optimization of variables

The variables affecting the system were optimized following the univariate method. All reported concentrations are initial concentrations in each syringe, which are twice the final concentrations in the reaction mixture at time zero. As indicated above, the analytical parameter used was the difference in the time necessary to observe a decrease in the fluorescence of indocyanine green in the presence and absence of GA, which was called induction time and expressed in seconds. Each result was the average of at least four measurements. **Table 1** summarizes the

variables studied, including the range assayed and the optimum values chosen.

The mixing cell volume chosen was 0.3 ml, as this variable had not any influence on the system in the range 0.1 - 0.5 mL. The pH dependence of the system was studied in the range of 6 – 8.5 because the indocyanine green solution was no stable at pH lower than 6.0, showing a decrease in its fluorescence intensity, whereas the laccase solution was unstable at pH above 8.5, as its catalytic effect through the decrease of the dye fluorescence was not observed. A pH 7.5 was selected as the optimum value, using a Tris buffer solution adjusted at this pH value. The study of the influence of the concentration of this buffer on the process, assayed in the range of 10 – 200 mmol L^{-1}, showed that a 25 mmol L^{-1} concentration was suitable to adjust the pH. Several sodium chloride concentrations, in the range of 0-200 mmol L^{-1}, were assayed to evaluate the influence of the ionic strength on the system, but the results obtained showed that this variable did not modify the behaviour of the system. The effect of the concentration of positive charged AuNPs on the system was studied in the interval 0.0 – 52.4 µmol L^{-1} (**Figure 4.A**), obtaining an optimum concentration close to 40 µmol L^{-1}. Laccase activity was a critical variable, as can be seen in **Figure 4.B**, which shows its influence on the system in the interval 0.0 – 1.66 u.a. mL^{-1}. The optimum value was close to 0.1 u.a. mL^{-1}. **Figure 4.C** shows that the optimum indocyanine green concentration, assayed in the interval 0.0 – 20.8 µmol L^{-1}, was 5.2 µmol L^{-1}. The effect of the temperature on the system was studied in the range 10 – 40 °C, obtaining only a slight decrease on the analytical parameter up to a temperature of 22ºC, but it was sharper at higher temperatures. Thus, a temperature of 20ºC was chosen.

Table 1. Optimization of variables

Type of variable	Variable	Range studied	Optimum value
Instrumental	Excitation wavelength (nm)	500 – 800	764
	Emission wavelength (nm)	780 – 850	806
	Ex/em slits (nm)	4 – 16	4/4
	Power energy (V)	400-800	700
	Average time (s)	0.012 – 0.15	0.1
Stopped-flow device	Reaction volume (mL)	0.1 – 0.5	0.3
	Temperature (ºC)	5 – 40	20
Chemical	pH	2 – 9	7.5
	[Tris] buffer (mmol L^{-1})	10 – 200	25
	[AuNPs] (µmol L^{-1})	0.0 – 52.4	39.4
	[indocyanine green] (µmol L^{-1})	0.0 – 20.8	5.2
	[laccase] (u.a. mL^{-1})	0.0 – 1.66	0.1

2.3. Analytical features

Calibration graphs for five polyphenols (CA, HY, HH, GA, PY) were run under the optimal experimental conditions summarized in **Table 1**, using the induction time above defined as the analytical parameter, and assaying each standard in triplicate. **Table 2** summarizes the figures of merit of the method, including the calibration equations, linear ranges, LODs and precision data. The LODs obtained, calculated according to IUPAC recommendations [20], ranged between 0.01 and 0.04 µmol L^{-1}. These values are lower than most of the LODs described for GA using amperometric biosensors, which range between 0.19 and 50 µmol L^{-1} [7,10,11,13]. The LODs were also studied in the absence of AuNPs, with the aim of checking their influence on the features of the method, obtaining values about three-times higher. The study of the effect of ascorbic acid (2.0 µmol L^{-1}) on the system showed that the analytical parameter was not affected. The precision, expressed as relative standard deviation (RSD %) was studied at two concentrations of each analyte, 0.2 and 1 µmol L^{-1}. The values (n = 11) ranged between 1.8 and 5.6 %.

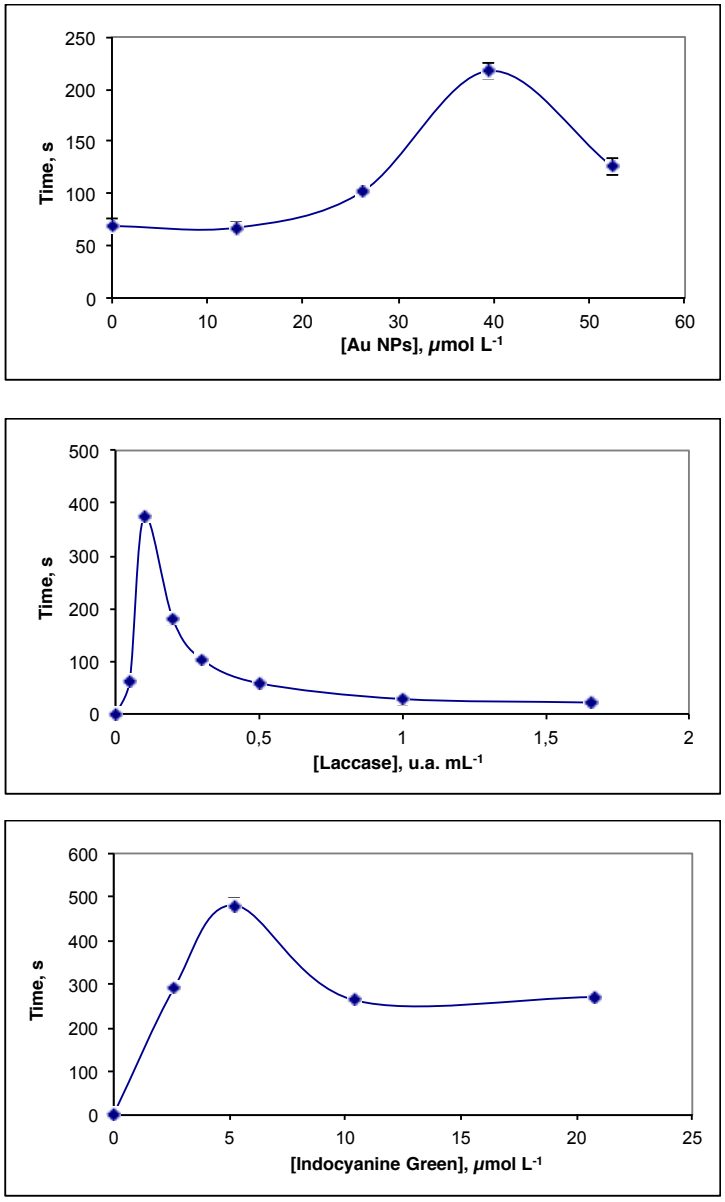

Figure 4. Study of the influence of AuNPs (A), laccase (B) and indocyanine green (C) concentrations on the analytical parameter in the presence of 2 µmol L^{-1} GA. [AuNPs] = 40.3 µmol L^{-1} on Figures B and C; [laccase] = 0.5 u.a. mL^{-1} on Figure A and 0.1 u.a. mL^{-1} on Figure C; [indocyanine green] = 10.4 µmol L^{-1} on Figure A and B; temperature = 20 ºC, pH = 7.5 and [Tris] = 25 mmol L^{-1} in all instances.

Table 2. Analytical features of the method

Analyte	Equation [1]	r^2	$\sigma_{y/x}$ [2]	Linear range (µmol L^{-1})	Detection Limit (µmol L^{-1})	RSD% [3]	
						low level	high level
Catechol	y=(246 ± 2) x + (4 ± 2)	0,998	0.33	0.08 – 5	0.01	4.4	5.5
Hydroquinone	y=(583 ± 3) x – (4 ± 2)	0.999	0.50	0.05 – 2	0.01	3.6	5.6
Hydroxyhydroquinone	y=(92.8 ± 0.7) x + (13.3 ± 0.8)	0.999	0.11	0.09 – 5	0.03	3.5	1.8
Gallic Acid	y=(227 ± 2) x + (15 ± 3)	0.999	0.76	0.13 – 5	0.04	3.9	5.4
Pyrogallol	y=(163 ± 1) x + (23 ± 2	0,999	0.54	0.17 – 5	0.04	3.5	4.6

[1] y denotes the analytical signal, and x is the analyte concentration (µmol L^{-1}).

[2] Residual standard deviation (or standard error of estimate).

[3] Relative standard deviation values achieved at two concentration levels of each analyte (0.2 and 1 µmol L^{-1}).

Table 3. Application of the method

Sample	Commercial name	Foodstuff type	Reference Method	Proposed Method		
			Conc. Found (±SD)[1]	Conc. Found (±SD)[1]	Recovery % [2]	
					1st	2nd
1	Sunny Delight	Orange juice	394 ± 80	344 ± 116	90.0	100.2
2	Salobreña	Grape juice	166 ± 2	159 ± 17	89.9	97.1
3	Alteza Tea	Green tea	31 ± 2	24± 8	91.5	99.9
4	Twinings Tea	Cinnamon tea	58 ± 2	48 ± 6	90.4	97.3

[1] Polyphenols measured as GA, concentration in mg L^{-1} for juices and mg g^{-1} for teas (2) Recoveries after the first (0,2 μmol L^{-1}) and second addition (1 μmol L^{-1}), respectively.

2.4. Application of the method

The proposed method was applied to the analysis of four commercial foodstuffs, orange juice, red grape juice and teas, to determine their corresponding polyphenol content, which was expressed as GA concentration. The samples were analysed following the procedure above described. Each sample was diluted with distilled water to match the linear range of the calibration graph and analysed using both the proposed and the Folin-Ciocalteu official methods. The method was tested in two ways: 1) by determining the polyphenolic concentration in the samples, and 2) by studying the recovery after addition of two standard aliquots of GA at two concentration levels (0.2 and 1μmol L^{-1}) which covered the linear range of the calibration graph. The results achieved are summarized in **Table 3**, which include the commercial name of the samples, the foodstuff type, the concentrations obtained by both reference and proposed methods and the recovery data. As can be seen, the results obtained by the proposed method are slightly lower than those obtained by the reference method, which could be ascribed to the lower selectivity of the later. The recovery percentages, calculated by subtracting the results obtained from similar unspiked samples, ranged between 89.9 and 100.2 %.

3. Conclusions

The new approach described, involving kinetic methodology, long wavelength fluorescence measurements and AuNPs, has shown its usefulness for the determination of polyphenol compounds in food samples. Stopped-flow mixing technique is used for the fast and automatic mixture of the reactants, which simplify the development of the method. Although the consume of enzyme is higher than in the methods involving its immobilization, the enzyme amount required for each measurement is very low owing to the high sensitivity of the method. It has been also shown that the activity of the enzyme is decreased by its interaction with AuNPs, which allows the improvement of the LODs of the method. The values obtained are lower than those obtained in most of the methods based on amperometric biosensors [7,10,11,13]. Also, the use of long wavelength fluorimetry avoids potential background signals from the sample matrix, which can appear at lower wavelengths.

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CAPITULO 4

NUEVAS INVESTIGACIONES CON NANOPARTICULAS DE ORO

1. Introducción

Con objeto de adquirir nuevos conocimientos sobre la síntesis y caracterización de las nanopartículas de oro (AuNPs) y sobre el uso de técnicas instrumentales en las que se utilizan estas NPs, se realizó una estancia breve de tres meses en el "Institute of Analytical Chemistry, Chemo- and Biosensors" de la Universidad de Regensburg (Baviera, Alemania), bajo la tutela del Profesor Otto Wolfbeis.

La realización de esta estancia ha permitido también cumplir uno de los requisitos necesarios para poder optar a la Mención de Doctorado Europeo.

2. Objetivos

Se plantearon los dos objetivos siguientes:

1) Síntesis de AuNPs a partir de antioxidantes de uso alimentario.
2) Desarrollo de un sensor selectivo de AuNPs basado en la resonancia del plasmón de superficie (SPR).

3. Conocimientos adquiridos

3.1. Síntesis de nanopartículas de oro

Entre todos los métodos convencionales de síntesis de AuNPs basados en la reducción de derivados de oro(III), el más popular de ellos ha sido él que emplea como agente reductor citrato sódico sobre $HAuCl_4$ en medio acuoso, introducido por Turkevich en 1951 [1]. Es un método sencillo que requiere solamente un equipo de reflujo donde se calienta y mantiene a una temperatura constante una disolución de $HAuCl_4$, a la que se adiciona una cantidad adecuada de una disolución de citrato sódico. La disolución inicialmente amarilla va virando a azul-violeta y finalmente, después de unos 30 minutos, presenta coloración roja intensa debido a la presencia de AuNPs de unos 20 nm de diámetro. Posteriormente en 1973 Frens intentó obtener nanopartículas de un tamaño predeterminado (entre

16 y 147 nm) controlando su formación. Para ello propuso un método donde la relación citrato trisódico – Au(III) iba variando según el diámetro deseado. En 1999 Kunitake desarrolló un método para obtener AuNPs estabilizadas por 3-mercaptopropionato de sodio, en donde se produce la adición simultanea de la sal de citrato y de un surfactante anfifílico (**Figura 1**); el tamaño de la nanopartícula puede ser controlado mediante la variación de la relación estabilizador/oro [2].

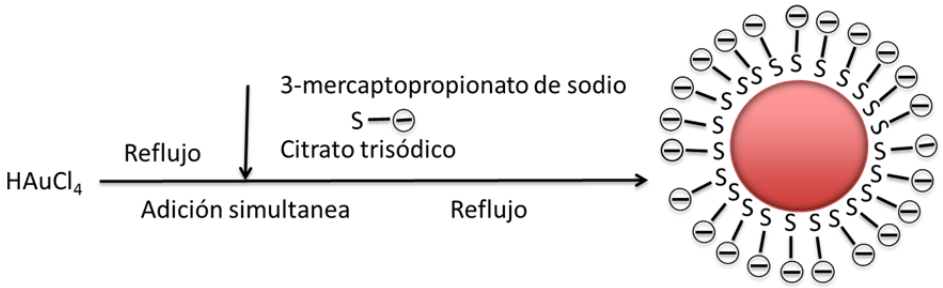


Fig 1. Síntesis de Kunitake.

En la investigación realizada se utilizó la síntesis de Turkevitch ya que es muy sencilla y reproducible. Las nanopartículas fueron caracterizadas mediante fotometría y microscopía TEM.

3.2. Técnica de resonancia del plasmón de superficie (SPR)

Desde su primera observación a principios del siglo XX, el fenómeno físico de la resonancia del plasmón de superficie (SPR) ha encontrado un lugar destacable en aplicaciones de tipo práctico tales como el desarrollo de detectores sensibles, que responden a la adsorción de macromoléculas sobre su superficie. Posteriormente se observó un patrón "anómalo" de bandas oscuras y claras en la luz reflejada, cuando se hizo incidir un haz de luz polarizada en un espejo con una red de difracción en su superficie. La interpretación física del fenómeno fue iniciada por Rayleigh, pero no fue hasta 1968, cuando Otto, Kretschmann y Raether descubrieron la excitación de los plasmones de superficie [3,4]. La aplicación de sensores

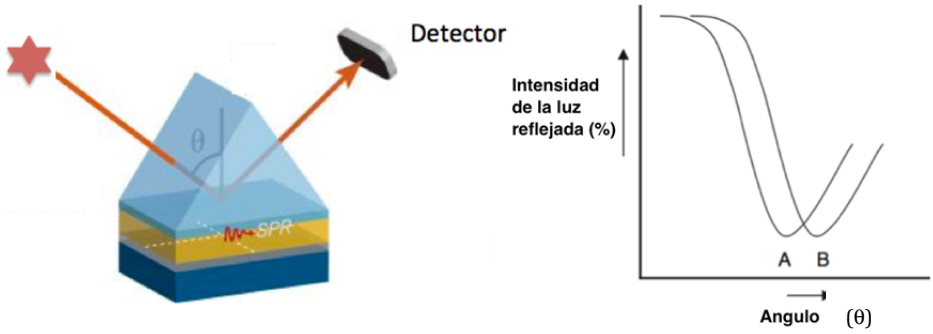
basados en SPR para monitorizar interacciones biomoleculares fue demostrada por primera vez en 1983 por Liedberg [5].

Para explicar la excitación de los plasmones de superficie, puede utilizarse el montaje experimental de la **Figura 2**. Un haz de luz polarizada se hace incidir a través de un prisma sobre un chip sensor recubierto con una delgada película de oro, la cual actúa como un espejo reflejando la radiación. Al ir cambiando el ángulo de incidencia y monitorizar la intensidad de la luz reflejada, ésta alcanza un valor mínimo (**Figura 2, línea A**), cuando la radiación incidente excita el plasmón de superficie induciendo su resonancia. Los fotones de la luz p-polarizada pueden interactuar con los electrones libres de la película metálica, produciendo oscilaciones en forma de ola de los electrones libres y reduciendo de este modo la intensidad de la luz reflejada.

El ángulo al cual ocurre la máxima pérdida de la intensidad de la radiación reflejada se conoce como ángulo de resonancia o ángulo SPR. Este ángulo depende de las características ópticas del sistema, tales como los índices de refracción de los medios situados a ambos lados de la película de oro. Mientras se mantiene constante el índice de refracción en el lado del prisma, se producirá un cambio en el índice de refracción en la superficie del metal cuando se deposite algún tipo de masa sobre ella (por ejemplo proteínas). Por lo tanto, las condiciones a las que se produce la resonancia del plasmón de superficie pueden cambiar, produciéndose un desplazamiento del ángulo SPR (**Figura 2, línea B)**, que puede utilizarse para obtener información de la cinética de un proceso, por ejemplo la adsorción de proteínas en la superficie del metal.

La técnica SPR es una opción excelente para monitorizar los cambios del índice de refracción en las inmediaciones de la superficie del metal. La **Figura 3** muestra la variación del ángulo al cual se obtiene el mínimo con el tiempo, representación a la que se le denomina sensorgrama. Cuando el índice de refracción cambia, el ángulo al cual se obtiene el mínimo de intensidad se desplazará, como puede observarse en dicha figura, donde (A) muestra la representación real de la intensidad de la luz reflejada frente al ángulo incidente y (B) es la representación después del cambio en el índice de refracción. El proceso de adsorción-desorción puede ser monitorizado en tiempo real y las cantidades de las especies

adsorbidas pueden ser determinadas. La técnica SPR no sólo es adecuada para medir la diferencia entre estos dos estados, si no que también se puede monitorizar el cambio en el tiempo, si se monitoriza el tiempo que dura el desplazamiento del ángulo de resonancia al cual el mínimo es observado.



(θ) **Figura 2**. Montaje experimental SPR. Un chip sensor con un recubrimiento de oro es colocado en un prisma. La fuente de luz (estrella) emite una radiación polarizada e incide sobre el chip sensor. La intensidad de la luz reflejada es medida en el detector (disco). A un determinado valor del ángulo de incidencia (θ) , ocurre la excitación del plasmón de superficie, lo que conlleva una disminución en la intensidad de la luz reflejada (A). Un cambio en el índice de refracción en la superficie de la película de oro causará un desplazamiento en el ángulo de A a B.

Si el cambio observado es debido a una interacción molecular, la cinética de esta interacción puede ser medida en tiempo real. Los sensores SPR miden solamente una región o volumen muy limitado en la superficie del metal. La profundidad de la penetración del campo electromagnético (también llamado campo evanescente) en la película de oro a la cual se observa la señal, normalmente no excede unos pocos cientos de nanómetros.

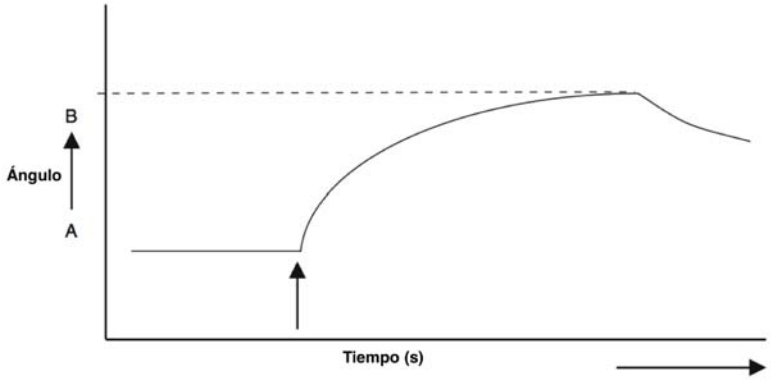


Figura 3. Esquema de un sensorgrama en el que se monitoriza el ángulo al cual el mínimo es observado frente al tiempo. Al principio no ocurre ningún cambio en el sensor obteniendo una línea de base para el mínimo en el ángulo SPR (A). Después de la inyección de una muestra (flecha) las biomoleculas se adsorberán en la superficie resultando un cambio en el índice de refracción y un desplazamiento en el ángulo SPR hacia la posición B.

Una limitación de los sensores SPR es su falta de selectividad intrínseca ya que todos los cambios en el índice de refracción en el campo evanescente serán reflejados en un cambio en la señal. Estos cambios en el índice de refracción pueden deberse, por ejemplo, a variaciones en la composición o concentración de la disolución reguladora. También, la adsorción de material sobre la superficie del sensor puede afectar al índice de refracción. La cantidad de especies adsorbidas puede ser determinada después de la inyección de la disolución reguladora inicial que originó la línea de base, como se muestra en la **Figura 3**. Para conseguir la detección selectiva en un sensor de SPR, su superficie debe ser modificada con ligandos adecuados que reaccionen o interaccionen selectivamente con los compuestos de interés, pero que no faciliten la adsorción de otros componentes presentes en la muestra o de la disolución reguladora.

En el caso más simple de una medida de SPR, el analito es capturado por el ligando, por ejemplo un anticuerpo (**Figura 4**), el cual está inmovilizado en la superficie del sensor previamente a la medida. Existen numerosos sensores con diferentes ligandos inmovilizados que son comercialmente disponibles, aunque también se puede inmovilizar el ligando fácilmente en el laboratorio. En el caso más sencillo, denominado

detección directa, la captura de la sustancia analizada por el ligando da lugar a una señal medible. La **Figura 5** muestra la señal del sensor paso a paso en el ciclo de medición con detección directa.

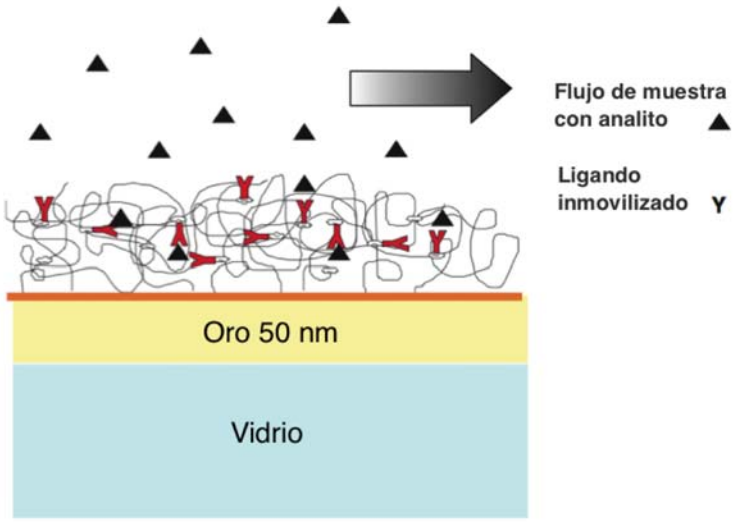


Figura 4. Representación esquemática de la detección directa: el analito es capturado por los ligandos (Y) inmovilizados en la superficie del sensor, por ejemplo, un hidrogel. La acumulación de los analitos da lugar a un cambio del índice de refracción en el campo evanescente cambiando el ángulo de SPR.

Cada medida se inicia con el acondicionamiento de la superficie del sensor utilizando una disolución reguladora adecuada (1). Un aspecto crítico es la disponibilidad de una referencia fiable antes de que se inicie la medida. En este punto, la superficie del sensor contiene los ligandos activos, preparados para capturar los analitos. Al inyectar la solución que contiene los analitos (2), éstos son capturados en la superficie. Aunque otros componentes de la muestra podrían adherirse a la superficie del sensor, podrían eliminarse fácilmente ya que no existiría un enlace selectivo. En este paso, la cinética de adsorción del analito puede ser determinada en tiempo real. A continuación, se inyecta disolución reguladora en el sensor y los componentes que no están enlazados selectivamente son desplazados (3). Como se indica en la **Figura 5**, la cantidad acumulada de analito se puede determinar mediante la respuesta

del SPR (ΔR). También se puede producir en este paso la disociación de la sustancia analizada, lo que permite estudiar la cinética del proceso de disociación. Finalmente, se inyecta una disolución de regeneración, la cual rompe la unión selectiva entre el analito y el ligando (4). Si se ha hecho una adecuada inmovilización de los ligandos, éstos permanecerán en el sensor, mientras que los analitos son cuantitativamente eliminados. Esta etapa es básica para realizar múltiples medidas con el mismo chip sensor ya que la utilización de una disolución regenerante que mantenga a los ligándos activos permitirá realizar cientos e incluso miles de medidas. La etapa de regeneración mediante la disolución regeneradora permite acondicionar la superficie del sensor para el siguiente análisis. Si la regeneración es incompleta, la masa acumulada provocará un aumento en el nivel de referencia.

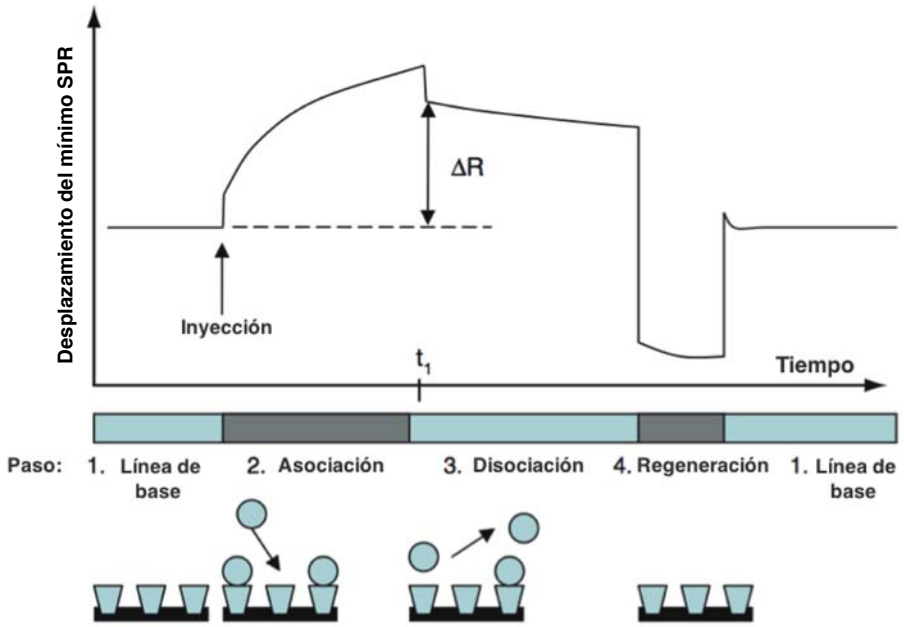


Figura 5. Sensorgrama que muestra las etapas de un ciclo de análisis: 1, la disolución acondicionadora está en contacto con el sensor (línea de base); 2, inyección de la disolución de la muestra (asociación); 3, inyección de la disolución reguladora (disociación); ΔR indica la respuesta medida debido al analito; 4, inyección de la disolución regenerante para la eliminación de los analitos retenidos (regeneración), y preparación del sistema para otro ciclo de medida. Como puede observarse, en t_1 se produce un cambio en el índice de refracción.

Frecuentemente, las mediciones de SPR se utilizan para estudiar la cinética de un proceso de enlace, por lo que es básico para obtener resultados reales que el proceso de inmovilización del ligando no afecte a su afinidad hacia el analito.

Además de la realización de estudios cinéticos y termodinámicos, las medidas SPR también pueden ser utilizadas para la determinación de la concentración de un analito en una muestra (análisis cuantitativo). En este caso, en primer lugar se debe obtener la calibración midiendo las señales para distintas concentraciones de disoluciones estándar del analito. Los resultados obtenidos al superponer los diferentes sensorgramas se muestran en la **Figura 6**, en la que se observa que las mesetas debidas a la etapa de asociación van aumentando al aumentar la concentración del analito [6]. Se puede construir una recta de calibrado representando la respuesta analítica (ΔR) después de un cierto intervalo de tiempo (t_1) frente a la concentración.

Para determinar la concentración desconocida de un analito en una muestra pueden realizarse diluciones secuenciales, por ejemplo por 10, 100 y 1000. Para determinaciones más precisas pueden utilizarse diluciones que varíen en un factor de 2. Si la concentración del analito en la muestra es muy alta, la muestra sin diluir dará resultados por encima de la zona superior de la recta de calibrado. Las disoluciones diluidas, sin embargo, darán lugar a señales situadas en la zona inferior de la recta de calibrado y se podrá determinar la concentración del analito.

Como se mencionó anteriormente, la técnica SPR detecta los cambios que se producen en el índice de refracción en la superficie del sensor debidos a la cantidad de masa depositada. La detección directa sólo es posible si la retención del analito produce cambios medibles del índice de refracción. Esta situación se consigue con mayor facilidad si el peso molecular de la sustancia determinada es elevado (alrededor de 1000 Da o superior). Sin embargo, para moléculas pequeñas, sería necesario una cantidad elevada de las mismas para producir un cambio medible del índice de refracción, por lo que el análisis sería poco sensible. Si el analito es una molécula pequeña (peso molecular<1000 Da), la detección se realiza mediante un formato de ensayo competitivo o de inhibición de tipo sándwich. En todos los formatos de ensayo, no sólo la mínima

concentración detectable es limitada, sino también el número físico de los elementos que pueden ser inmovilizados en la superficie del sensor, lo que proporciona un valor límite máximo.

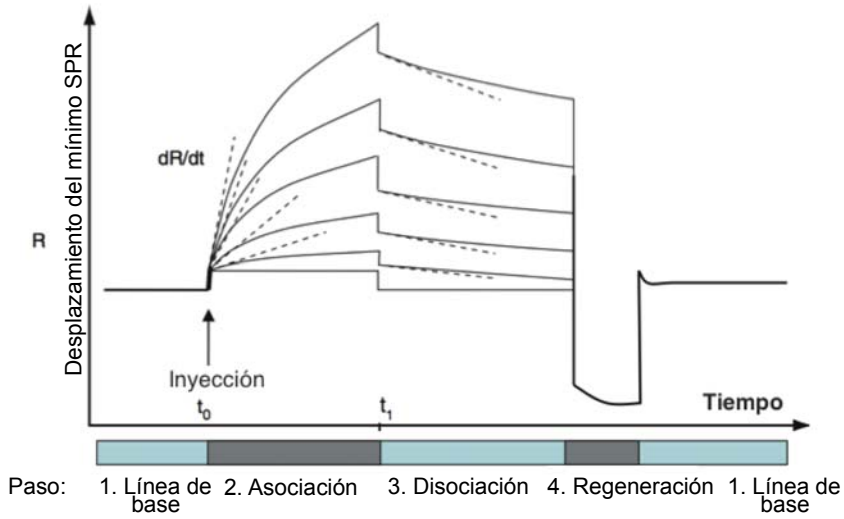


Figura 6. Sensorgramas obtenidos para distintas concentraciones de analito. Justo después de la inyección en t_0 se produce un enlace selectivo de la sustancia analizada. El transporte de masa a la superficie es limitante de la velocidad y depende linealmente de la concentración. La medida de las pendientes (dR / dt) permite construir la recta de calibrado y, por tanto, puede determinar la concentración de analito en una muestra. La fase de disociación comienza en t_1, al inyectar la disolución disociante, provocando la ruptura del enlace analito-ligando. Los datos obtenidos en este proceso pueden utilizarse para determinar la constante de disociación (k_d).

4. Trabajo experimental

La primera etapa del trabajo experimental realizado consistió en aprender a sintetizar AuNPs de tamaño controlado utilizando procedimientos bien establecidos, así como a utilizar la técnica SPR, aspectos en los que el grupo de investigación del Profesor Wolfbeis tiene una gran experiencia. Posteriormente se procedió a desarrollar los dos objetivos planteados que consistieron en:

1) Investigar la posibilidad de conseguir la síntesis de AuNPs mediante procedimientos alternativos a los ya establecidos.
2) Utilizar la técnica SPR para la cuantificación de AuNPs.

Aunque el breve tiempo disponible no permitió completar satisfactoriamente estos objetivos, cabe destacar que el trabajo experimental realizado ha contribuido positivamente a la formación científica del doctorando.

A continuación se describen brevemente las investigaciones realizadas.

4.1. Síntesis de nanopartículas de oro a partir de antioxidantes de uso alimentario.

Partiendo de la síntesis de Turkevich [1], donde se emplea citrato sódico cómo reductor del $HAuCl_4$ para la formación de nanopartículas de oro, se planteó la posibilidad de utilizar otro tipo de reductores para llevar a cabo esta síntesis. Como muestra la **Figura 7**, la utilización de este reductor permite obtener AuNPs con máximo de absorción a unos 520 nm **(Figura 7.1)** y con un tamaño medio de partícula próximo a 20 nm **(Figura 7.2)**. Como reductores alternativos se ensayaron antioxidantes alimentarios, tales como las sales sódicas de los galatos de propilo (E-310), octilo (E-311) y dodecilo (E-312), butilhidroxianisol (BHA) y butilhidroxitolueno (BHT).

Se realizaron diferentes ensayos con todos estos compuestos para poder obtener disoluciones monodipersas de nanopartículas de oro de tamaño controlado.

Se utilizaron cantidades variables de $HAuCl_4$ y antioxidantes, modificando también la temperatura y el tiempo de sintesis. En ninguno de los ensayos realizados se consiguieron resultados satisfactorios. No obstante, en algunos casos se observó la formación de nanopartículas de tamaño relativamente grande, así como su agregación, ya que los espectros de absorción obtenidos presentaban bandas situadas a longitudes de onda

mayores que la banda característica obtenida para una disolución con nanopartículas dispersadas. Asimismo, las disoluciones presentaban una coloración violeta-azulada, indicativo de su elevado tamaño.

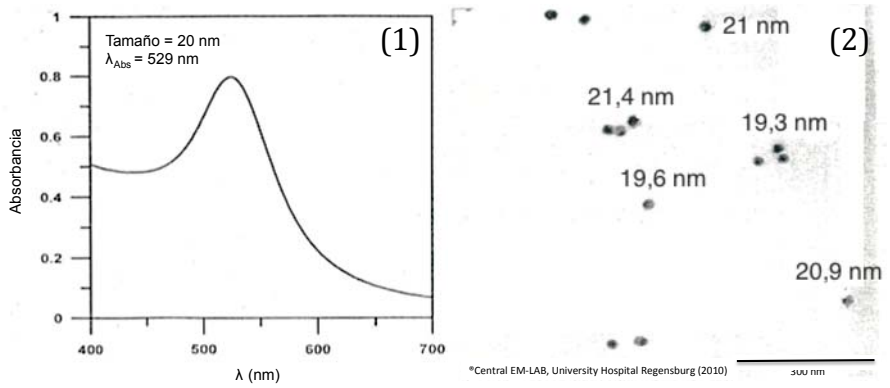


Figura 7. Espectro de absorción (1) e imagen TEM obtenida por un microscopio electrónico Leo 912 AB (2) de AuNPs mediante una síntesis de Turkevich empleando citrato sódico como agente reductor.

4.2. Sensor SPR

Con objeto de desarrollar un sensor SPR sensible a AuNPs, se investigó la posibilidad de que la albúmina de suero bovino (BSA) formara un enlace selectivo con las AuNPs. La primera etapa de este estudio fue la inmovilización de BSA en la superficie de la lámina de oro del sensor. Para ello se deposito una fina capa de ácido 16-mercaptohexadecanoico ($SH(CH_2)_{15}COOH$), de forma que los grupos carboxílicos interaccionasen con la BSA. Una vez anclados los grupos carboxílicos a la lámina de oro, se pasó una corriente de 1-etil-3-(dimetilaminopropilcarbodiimida) (EDC), reactivo que se usa generalmente como un agente activador de grupos carboxílicos para el acople con aminas primarias que conduzcan a enlaces amida. Al pasar una corriente de BSA se produjo su inmovilización quedando preparado el sistema para estudiar su respuesta frente a las AuNPs.

Las AuNPs como era de prever, se enlazaron a BSA, dando mayores señales al aumentar su concentración, obteniendo así la correspondiente recta de calibrado. Sin embargo, el enlace BSA-AuNPs era demasiado fuerte,

ya que la señal del SPR apenas disminuía al pasar corrientes de lavado con agua o NaCl. La regeneración se intentó también haciendo pasar diferentes corrientes de disoluciones reguladoras de pHs entre 1,5 y 10,1, pero sin resultado satisfactorio.

El siguiente paso de la investigación fue el estudio del comportamiento del sistema en ausencia de BSA. Aunque las señales obtenidas fueron menores, también variaron con la concentración de AuNPs dando lugar a una recta de calibrado con un intervalo dinámico y coeficiente de regresión similar a los obtenidos en presencia de BSA, aunque la reproducibilidad de las medidas fue peor.

Este hecho pone de manifiesto la existencia de enlaces inespecíficos cuando se utilizala BSA. Se ensayó una alternativa más selectiva para enlazar las AuNPs, usando cisteína, un α-aminoácido con grupo tiol afín al oro. El estudio se inició recubriendo la superficie de la lámina de oro del SPR con una capa de HS-$(CH_2)_{15}$-COOH y HS-$(CH_2)_{15}$-CH_3, en diferentes proporciones para detectar la posible existencia de enlaces inespecíficos. Una vez seleccionada la más adecuada, se activaron los grupos carboxilo con EDC para su unión con la N-hidroxisulfosuccinimida (Sulfo-NHS) y por último se enlazó la cisteína que sería la encargada de retener a las AuNps. Sin embargo no se obtuvo ningún resultado satisfactorio ya que no se pudo inmovilizar la cisteína adecuadamente.

Se ensayó una estrategia alternativa a la utilizada en la experiencia anterior, en la que se hacía fluir una corriente de cisteína sobre la lámina de oro del sensor durante unos minutos. Considerando que probablemente la cinética de la reacción fuera lenta y no se produjera el enlace debido al caudal de la corriente, se utilizó un compuesto con doble grupo tiol (HS-(C_{15})-COONHS), de tal manera que por un extremo se uniera a la lámina de oro y el otro extremo tiol quedase libre para reaccionar con la cisteína. En esta ocasión en vez de pasar una corriente de cisteína, la lámina se sumergió en la disolución durante 3 h, para favorecer así la fijación de la cisteína sobre la superficie del sensor (**Figura 8**).

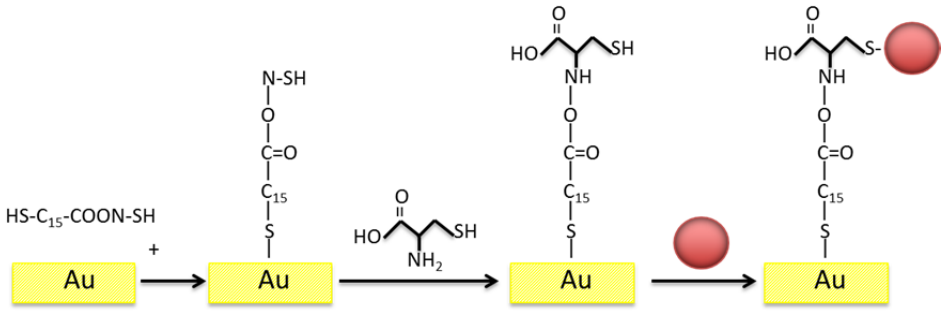


Figura 8. Esquema de la estrategia seguida para la inmovilización de Au-NPs

Las bajas señales SPR obtenidas pusieron de manifiesto que las AuNps no se inmovilizaban en la cisteína o que la interacción no se producía porque la cinética fuera lenta. Incluso pasando la disolución de nanopartículas a caudales bajos no se mejoró la señal. Como alternativa, en lugar de pasar una corriente de nanopartículas, se dejó un pequeño volumen de disolución en contacto con uno de los canales del sensor durante 48 horas, mientras que el otro canal se dejó como referencia. Si existiera depósito de nanopartículas se detectaría comparando los mínimos de resonancia de ambos canales. Lamentablemente las medidas dieron valores casi idénticos, lo que indicó que no existe inmovilización.

La falta de respuesta del sistema ensayado podría atribuirse a que, al inmovilizar el compuesto con doble grupo tiol, ambos tioles terminales se enlazaran a la lámina de oro, impidiendo su interacción con las AuNPs.

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CAPITULO 5

DISCUSIÓN DE LOS RESULTADOS
DISCUSSION OF THE RESULTS

Introducción

En este capítulo se aborda una discusión detallada de los resultados obtenidos a lo largo de esta Tesis Doctoral. Se pondrán de manifiesto los puntos fuertes y débiles de las metodologías desarrolladas así como la comparación con otras metodologías empleadas en el ámbito científico para la misma finalidad.

Este capítulo se divide en dos secciones, discutiendo en la primera los resultados obtenidos con las metodologías desarrolladas basadas en la separación cromatográfica, derivatización y detección fluorescente, y en la segunda las metodologías automáticas no cromatrográficas propuestas mediante el uso de la técnica de flujo detenido y nanopartículas.

1. Metodologías basadas en separación cromatográfica, derivatización postcolumna y detección fluorescente

En este bloque se encuadran los diferentes métodos cromatográficos desarrollados para la determinación de compuestos fenólicos en muestras de origen alimentario. El objetivo general de estos métodos fue el establecimiento de nuevas metodologías que permitieran mejorar las propiedades analíticas básicas (precisión, selectividad y sensibilidad) y complementarias (rapidez, costes y factores personales) de los métodos ya existentes, de forma que los nuevos métodos propuestos puedan ser una alternativa real para la determinación de estos compuestos de gran interés en el control de la calidad alimentaria.

Para alcanzar el objetivo propuesto se ha utilizado la cromatografía de líquidos como técnica de separación y la luminiscencia como sistema de detección. No obstante, la innovación de los métodos propuestos radica en los diversos sistemas de derivatización post-columna empleados en cada uno de ellos para poder dotar a los analitos (carentes de fluorescencia nativa) de propiedades luminiscentes. Así, se han desarrollado:

- Un método de análisis que emplea un sistema de derivatización post-columna basado en la formación de quelatos luminiscentes

de los analitos con terbio(III) en medio ligeramente básico y en presencia de TOPO y AEDT.

- Un método de análisis que hace uso de un sistema de derivatización basado en la capacidad de los analitos para formar complejos estables con aluminio(III) y terbio(III) en un medio submicelar originado por SDS.

- Un método de análisis basado en un sistema de derivatización donde los analitos interaccionan con cerio(IV) y un fluoróforo de larga longitud de onda.

Todas las metodologías desarrolladas en esta sección comparten una serie de elementos comunes que aportan diversas ventajas frente a otros métodos de análisis ya establecidos.

En primer lugar, la columna empleada para la separación en los tres métodos propuestos es una columna monolítica C_{18}, como alternativa a las columnas de fase reversa empaquetadas con micropartículas de sílice más frecuentemente utilizadas en la separación de compuestos fenólicos. El uso de este tipo de columnas permite trabajar con valores de caudal de fase móvil más altos sin que ello repercuta negativamente en la presión ejercida en el sistema. Por tanto, ofrece la posibilidad de trabajar con volúmenes de inyección de muestra relativamente elevados, obteniendo una mejor señal analítica ya que no se produce el ensanchamiento de los picos cromatográficos sino que se obtienen picos más estrechos y altos. Otra ventaja que aporta este tipo de columnas es la posibilidad de la inyección directa de la muestras o, en todo caso, la utilización de una baja dilución ya que la regeneración de la columna puede hacerse a una elevada velocidad.

En segundo lugar, el detector empleado en todos los casos es un espectrofluorímetro para obtener señales analíticas a larga, o relativamente larga, longitud de onda utilizando un fluoróforo adecuado o la formación de quelatos luminiscentes de los analitos con terbio(III), respectivamente. La detección en esta zona del espectro permite minimizar posibles interferencias debidas a la matriz de la muestra, ya

que suelen emitir a menores longitudes de onda. Además, la selectividad que ofrece la luminiscencia sensibilizada facilita la identificación de algunos compuestos que no pueden ser identificados fácilmente usando detección fotométrica.

A continuación se discutirá en mayor profundidad los resultados obtenidos para cada una de las metodologías desarrolladas.

Determinación de compuestos fenólicos en vino mediante luminiscencia sensibilizada de terbio.

Este método permite la determinación simultánea, empleando doble detección fotométrica y fluorimétrica, de 18 compuestos fenólicos diferentes entre los que se encuentran ácidos hidroxibenzoicos e hidroxicinámicos, catequinas, aldehídos fenólicos, flavonoides y estilbenos. El método se basa en la separación cromatográfica y derivatización post-columna de los analitos para transformar a diez de ellos en derivados fluorescentes. La derivatización utiliza formación de quelatos solubles y estables de los analitos-ligandos con terbio(III), en los que el ligando se excita absorbiendo la radiación y, a continuación, transfiere la energía absorbida al lantánido, el cual finalmente la emite en forma de energía luminosa.

Las ventajas del método presentado frente a otros métodos previamente descritos son las siguientes:

- Las derivadas del uso de una columna monolítica C_{18}, que se han comentado con anterioridad.
- La relativa larga longitud de onda a la que emite el terbio(III) puede minimizar posibles interferencias existentes en la matriz de la muestra, que suelen producirse a menores longitudes de onda. Además, la selectividad de la luminiscencia sensibilizada puede facilitar la identificación de algunos compuestos que no pueden detectarse fácilmente mediante fotometría.
- El tratamiento de muestra es prácticamente nulo, requiriendo sólo la dilución de la muestra.
- Se ha descrito por primera vez el uso de terbio(III) como reactivo para la determinación de aldehídos aromáticos.

Estas características han posibilitado la separación cromatográfica en menor tiempo que el requerido en otros métodos descritos para estos compuestos fenólicos, separando 15 compuestos fenólicos en muestras de vino en tan sólo 25 minutos. Por el contrario, otros métodos [1-2] que emplean columnas empaquetadas logran la separación de 14 a 25 fenoles en 60 minutos, o 32 compuestos en 90 minutos. También ha permitido mejorar los límites de detección, como se muestra en la Tabla 1, donde se comparan los límites de detección obtenidos frente a los descritos en otros métodos previamente publicados [2-3]. Se puede observar que en el 40% de los valores comparados el límite de detección fluorimétrico es menor que el fotométrico, por lo que no solamente la luminiscencia sensibilizada ayuda a aumentar la selectividad si no también la sensibilidad del método. Al comparar el método propuesto con otros métodos se puede concluir que se mejoran los niveles de detección fotométricos y fluorimétricos en la mayoría de los casos, poniendo de manifiesto la utilidad del método desarrollado.

Tabla 1. Comparación de los LODs entre diferentes métodos

Analito	Método propuesto		[2]	[3]	
	Fotometría LOD ($ng\ mL^{-1}$)	Fluorimetria LOD ($ng\ mL^{-1}$)	Fotometría LOD ($ng\ mL^{-1}$)	Fotometría LOD ($ng\ mL^{-1}$)	Fluorimetria LOD ($ng\ mL^{-1}$)
Ácido gálico	3	5	14	60	-
Ácido protocateuico	36	2	18	120	-
Ácido p-hidroxibenzoico	22	15	-	-	-
Ácido salicílico	14	14	-	-	-
Ácido vainíllico	30	17	11	60	68
Ácido siríngico	10	3	3	50	3
Catequina	8	114	51	110	93
Vainillina	19	40	-	-	-
Siringaldehído	3	15	-	40	-
Epicatequina	8	29	55	340	31

Aunque este método presenta diversas ventajas, también tiene algunas limitaciones, siendo probablemente la más destacable la imposibilidad de conseguir que todos los analitos ensayados originen derivados fluorescentes. Sólo los ácidos hidroxibenzoicos, catequinas y

aldehídos fenólicos han presentado fluorescencia, siendo imposible bajo las condiciones experimentales fijadas en el método que los ácidos hidroxicinámicos, flavonoides y estilbenos ensayados formaran derivados fluorescentes.

Determinación de compuestos flavonoides en zumos de naranja mediante luminiscencia sensibilizada de terbio.

El método propuesto permite determinar cinco flavonoides en zumos de naranja realizando su separación cromatrográfica y posterior derivatización mediante la formación simultanea de quelatos con los iones aluminio(III) y terbio(III) para obtener luminiscencia sensibilizada. La intensidad de estos quelatos es mayor que la obtenida utilizando sólo terbio(III), lo que puede atribuirse a que el aluminio(III) origina un efecto de co-luminiscencia [5].

Entre las innovaciones que presenta este método cabe destacar que es la primera vez que se describe el uso de una columna monolítica para la separación de estos compuestos en zumos de naranja y la formación simultanea de quelatos de dos iones metálicos para obtener luminiscencia sensibilizada. El aporte de estas innovaciones repercute directamente en las ventajas del método: 1) no se requiere una etapa previa de tratamiento de muestra, 2) el volumen de inyección de muestra (200μL) es superior al de otros métodos, y 3) el sistema de derivatización desarrollado permite obtener señales luminiscentes más intensas que las obtenidas previamente utilizando sólo terbio(III) como reactivo derivatizante [4].

En la **Tabla 2** se muestra la comparación de los límites de detección obtenidos con el método propuesto con los descritos en otros métodos ya establecidos [6, 7], donde se puede comprobar la excelente mejora de los mismos. Así, el límite de detección para la naringina es 1250 veces menor en el nuevo método que en el descrito utilizando detección fotométrica [6], y 1000 veces menor que con detección electroquímica [7]. Para el kaempferol se ha obtenido un límite de detección que es 1250, 500 y 100 veces menor que los descritos utilizando detección electroquímica, masas y fotométrica, respectivamente [7].

Tabla 2. Comparación de los LODs entre diferentes métodos

Analito	Método Propuesto LC-FD	[6]	[7]		
			LC-UV	LC-MS	LC-ED
	LOD (ng mL^{-1})	LOD (ng mL^{-1})	LOD (ng mL^{-1})		
Naringina	1.0	1250	250	150	1000
Hesperidina	2.7	1000	300	100	500
Quercetina	1.0	2500	150	200	300
Naringenina	1.5	-	-	-	-
Kaempferol	0.8	-	80	400	1000

Aunque este método presenta diversos aspectos positivos, los resultados podrían haber sido mejores si se hubiera podido extender a la determinación de un mayor número de compuestos fenólicos. También hubiera sido deseable la utilización de mayores caudales de fase móvil en la separación cromatográfica, pero el caudal se tuvo que adaptar a las características del sistema de derivatización que presentaba problemas de presión. No obstante, el tiempo necesario para la separación es menor que el requerido utilizando las tradicionales columnas de fase reversa.

Determinación de flavonoides en zumos de naranja mediante el uso de fluorescencía de larga longitud de onda.

Mediante este método se han podido determinar seis de los flavonoides más representativos presentes en los zumos de naranja mediante su separación cromatográfica y posterior derivatización utilizando cerio(IV) y el fluoróforo de larga longitud de onda violeta de cresilo.

El uso de fluoróforos de larga longitud de onda como reactivos es una opción interesante para mejorar la selectividad del análisis fluorimétrico y para evitar o minimizar la etapa del tratamiento de muestra en el proceso analítico. Estos fluoróforos permiten realizar medidas en una región del espectro electromagnético (>600 nm) donde la absorción o emisión de la matriz de la muestra es mínima. Además, la interferencia Raman es en gran medida evitada y la probabilidad de

procesos de inhibición no radiantes se reduce debido a la corta duración de la fluorescencia de estos fluoróforos.

Al igual que en los métodos anteriores, se trabaja con una columna monolítica disminuyendo la duración de la separación, la cual se consigue en unos 12 minutos, frente a los 35 minutos requeridos en otros métodos [8]. El análisis de zumos requiere frecuentemente recurrir a procesos de tratamiento de muestra como extracción, hidrólisis, centrifugación y/o filtración [6-7,9-12]. Sin embargo, en el método presentado destaca la inexistencia del tratamiento de muestra, salvo procesos de dilución, lo que disminuye la complejidad y duración global del proceso analítico. La comparación de los límites de detección obtenidos frente a los descritos para otros métodos, mayoritariamente fotométricos, propuestos para determinar estos compuestos (**Tabla 3**), permite comprobar la notable mejora lograda mediante el nuevo método ya que en todos los casos se consiguen límites de detección muy inferiores. Esta disminución varía entre 700 veces menor para naringina y 7 veces menor para naringenina.

Tabla 3. Comparación de los LODs entre diferentes métodos.

Analito	Método Propuesto LC-FD	[8] LC-UV	[6] LC-UV	[7] LC-UV	[7] LC-MS
	LOD (ng mL^{-1})				
Rutina	1.5	40	-	-	-
Naringina	1.8	30	1250	250	150
Hesperidina	3.0	-	1000	300	100
Quercetina	3.7	100	2500	150	200
Naringenina	2.0	14	-	-	-
Kaempferol	1.0	-	-	80	400

Una limitación del método propuesto ha sido la imposibilidad de compatibilizar el sistema de derivatización con caudales mayores de fase móvil en la separación cromatográfica. También hubiera sido deseable ampliar el método a la determinación de un mayor número de compuestos fenólicos. Se ensayaron diversos ácidos fenólicos e hidroxicinámicos como los ácidos gálico, cafeico y p-coumarico, pero no se consiguió una separación cromatográfica adecuada ya que se eluían con el frente de disolvente.

Comparación general de los métodos desarrollados

Una vez comparados individualmente cada uno de los tres métodos cromatográficos propuestos con otros métodos previamente descritos, se realiza a continuación la comparación de las características principales de estos métodos entre sí, ya que todos ellos implican separación, derivatización y detección fluorimétrica de analitos similares. Para simplificar la comparación se han numerado los métodos según el sistema derivatizante utilizado, como se indica a continuación:

- Método 1: terbio(III)
- Método 2: aluminio(III) y terbio (III)
- Método 3: cerio(IV) y violeta de cresilo

A) Proceso de separación

En la **Tabla 4** se comparan el número de analitos, la duración, el número de componentes de la fase móvil y el caudal utilizado en cada caso. Como puede observarse, el número de analitos separados en el método 1 es muy superior al de los otros dos métodos, aunque sólo 10 de los 18 analitos originan respuesta fluorescente. En los métodos 2 y 3 se han separado los mismos analitos pero incluyendo en el método 3 a la rutina. Es de destacar que la separación en este método se realiza en la mitad de tiempo que en el método 2. Aunque los componentes de la fase móvil son los mismos en ambos métodos (disolución reguladora de ácido acético (pH 4), acetonitrilo y metanol), el gradiente de elución utilizado en el método 3 disminuye notablemente la duración del proceso de separación. En lo referente al caudal de fase móvil, éste es bastante mayor en el método 1.

Tabla 4. Comparación de las características de la separación

Separación	Método 1	Método 2	Método 3
Nº analitos	18	5	6
Tiempo de separación (min)	25	25	12
Nº disolventes en la fase móvil	2	3	3
Caudal de fase móvil [mL min^{-1}]	2	0.7	0.7

B) Sistema de derivatización

En la **Tabla 5** se muestran los distintos componentes del sistema derivatización utilizado en cada método, en el que se incluye en todos los casos la presencia de agentes surfactantes. También se incluyen los caudales utilizados, siendo menor en el método 2 y bastante similares en los otros dos métodos.

El método 1 es el más complejo ya que implica el uso de un mayor número de reactivos en el sistema de derivatización. El AEDT fue utilizado principalmente para evitar la precipitación del terbio(III), debido al elevado pH utilizado, aunque también se comprobó que mejoraba la señal del ácido gálico. El uso de surfactantes se justifica principalmente por su efecto protector en la luminiscencia de los quelatos con terbio(III), aunque el Triton X-100 también aumentó la señal del ácido gálico, mientras que el SDS fue necesario para conseguir aumentar la señal luminiscente del siringaldehído, la cual fue muy baja en su ausencia.

En el método 2 no se requirió el uso de AEDT ya que el pH utilizado fue bastante menor (4.0) que en el método 1, por lo que no existía riesgo de que se produjera la precipitación del terbio(III). Además, se comprobó que su presencia no mejoraba las señales luminiscentes de los analitos. Tampoco fue necesario el uso de TOPO como agente sinergético pero, de los distintos surfactantes ensayados, el SDS mejoró notablemente las señales analíticas a nivel de concentración sub-micelar.

El sistema derivatizante utilizado en el método 3 permite la realización de las medidas a mayor longitud de onda que en los métodos anteriores, debido al uso del fluoróforo violeta de cresilo, lo que confiere al método de una mayor selectividad frente a la matriz de la muestra, como se ha discutido anteriormente. La obtención de picos cromatográficos negativos es el resultado de la inhibición que causan indirectamente los analitos en la fluorescencia del fluoróforo debido a una combinación de procesos redox, ácido-base e hidrofóbicos. Cabe destacar que el caudal utilizado en este sistema es bastante mayor que en el método 2, lo que es atribuible a que las cinéticas de los procesos implicados en la derivatización son más rápidas que en los procesos de co-luminiscencia

que requiere la formación de los quelatos de los analitos con aluminio(III) y terbio(III).

Tabla 5. Comparación del sistema de derivatización

Derivatización	Método 1	Método 2	Método 3
Componentes empleados	Tb(III) AEDT TOPO TRITON X-100	Tb(III) Al(III) SDS	Violeta de cresilo CTAB Ce(IV)
Caudal de disolución derivatizante (mL min^{-1}]	0.8	0.5	0.9

C) Limites de detección y precisión

La **Tabla 6** recoge los límites de detección obtenidos para los analitos determinados en los tres métodos. Aunque no se puede hacer una comparación directa de los resultados obtenidos, ya que la mayoría de los analitos del método 1 no coinciden con los de los otros dos métodos, es de destacar que en todos los casos se alcanzan límites de detección muy bajos, a niveles de ng mL^{-1}, aunque éstos son en general más elevados en el método 1. Para este método sólo se han incluidos los obtenidos mediante detección fluorimétrica, a excepción de los correspondientes a rutina, quercetina y kaempferol, obtenidos mediante detección fotométrica, ya que éstos también están incluidos en los otros dos métodos. Como puede observarse, los límites de detección para estos analitos son bastante menores cuando se utiliza detección fluorimétrica.

Tabla 6. Comparación de límites de detección

Analito	Método 1 LOD (ng mL^{-1})	Método 2 LOD (ng mL^{-1})	Método 3 LOD (ng mL^{-1})
Ácido gálico	5	-	-
Ácido protocateuico	2	-	-
Ácido p-hidroxibenzoico	15	-	-
Ácido salicílico	14	-	-
Ácido vainíllico	17	-	-
Ácido siríngico	3	-	-
Catequina	114	-	-
Vainillina	40	-	-
Siringaldehído	15	-	-
Epicatequina	29	-	-
Rutina	24*	-	1.5
Naringina	-	1.0	1.8
Hesperidina	-	2.7	3.0
Quercetina	60*	1.0	3.7
Naringenina	-	1.5	2.0
Kaempferol	18*	0.8	1.0

*Detección fotométrica

Si se comparan los resultados de los métodos 2 y 3, donde se han utilizado los mismos analitos, a excepción de la rutina, se observa que prácticamente se alcanzan los mismos valores de los límites de detección, aunque son ligeramente más bajos en el método 2.

En lo referente a la de precisión de los tres métodos, expresada como %RSD, estos valores oscilan entre 0,08-6,4%, 1,3-4,7% y 2,8-6,2% para los métodos 1, 2 y 3, respectivamente. Aunque en el método 1 se alcanzan valores bastante bajos en algunos casos, en general no existen diferencias muy significativas entre ellos.

Como resumen de la comparación de los tres métodos cromatográficos descritos en esta Memoria, y considerando que cada uno de ellos tiene sus correspondientes ventajas y limitaciones, pueden deducirse las conclusiones siguientes:

1) Los bajos límites de detección obtenidos en los tres casos permiten el análisis directo de las muestras mediante su adecuada dilución.
2) El método 1 es el que presenta mayor resolución debido al elevado número de analitos separados, aunque el sistema de derivatización es más complejo y los límites de detección son más elevados.

3) Los mejores límites de detección se obtienen con el método 2, pero la separación es más lenta que en el método 3.
4) El método 3 es el que utiliza probablemente un sistema de derivatización más original y que permite conseguir una separación más rápida.

Método quimiométrico de clasificación

Como consecuencia de la información analítica obtenida en el método propuesto para la determinación compuestos fenólicos en vinos mediante luminiscencia sensibilizada de terbio, se ha desarrollado un método de clasificación de vinos mediante técnicas quimiométricas basado en el contenido en compuestos fenólicos, variedad de vino y origen geográfico.

La matriz de datos construida con la concentración de cada uno de los compuestos fenólicos hallados en cada muestra ha sido sometida a diferentes algoritmos matemáticos para caracterizar y clasificar adecuadamente las muestras de vino. Se ha hecho uso de métodos supervisados (análisis discriminante lineal) y no supervisados (análisis del factor) de pautas.

Se han descrito diversos métodos de clasificación de vinos. Como ejemplos caben citar dos métodos que requieren un número elevado de compuestos fenólicos, 19 o más, y que utilizan el origen geográfico y la variedad de uva [13] o el año de cosecha [14], obteniendo en ambos casos un 95% de efectividad en la clasificación de vinos. En el método propuesto se ha demostrado que, aunque el método de análisis aporta información sobre 18 analitos utilizando detección fotométrica y sobre 10 de ellos utilizando detección fluorimétrica, sólo con esta última información se puede establecer un procedimiento quimiométrico que alcanza un 100% de efectividad para la clasificación de las muestras.

2. Metodologías automáticas no cromatográficas basadas en el uso de la técnica de flujo detenido y nanopartículas

En este apartado se comentan y discuten los resultados obtenidos en el establecimiento de dos métodos para la determinación de compuestos fenólicos y otros antioxidantes en muestras de alimentos. En ambos métodos se ha utilizado la técnica de flujo detenido para automatizar la mezcla de los reactantes y la obtención de resultados analíticos. El objetivo básico de este estudio ha sido el desarrollo de nuevas metodologías de análisis introduciendo en el sistema analítico el uso de nanomateriales y/o enzimas que permitan mejorar las propiedades analíticas de los métodos ya existentes. Los métodos establecidos utilizan dos parámetros cinéticos, velocidad inicial de la reacción y periodo de inducción, para la cuantificación de los analitos mediante medidas de radiación dispersada de resonancia en el primer caso y de fluorescencia de larga longitud de onda en el segundo. Así, se han desarrollado:

- Un método para la determinación de antioxidantes mediante medida directa de la formación de nanopartículas de oro (AuNPs) utilizando la radiación dispersada de resonancia como sistema de detección.
- Un método enzimático-fluorimétrico para la determinación de polifenoles basado en la inhibición temporal que ejercen estos compuestos en la oxidación de un fluoróforo de larga longitud de onda.

Determinación de aditivos antioxidantes en muestras alimentarias mediante la medida directa de la formación de nanopartículas de oro por dispersión de la radiación.

La determinación de antioxidantes alimentarios se realiza generalmente mediante cromatografía de líquidos y usando fotometría o espectrometría de masas como sistemas de detección [15,16]. También se han descrito métodos basados en cromatografía de gases con detector de ionización de llama o espectrometría de masas, y en electroforesis capilar con detección fotométrica o electroquímica [15]. En general, los límites de detección alcanzados en estos métodos se encuentran a niveles de μmol L^{-1}.

Aunque el método propuesto no incluye la separación de antioxidantes y, por tanto, no permite su cuantificación individual, constituye una alternativa original y rápida como método de screening para detectar la presencia de antioxidantes naturales y/o sintéticos en alimentos. Se demuestra por primera vez la posibilidad de utilizar con fines analíticos la formación de AuNPs inducida por antioxidantes, usando la velocidad inicial del proceso, obtenida midiendo la variación de la dispersión de la radiación de resonancia con el tiempo, como parámetro analítico. La medida de este parámetro se realiza en unos cinco segundos, sin necesidad de utilizar ninguna etapa previa de incubación. Es de destacar la necesidad de llevar a cabo el proceso en presencia del surfactante catiónico CTAB, lo que se atribuye a que la formación de un par iónico con el anión $AuCl_4^-$ favorece la reacción redox.

Los límites de detección alcanzados varían entre 0.007 μmol L^{-1} para galato de dodecilo y 0.03 μmol L^{-1} para ácido ascórbico. Al comparar estos valores con los límites obtenidos por otros métodos, con diversos sistemas de detección [15], se puede establecer que el método desarrollado mejora los valores descritos. Por ejemplo, en el caso de los galato de propilo y octilo, los límites de detección descritos son 0.29 y 2.3 μmol L^{-1}, respectivamente, mientras que para el método propuesto los valores obtenidos para estos analitos son 0.01 y 0.008 μmol L^{-1}, respectivamente.

El método se ha aplicado satisfactoriamente al análisis de varios alimentos, obteniendo resultados similares a los obtenidos mediante cromatografía de líquidos y, en el caso del ácido ascórbico, mediante valoración redox.

Determinación del contenido de polifenoles en bebidas empleando laccasa, AuNPs y fluorimetría de larga longitud de onda.

Como se ha indicado en la introducción de esta Tesis, el desarrollo de métodos para determinar el contenido de polifenoles totales en alimentos continua siendo un área de investigación de gran interés. Los métodos más empleados son el método fotométrico oficial de la AOAC (Folin-Ciocalteu) [17] y métodos basados en biosensores amperométricos [18,19]. El método propuesto ofrece una alternativa innovadora mediante

cuatro elementos que lo diferencian de los métodos anteriormente descritos:

1) Utiliza fluorimetría de larga longitud de onda como sistema de detección lo que, como previamente se ha discutido, confiere al método cierta selectividad para evitar posibles interferencias de la matriz de la muestra.
2) En lugar de un reactivo que actúa frente a cualquier reductor presente en la muestra, como ocurre con el reactivo Folin-Ciocalteu, utiliza la enzima laccasa, que ofrece un mayor grado de selectividad para la oxidación de polifenoles.
3) El método se basa en la inhibición temporal que causan los polifenoles en la oxidación del fluoróforo verde de indocianina (λ_{ex} 764 nm, λ_{em} 806 nm), utilizado un módulo de flujo detenido para automatizar la mezcla de los reactantes y la medida del periodo de inducción obtenido, proporcional a la concentración de analito.
4) Incluye la utilización de AuNPs cargadas positivamente, las cuales interaccionan con la enzima y disminuyen su actividad, consiguiendo mejorar los límites de detección obtenidos.

Estos elementos han permitido el desarrollo de un método que presenta límites de detección bastante menores que los obtenidos en la mayoría de los métodos que emplean biosensores amperométricos, como muestra la **Tabla 7**.

Tabla 7. Comparación de los LODs entre diferentes métodos

Analito	Método propuesto FLD	[18] Biosensor	[19] Biosensor	[20] Biosensor
	LOD (μmol L^{-1})	LOD (μmol L^{-1})	LOD (μmol mL^{-1})	LOD (μmol mL^{-1})
Catecol	0.01	10	-	-
Hidroquinona	0.01	-	-	-
Hidroxihidroquinona	0.03	-	-	-
Ácido gálico	0.04	380	0.41	17.01
Pirogalol	0.04	-	-	-

Una limitación del método, al igual que ocurre en los otros métodos para la determinación de polifenoles totales, es que sólo se obtiene una

estimación aproximada ya que los resultados están referidos a un polifenol que se utiliza como modelo, generalmente ácido gálico. Otro aspecto criticable es el uso de la enzima en medio homogéneo, a diferencia de los biosensores enzimáticos que implican su inmovilización, lo que posibilita su reutilización. No obstante, la cantidad de enzima utilizada en cada medida es extraordinariamente baja, debido a la elevada sensibilidad del método.

Introduction

The results obtained throughout this Doctoral Thesis are discussed in this chapter, including the strengths and weakness of the methodologies developed. Also, a comparison of their main features with those obtained for other methods described for the same purpose is made.

This chapter is divided in two sections. In the first one, the results obtained from the methodologies based on chromatographic separation, derivatization, and fluorescence detection are discussed and, in the second one, the results from the automated non-chromatographic methodologies proposed by the use of the stopped-flow technique are discussed.

1. Methodologies based on chromatographic separation, post-column derivatization and fluorescence detection

This section deals with the different chromatographic methods developed for the determination of phenolic compounds in food samples. The aim of these methods was the establishment of new methodologies which improve the basic (precision, selectivity and sensitivity) and the complementary (speed, cost and personal factors) analytical properties of the existing methods, so that the new proposed methods could be a real alternative for the determination of these compounds, which are of great interest in food quality control.

Liquid chromatography as separation technique and luminescence as detection system have been used to achieve the proposed objectives. However, the innovation of the proposed methodology lies in the several post-column derivatization systems used in each of them to provide the analytes (with no native fluorescence) with luminescent properties. Thus, the following methods have been developed:

- An analytical method that involves a post-column derivatization system based on the formation of luminescent chelates of the analytes with terbium(III) at a slightly basic medium, in the presence of TOPO and EDTA.

- An analytical method that involves a post-column derivatization system based on the capability of flavonoids to form stable chelates with aluminum(III) and terbium(III) in the presence of a sub-micellar medium provided by SDS.

- An analytical method based in a derivatization system in which the analytes interact with cerium(IV) and a long wavelength fluorophor.

All the methodologies developed in this section share some common elements that provide several advantages over other analytical methods previously established.

First, the chromatographic column used in the three proposed methods is a monolithic C_{18} column, in contrast with the reversed-phase columns packed with silica microparticles that are most frequently used for the separation of phenolic compounds. The use of this kind of columns provides the possibility of operate at higher flow rates with lower back-pressure. Thus, they allow the analysis of samples using high sample volumes, achieving a better analytic signal in form of high and narrow chromatographic peaks. Another advantage provided by this type of columns is that they allow the analysis of samples using direct injection or low sample dilutions, because the cleaning and regeneration of the column can be done at a high speed.

Secondly, a spectrofluorimeter detector is used in all instances to measure the analytical signals at long or relative long wavelength, using an appropriate fluorophor or the luminescent chelates formation of the analytes with terbium(III), respectively. The detection in this part of the spectrum minimizes potential interferences from background sample matrix as they tend to emit at shorter wavelengths. In addition, the selectivity offered by sensitized luminescence facilitates the identification of some compounds that cannot be easily identified using photometric detection.

Below, the results for each of the methodologies developed are discussed with more detail.

Determination of phenolic compounds in wine by terbium-sensitized luminescence

This method allows the simultaneous determination of 18 different phenolic compounds, including hydroxybenzoic and hydroxycinnamic acids, catechins, phenolic aldehydes, flavonols and stilbenes, using photometric and fluorimetric detection. The method is based on the chromatographic separation and post-column derivatization of the analytes to transform them into fluorescent derivatives. The derivatization system is based on the formation of soluble and stable chelates of the analyte-ligands with terbium(III), in which the ligand is excited and then the absorbed energy is transferred to the lanthanide, which finally emits luminescence.

The advantages of the method presented are the following:

- Those derived from the use of a C_{18} monolitic column, which have discussed earlier.
- The relatively long emission wavelength of terbium(III) chelates can minimize existing interferences in the sample matrix, which typically emit at lower wavelength. Furthermore, the selectivity of the sensitized luminescence can facilitate the identification of some compounds that are not be easily detected by photometry.
- The only treatment needed is sample dilution.
- This is the first time that aromatic aldehydes have been determined using terbium-sensitized luminescence.

These features have allowed the chromatographic separation in less time than that described in other methods for these phenolic compounds. Fifteen phenolic compounds in wines samples have been separated in just 25 minutes. In contrast, other methods [1-2] that use packed columns achieve the separation of 14 to 25 phenols in 60 minutes, or 32 compounds in 90 minutes. Detection limits have been also improved, as shown in **Table 1**, in which a comparison with other methods previously described [2-3] is included. The detection limits achieved using fluorimetric detection are lower than those obtained using photometric detection in the 40% of the cases, which shows that sensitized luminescence helps not only to increase

the selectivity but also the sensitivity. Comparing the proposed method with others, it can be concluded that both photometric and fluorimetric detection limits are improved in most cases, demonstrating the utility of the method developed.

Table 1. Comparison of LODs between different methods

Analyte	Proposed Method		[2]	[3]	
	Photometry LOD (ng mL^{-1})	Fluorimetry LOD (ng mL^{-1})	Photometry LOD (ng mL^{-1})	Photometry LOD (ng mL^{-1})	Fluorimetry LOD (ng mL^{-1})
Gallic acid	3	5	14	60	-
Protocatechuic acid	36	2	18	120	-
p-hydroxy benzoic acid	22	15	-	-	-
Salicylic acid	14	14	-	-	-
Vanillic acid	30	17	11	60	68
Syringic acid	10	3	3	50	3
Catechin	8	114	51	110	93
Vanillin	19	40	-	-	-
Syringaldehyde	3	15	-	40	-
Epicatechin	8	29	55	340	31

Although this method has several advantages, it has also some limitations, being probably the most remarkable the impossibility to get fluorescent derivatives of all the analytes. Only hydroxybenzoic acids, catechins and phenolic aldehydes have presented fluorescence, but it was impossible to obtain fluorescence signals from hydroxycinnamic acids, flavonoids and stilbenes under the experimental conditions set in the method.

Determination of flavonoids compounds in orange juice using terbium sensitized luminescence

The proposed method can identify five flavonoids in orange juices by their chromatographic separation and derivatization, which is based on the capability of flavonoids to form stable chelates with aluminum(III) and terbium(III), and the measurement of terbium sensitized luminescence. The luminescence intensity of these chelates is higher than that obtained using only terbium (III), which can be ascribed to a co-luminescence effect [5].

The two main innovations of this method are the use of a monolithic column for the separation of these compounds in orange juices and the simultaneous formation of chelates using two different metal ions to obtain sensitized luminescence. These innovations are directly related with the benefits of the method: 1) sample pre-treatment is not required, 2) the sample injection volume (200 µL) is higher than that used in other methodologies, and 3) the luminescence signals obtained by the proposed derivatization system are more intense than those obtained using only terbium(III) as the derivatizing reagent [4].

Table 2 shows a comparison between the detection limits achieved with the proposed method and those described in other existing methods [6, 7]. As can be seen, an excellent improvement on the detection limits is obtained. For instance, the naringin detection limit is 1250 and 1000 times lower than those reported using photometric [6] and electrochemical (ED) detection [7], respectively. For kaempferol, the detection limit is 1250, 500 and 100 times lower than those obtained in the methods described using electrochemical, mass spectrometry and photometric detection, respectively [7].

Table 2. Comparison of LODs between different methods

Analyte	Proposed Method LC-FD	[6] LC-UV	[7]		
			LC-UV	LC-MS	LC-ED
	LOD (ng mL^{-1})	LOD (ng mL^{-1})	LOD (ng mL^{-1})		
Naringin	1.0	1250	250	150	1000
Hesperidin	2.7	1000	300	100	500
Quercetin	1.0	2500	150	200	300
Naringenin	1.5	-	-	-	-
Kaempferol	0.8	-	80	400	1000

Although this method has several positive aspects, it also shows two main drawbacks: 1) the number of phenolic compounds determined is relatively low, and 2) A higher flow rate of mobile phase in the chromatographic separation would have been desirable, but the flow had to be adapted to the characteristics of the derivatization system to avoid pressure problems. However, the time required for the separation is lower than that reported in other methods, which involve the use of traditional reversed phase columns.

Determination of flavonoids in orange juice using long wavelength fluorescence

A new method for the determination of six of the most representative flavonoids present in orange juice has been developed. This method involves a chromatographic separation and a post-column derivatization system using cerium(IV) and the long wavelength fluorophor cresyl violet.

The use of long wavelength fluorophores as analytical reagents is an interesting option to improve the selectivity of fluorimetric analysis and avoid or minimize the sample treatment step of the analytical process. These fluorophores allow measurements in a region of the electromagnetic spectrum (>600nm) in which the potential absorption or emission associated to sample matrix is minimized. Also, Raman interference is greatly diminished and the probability of non-radiative quenching processes is decreased due to the usually short fluorescence lifetime of these fluorophores.

As in previous methods, the use of a monolithic column decreases the length of the separation, which is achieved in about 12 minutes, in contrast with the 35 minutes required in other methods [8]. Juice analysis often requires sample treatment processes such as extraction, hydrolysis, centrifugation and/or filtration [6-7,9-12]. However, these processes are avoided in the proposed method, except a dilution step, so the complexity and the overall length of the analytical processes are diminished.

Comparing the detection limits obtained in the proposed method to those described in other methods, mainly photometric methods, described for the determination of these compounds (**Table 3**), it can be checked the remarkable improvement achieved in all instances. Thus, the decrease obtained for the detection limits ranges between 700 and 7 times lower for naringin and naringenin, respectively.

Table 3. Comparison of LODs between different methods

Analyte	Proposed Method LC-FD	[8] LC-UV	[6] LC-UV	[7] LC-UV	[7] LC-MS
	LOD (ng mL^{-1})				
Rutin	1.5	40	-	-	-
Naringin	1.8	30	1250	250	150
Hesperidin	3.0	-	1000	300	100
Quercetin	3.7	100	2500	150	200
Naringenin	2.0	14	-	-	-
Kaempferol	1.0	-	-	80	400

In spite of the positive aspects of the proposed method, the determination of a higher number of phenolic compounds would be desirable. However, several phenolic and hydroxycinnamic acids, such as gallic, caffeic and p-coumaric acids were tested but the chromatographic separation was not possible as they eluted with the solvent front. In addition, it would have been desirable the use of higher flow rates of mobile phase in the chromatographic separation, but the flow had to be adapted to the characteristics of the derivatization system to avoid pressure problems.

General comparison of the methods developed

After the individual comparison of the three proposed chromatographic methods with other methods previously described, now the direct comparison of the main features of the new methods is presented, as all of them involve the use of chromatographic separation, derivatization and fluorimetric detection of similar analytes. To simplify the comparison, the methods have been numbered according to the derivatizing system, as follows:

- Method 1: terbium(III)
- Method 2: aluminium(III) and terbium(III)
- Method 3: cerium(IV) and cresyl violet

A) Separation process

The number of analytes, the length of each separation, the number of mobile phase components and its flow rate are compared in **Table 4**. As shown, the number of analytes separated in method 1 is much higher than in the other two methods, although only 10 of the 18 analytes originate a fluorescent response. In methods 2 and 3 the same analytes have been separated, but including rutin in method 3. It is remarkable that the separation in method 3 is performed in half time than in method 2. Although the components of the mobile phase are the same in both methods [acetic buffer solution (pH 4), acetonitrile and methanol], the elution gradient used in method 3 significantly decreased the length of the separation process. Regarding the mobile phase flow, it is considerably higher in method 1.

Table 4. Comparison of the separation characteristics

Separation	Method 1	Method 2	Method 3
Analytes	18	5	6
Separation time (min)	25	25	12
Mobile phase components	2	3	3
Mobile phase flow [mL min^{-1}]	2	0.7	0.7

B) Derivatization system

Table 5 shows the different components of the derivatization system used in each method in which the use of surfactants is necessary in all instances. The flows used are also included, being lower in method 2 and quite similar in the other two methods.

Method 1 is the most complex because it involves the use of a greater number of reagents in the derivatization system. EDTA was mainly used to prevent terbium(III) precipitation due to the high pH used, but it improves also the signal of gallic acid. The use of surfactants is mainly justified by its protective effect on the luminescence of terbium(III) chelates. Triton X-100 also increased the signal of gallic acid, while SDS was

necessary to achieve an increase in the luminescence signal of syringaldehyde, which was very low in its absence.

The use of EDTA was not required in method 2, as the pH used was significantly lower (4.0) than in method 1, so there is no risk about terbium(III) precipitation. In addition, it was found that its presence did not improve the luminescent signal of the analytes and, also, the use of TOPO as synergetic agent was no necessary. However, the assay of the potential effect of different surfactants on the system showed that SDS greatly improved the analytical signals at a sub-micellar concentration.

The derivatizing system used in method 3 allows the measurement at a longer wavelength that in the previous methods because of the use of the fluorophor cresyl violet endows the method with a better selectivity, as it has been discussed before. The negative chromatographic peaks are the results of the indirect inhibition in the cresyl violet fluorescence by the analytes due to a combination of redox, acid-base and hydrophobic processes. The flow used in this system is higher than in method 2, which is ascribed to the kinetics of the process involved in the derivatization, which are faster than those involved in the co-luminescence processes requiring the chelates formation with aluminium(III) and terbium(III).

Table 5. Comparison of the derivatization systems

Derivatization	Method 1	Method 2	Method 3
Components	Tb(III) AEDT TOPO TRITON X-100	Tb(III) Al(III) SDS	Cresyl violet CTAB Ce(IV)
Derivatizing solution flow (mL min^{-1}]	0.8	0.5	0.9

C) Detection limits and precision

Table 6 shows the detection limits obtained in the three methods. A direct comparison of all the results obtained cannot be made because most of the analytes in method 1 do not coincide with those in the other methods. However, it can be seen that the detection limits are very low in all instances, reaching levels of ng mL^{-1}, although these values are generally

higher for method 1. For this method only those obtained by fluorimetric detection have been included, with the exception for rutin, quercetin and kaempferol, obtained with photometric detection. As shown, the detection limits for these analytes are significantly lower using fluorimetric detection.

Table 6. Comparison of detection limits

	Method 1	Method 2	Method 3
Analito	LOD (ng mL^{-1})	LOD (ng mL^{-1})	LOD (ng mL^{-1})
Gallic acid	5	-	-
Protocatechuic acid	2	-	-
p-Hydroxybenzoic acid	15	-	-
Salicylic acid	14	-	-
Vanillic acid	17	-	-
Syringic acid	3	-	-
Catechin	114	-	-
Vanillin	40	-	-
Syringaldehyde	15	-	-
Epicatechin	29	-	-
Rutin	24*	-	1.5
Naringin	-	1.0	1.8
Hesperidin	-	2.7	3.0
Quercetin	60*	1.0	3.7
Naringenin	-	1.5	2.0
Kaempferol	18*	0.8	1.0

* Photometric detection

Comparing the results of methods 2 and 3, in which the same analytes are involved, except for rutin, the detection limit levels reached are very similar, although it seems to be lower in method 2.

Regarding the precision of the three methods, expressed as % RSD, these values ranged between 0.08 - 6.4%, 1.3 - 4.7 % and 2.8 - 6.2% for methods 1, 2 and 3 respectively. Although method 1 reaches very low values in some cases, there are no significant differences between them.

To summarize this comparison between methods and considering that each one has its advantages and limitations, the following conclusions can be established:

1) The low detection limits obtained in the three methods allow the direct sample analysis by the appropriate dilution.
2) Method 1 achieves the highest resolution due the high number of analytes separated, although the derivatization system is more complex and its detection limits are higher.
3) Method 2 gives the best detection limits, but the separation is slower than method 3.
4) Method 3 involves probably the most original derivatization system and allows a faster separation.

Chemometric classification method

As a result of the analytical information obtained from the proposed method for the determination of phenolic compounds in wine by terbium-sensitized luminescence, a chemometric classification method has been developed. This method involves the use of chemometric techniques to classify wines and it is based on the phenolic content, variety and geographical origin.

The pattern data matrix, constructed by the concentration of each analyte present in each wine sample, was subjected to different algorithms in order to classify and characterize the wine samples adequately. Supervised (linear discriminant analysis) and un-supervised (factor analysis) pattern recognition methods have been used.

Several classification methods of wines have been described. For example, there are two methods that require a high number of phenolic compounds, 19 or more, and use the geographical origin and the grape variety [13] or the vintage year [14] as classification pattern, obtaining in both cases a 95% effective classification. The information provided by the proposed method is obtained from 18 analytes, using photometric detection, and only 10 of them give fluorimetric results. However, a chemometric procedure for the classification of the samples that reaches 100% effectiveness can be established using only the fluorimetric information.

2. Automatic non-chromatographic methodologies based in the used of stopped-flow technique and nanoparticles.

The results obtained in two methods for the determination of phenolic compounds and other antioxidants in food samples are discussed in this section. The stopped-flow technique has been used in both methods to automate the mixing of the reactants and to obtain analytical results. The aim of this study has been the development of new analysis methods with the use of nanomaterials and/or enzymes, which allow the improvement of the analytical properties of the existing methods. Two kinetic parameters, initial reaction rate and induction period, have been used in these methods to quantify the analytes, and resonance light scattering and long wavelength fluorescence have been used as the detection systems. Thus, it has been developed:

- A method for antioxidant determination by the direct measurement of gold nanoparticles (AuNPs) formation using resonance light scattering as detection system.
- An enzymatic-fluorimetric method for the determination of polyphenols based on the temporary inhibition caused by these compounds on the oxidation of a long wavelength fluorophor.

Determination of antioxidant additives in food stuffs by direct measurement of gold nanoparticles formation using resonance light scattering detection.

The determination of food antioxidants is mainly carried out by using liquid chromatography with photometry or mass spectrometry as detection systems [15,16]. Methods based on gas chromatography with flame ionization detector or mass spectrometry, and capillary electrophoresis with photometric or electrochemical detection have also been described [15]. The limits of detection reported for most of these methods are at level of $\mu mol\ L^{-1}$.

The proposed method does not include the separation of the antioxidants so their individual quantification is not allowed. However, it

can be seen as an original and quick alternative as a screening method to detect the presence of natural and/or synthetic antioxidants in food. The usefulness of kinetic methodology for monitoring the formation of AuNPs induced by antioxidant compounds is shown for the first time. The initial rate of the process, which is obtained by measuring the variation of the resonance light scattering signal with time, is used as the analytical parameter. The measurement of this parameter takes about five seconds and a previous stage of incubation is not needed. It is remarkable the need of the cationic surfactant CTAB to carry out the process because the formation of an ionic pair between $AuCl_4^-$ and the cationic surfactant is necessary, under the experimental conditions used, to obtain the AuNPs.

The detection limits achieved ranged from 0.007 μmol L^{-1} for dodecyl gallate and 0.03 μmol L^{-1} for ascorbic acid. The comparison of these values with those reported in other methods involving different detection systems [15], shows that the developed method notably improves these values. For instance, the detection limits described for propyl and octyl gallates are 0.29 and 2.3 μmol L^{-1}, respectively, while the values obtained in the proposed method are 0.01 and 0.008 μmol $^{-1}$, respectively.

The method has been successfully applied to the analysis of several foodstuffs achieving similar results that those obtained by liquid chromatography and, in the case of ascorbic acid, by redox titration.

Determination of polyphenolic content in beverages using laccase, gold nanoparticles and long wavelength fluorimetry

The development of methods to determine total polyphenol content in foods is an area of great interest. The most common methods are the official AOAC photometric method (Folin-Ciocalteu) [17] and methods based on amperometric biosensors [18-19]. The proposed method offers an innovative alternative that is based in four elements that differentiate it from the methods previously described:

1) The use of long wavelength fluorimetry as detection system, which improves the selectivity of the method avoiding interferences from background sample matrix.

2) The use of the enzyme laccase that offers higher selectivity for the oxidation of polyphenols, instead of reagents that act against any reductant present in the sample, such as the Folin-Ciocalteu reagent.
3) The method is based on the temporal inhibition caused by polyphenolic compounds on the oxidation of the long wavelength fluorophor indocyanine green (λ_{ex} 764 nm, λ_{em} 806 nm), using a stopped-flow module to automate the mixing of the reactants and the measurement of the induction time, which is proportional to the analyte concentration.
4) The use of positively charged AuNPs, which interact with the enzyme and decrease its activity, allowing the improvement of the detection limits obtained.

These elements have given rise to the development of a method that shows lower detection limits than those obtained in most of the methods involving the use of amperometric biosensors (**Table 7**).

Table 7. LODs comparison between several methods

Analyte	Proposed method	[18]	[19]	[20]
	LOD (μmol L^{-1})	LOD (μmol L^{-1})	LOD (μmol mL^{-1})	LOD (μmol mL^{-1})
Catechol	0.01	10	-	-
Hydroquinone	0.01	-	-	-
Hydroxyhydroquinone	0.03	-	-	-
Gallic acid	0.04	380	0.41	17.01
Pyrogallol	0.04	-	-	-

One limitation of the method, as it occurs in other methods for total polyphenol determination, is that only a rough estimate can be achieved because the results are referred to a polyphenol that is used as a model, in this case gallic acid. Another critical aspect is the use of the enzyme in a homogeneous medium, unlike enzymatic biosensors in which the enzyme is immobilized and allows its reusability. However, the amount of enzyme used in each measurement is extremely low due to the high sensitivity of the method.

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CONCLUSIONES

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En la Memoria presentada se han propuesto diversas metodologías analíticas, cromatográficas y no cromatográficas, para la determinación de antioxidantes naturales y sintéticos en alimentos con el fin de ampliar la disponibilidad de métodos aplicables al control de la calidad alimentaria. A continuación se resumen las principales conclusiones del trabajo realizado:

1. Se ha desarrollado un método mediante cromatografía de líquidos para la determinación de dieciocho compuestos fenólicos en vinos utilizando medidas de absorbancia a cuatro longitudes de onda, y de luminiscencia sensibilizada de terbio. Se ha demostrado la utilidad de este último sistema de detección, mediante derivatización postcolumna de los analitos, para mejorar la selectividad de estas determinaciones.

2. Se ha descrito un procedimiento para el análisis clasificatorio de vinos utilizando su contenido en compuestos fenólicos, su variedad y su origen geográfico. Los datos obtenidos mediante la separación cromatográfica de los analitos y su detección fotométrica y luminiscente se han sometido a diversos algoritmos, aplicando métodos supervisados y no supervisados de pautas, consiguiendo la correcta discriminación entre los distintos grupos establecidos.

3. Se ha propuesto un método para la determinación de flavonoides en zumos de naranja basado en su separación mediante cromatografía de líquidos, derivatización postcolumna de los analitos con aluminio(III) y terbio(III) y detección de la luminiscencia sensibilizada. Se demuestra por primera vez la utilidad del uso conjunto de dos iones metálicos como derivatizantes para mejorar los límites de detección previamente obtenidos para la cuantificación de estos analitos mediante otros sistemas de detección, tales como fotometría, coulombimetría o espectrometría de masas.

4. Se ha descrito por primera vez el uso conjunto del fluoróforo de larga longitud de onda violeta de cresilo y cerio(IV) en medio micelar como sistema de derivatización postcolumna en cromatografía de líquidos para la determinación de flavonoides en zumos de naranja. La metodología desarrollada presenta las características siguientes: 1) evita posibles interferencias de la

matriz de la muestra, debido a la larga longitud de onda utilizada para realizar las medidas, 2) mejora los límites de detección obtenidos mediante detección fotométrica, y 3) permite el análisis directo de las muestras debido al elevado factor de dilución que puede utilizarse.

5. Se ha estudiado la capacidad de diversos compuestos antioxidantes utilizados como aditivos naturales o sintéticos en alimentos para formar nanopartículas de oro. El estudio realizado se ha aplicado a la determinación cinética de estos compuestos, utilizando un sistema de flujo detenido, mediante monitorización de la radiación dispersada producida por las nanopartículas formadas. El método propuesto evita las etapas de incubación requeridas en métodos de equilibrio y permite obtener resultados analíticos en pocos segundos. El método se ha aplicado al análisis de diversos alimentos y los resultados se han validado utilizando dos métodos de referencia.

6. Se ha investigado la utilidad analítica de la fluorimetría de larga longitud de onda para la determinación de polifenoles en bebidas utilizando la inhibición temporal producida por estos compuestos en la oxidación del fluoróforo verde de indocianina, en presencia de la enzima laccasa y de nanopartículas de oro. Se ha demostrado que la interacción electrostática entre la enzima y las nanopartículas mejora los límites de detección obtenidos, consiguiendo valores inferiores a los descritos en la mayoría de los biosensores amperométricos previamente propuestos para la determinación de estos compuestos.

CONCLUSIONS

Several analytical chromatographic and non chromatographic methodologies for the determination of natural and synthetic antioxidants in foods have been proposed in this study. The purpose has been to expand the availability of methods for the control of food quality. The main conclusions of the research performed are summarized below:

1. A liquid chromatographic method with online photometric and luminescent detection for the determination of eighteen phenolic compounds in wines has been developed. Photometric detection has been performed at four different wavelengths and the luminescent detection has been achieved using terbium(III) as a postcolumn derivatization reagent. The usefulness of the last detection system to improve the selectivity of these determinations has been demonstrated.

2. A method for wine classification based on the phenolic compound content, wine variety and geographical area has been described. The data obtained from the chromatographic separation of the analytes and their photometric and fluorimetric detection have been subjected to different algorithms, applying supervised and un-supervised pattern recognition methods, achieving the correct discrimination between the different groups established.

3. A method for the determination of flavonoids in orange juices, involving liquid chromatographic separation, post-column derivatization of the analytes with terbium(III) and aluminium(III) and sensitized luminescence detection, has been described. The usefulness of two metal ions as derivatizing agents to improve the detection limits achieved by others detection systems, such as photometry, coulombimetry and mass spectrometry, is shown for the first time.

4. A new method for the determination of flavonoids in orange juices has been described, which is based on the simultaneous use of the long wavelength fluorophor cresyl violet and cerium(IV) in a micellar medium as a post-column derivatizing system in liquid chromatography. The main features of the

method are the following: 1) the use of long wavelength measurements to obtain analytical signals avoids the interference of potential signals from the sample matrix, 2) the detection limits achieved in this method are better than those obtained using photometric detection, and 3) the method allows the direct analysis of the samples owing to the high dilution factors that can be used.

5. The capability of several antioxidant compounds, used as natural or synthetic additives in food, to reduce gold(III) to gold nanoparticles has been studied. This study has been applied to the kinetic determination of these compounds using stopped-flow mixing technique and monitoring the formation of gold nanoparticles using resonance light scattering as the detection system. The proposed method avoids incubation steps in contrast to equilibrium methods and the analytical results can be achieved in few seconds. The method has been applied to the analysis of several food samples and the results obtained were validated using two reference methods.

6. The analytical utility of long wavelength fluorimetry for the determination of poliphenols compounds in beverages has been studied. A method based on the temporal inhibition caused by these compounds on the oxidation of the fluorophor indocyanine green in the presence of the enzyme laccase and positive charged gold nanoparticles has been developed. It has been shown that the electrostatic interaction between the enzyme and the nanoparticles improves the detection limits obtained, achieving values that are lower than those obtained in most of the methods based on amperometric biosensors proposed for the determination of these compounds.

ANEXO

PRODUCCIÓN CIENTÍFICA

Producción científica derivada de esta Tesis Doctoral

Publicaciones:

1. Analytical innovations in the detection of phenolics in wines.
   Russo, P.; Andreu-Navarro, A.; Aguilar-Caballos, M. P.; Fernández-Romero, J. M.; Gómez-Hens, A.
   Journal of Agricultural and Food Chemistry. 2008, 56, 1858–1865.

2. Usefulness of terbium-sensitised luminescence detection for the chemometric classification of wines by their content in phenolic compounds.
   Andreu-Navarro, A; Russo, P.; Aguilar-Caballos, M. P.; Fernández-Romero, J. M.; Gómez-Hens, A.
   Food Chemistry, 2011, 124, 1753–1759.

3. Luminescent determination of flavonoids in orange juices by LC with post-column derivatization with aluminum and terbium.
   Andreu-Navarro, A.; Fernández- Romero, J. M.; Gómez-Hens, A.
   Journal of Separation Science, 2010, 33, 509–515.

4. Long-Wavelength Fluorescence Detection of Flavonoids in Orange Juices by LC.
   Andreu-Navarro, A.; Fernández- Romero, J. M.; Gómez-Hens, A.
   Chromatographia, 2010, 72, 1115-1120.

5. Determination of antioxidant additives in foodstuffs by direct measurement of gold nanoparticle formation using resonance light scattering detection.
   Andreu-Navarro, A.; Fernández- Romero, J. M.; Gómez-Hens, A.
   Analytica Chimica Acta 695 (2011) 11–17.

6. Determination of polyphenolic content in beverages using laccase, gold nanoparticles and long wavelength fluorimetry.
   Andreu-Navarro, A.; Fernández- Romero, J. M.; Gómez-Hens, A.
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Analytical Innovations in the Detection of Phenolics in Wines

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A liquid chromatographic method with online photometric and luminescent detection for the determination of 18 phenolic compounds in wines is reported. Photometric detection is performed at four wavelengths, namely, 256, 280, 320, and 365 nm, using a diode array detection system. The luminescent detection is achieved by means of a postcolumn derivatization reaction of 10 of these compounds with terbium(III) in the presence of synergistic agents, such as ethylenediaminetetraacetic acid (EDTA) and *n*-octyltriphosphine oxide (TOPO). A micellar medium provided by the surfactants sodium dodecyl sulfate and Triton X-100 was used for the determination of the luminescent chelates at λ_{ex} 317 and λ_{em} 545 nm. The long wavelength emission of lanthanide chelates can minimize interferences from background sample matrix, which usually emit at shorter wavelengths. The analytical features of the photometric and fluorometric methods, such as dynamic ranges of the calibration graphs, detection limits, and precision data, have been obtained. The practical usefulness of the developed methods is demonstrated by the analysis of Spanish and Italian wine samples (red, rosé, oloroso, and white), which were diluted and directly injected into the chromatographic system. The accuracy of both methods was checked by assaying a recovery study, which was performed at three different analyte levels for each type of sample.

KEYWORDS: Phenolic compounds; postcolumn derivatization; terbium-sensitized luminescence; wine samples

INTRODUCTION

Phenolics are a wide group of compounds constituted by phenolic aldehydes, hydroxybenzoic and hydroxycinnamic acids, catechins, flavonols, and stilbenes, in their monomeric form or conjugated to some species, such as tartaric acid in the case of cinnamic acids (*1*), among others. These compounds are present in wines because they are secondary metabolites of plants. The composition of phenolics and their concentration depend on grape variety, geographical origin, soil type, collection system, and grape processing. These compounds are responsible of the sensory properties of the wines, and, also, they are anticarcinogenic and have an anti-inflammatory action when they are regularly ingested. A particular example of the importance of monitoring phenolic concentration to control the quality of wines is the presence of aromatic aldehydes, formed by a group of volatile compounds that are extracted from wood lignin during the winemaking process. The presence of these aldehydes in wines is an indicator of fermentation and aging in oak barrels, their absence being indicative of counterfeit aged wines. Vanillin and syringaldehyde are the most abundant aromatic aldehydes in wines.

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The occurrence of phenolics has been extensively studied by liquid chromatographic methods (*1–18*). In most of them, conventional reversed-phase columns, constituted by packed microparticulate bonded silica, have been used (*1–5, 7–15, 18*), which generally feature separations of 14–25 compounds in almost 1 h or 32 phenolics in 90 min, which makes routine analysis of these compounds very tedious. The use of other materials such as mesoporous silica has given rise to monolithic columns, which operate at higher flow rates with lower backpressures than conventional columns (*16, 17*). Thus, they allow the analysis of samples using direct injection or low sample dilutions, because the cleaning and regeneration of the column can be done more quickly than in the conventional ones due to the high flow rates afforded. Monolithic columns have been used for the determination of 15 phenolics in red and white wine samples (*16*) with separation times around 30 min and, also, for the direct analysis of cider samples (*19*).

Diode array detection (*1, 3–17*) has been extensively used for the development of liquid chromatography methods, whereas fluorometric (*8, 12*) and mass spectrometry (*2*) detection systems have been used to a lesser extent. Most fluorometric methods proposed are based on measurements of the intrinsic fluorescence of some phenolics. Although these methods feature generally lower detection limits than photometric methods, the

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Analytical Methods

Usefulness of terbium-sensitised luminescence detection for the chemometric classification of wines by their content in phenolic compounds

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ABSTRACT

A method for wine classification based on the phenolic compound content, wine variety and geographical area is described. The method involves the use of the results obtained from the analysis of fifteen samples of Italian and Spanish wines from different geographical origins [Sicilia (Italy) and Córdoba (Spain)] using liquid chromatography (LC) with photometric and fluorimetric detection, in which eighteen phenolics were determined: gallic acid, protocatechuic acid, p-hydroxybenzoic acid, salicylic acid, vanillic acid, caffeic acid, syringic acid, catechin, vanillin, p-coumaric acid, syringaldehyde, epicatechin, ferulic acid, rutin, trans- and cis-resveratrol, quercetin and kaempferol. Photometric measurements were performed selecting four wavelengths (256, 280, 320 and 365 nm), using a diode-array detection system. The fluorimetric detection was achieved by measuring the sensitised luminescence provided by the chelates formed between each analyte and terbium (III). All samples were commercial wines bought in local markets and analysed immediately after they were opened. The pattern data matrix was constructed by the concentration of each analyte present in wine, which was determined by the most adequate method, namely LC-photometric or LC-fluorimetric method. This data matrix was subjected to different algorithms in order to classify and characterise the wine samples adequately. Supervised (LDA) and un-supervised (FA) pattern recognition methods were used. The wine pattern generation with LC separation and dual detection approach to determine eighteen phenolic compounds and the chemometric treatment provide an appropriate way with recognition and prediction rates. The values obtained for these rates were 100% when fluorimetric detection was used. These results can be considered satisfactory, which proves the usefulness of the selected variables.

1. Introduction

In recent years, there has been an increasing interest in the development of chemometric applications in order to establish the variety, geographical origin, manipulation and other technological features, which define the taste of foods and beverages from agricultural origin (Ariyama & Yasui, 2006; Marcos, Fischer, Rea, & Hill, 1998; Sanz, Pérez, Herrera, Sanz, & Juan, 1995; Smith, 2005). The reasons for this interest are: (1) laws enforce labelling of the geographical origin of the foodstuff and beverages in many countries due to demands of more information from consumers and improvement of their domestic production; (2) producers have begun to advertise their brands of high-quality products by including geographical origin and other features in the label for economic reasons; (3) prevention of frauds and adulteration on the label, production and commercialisation. Thus, the development of methods giving acceptable information is highly desirable for consumers, producers and administrative authorities.

Wine industry and the market sector are particular examples in which the development of sophisticated chemometric techniques are essential for the improvement of the analytical information about wine composition and for assessing wine authenticity (Arvanitoyannis, Katsota, Psarra, Soufleros, & Kallithraka, 1999; Horwitz, 1995). Several chemometric procedures have been used as the basis for discrimination of wines according to vinification technology and classification according to region, type and variety. Different pattern recognition techniques (PRT), such as Principal Component Analysis (PCA) (Boselli, Giomo, Minardi, & Frega, 2008; Recamales, Sayago, González-Miret, & Hernanz, 2006), Linear and Canonical Discriminant Analysis (LDA and CDA) (Hernández, Estrella, Dueñas, Fernandez de Simón, & Cadahía, 2007; Makris, Kallithraka, & Mamalos, 2006; Villiers et al., 2005), Probabilistic Neural Network (PNN) (Díaz, Conde, Estévez, Pérez-Olivero, & Pérez Trujillo, 2003), K-Nearest Neighbours (KNN) (Beltrán et al., 2006), Cluster Analysis (CA) (Villiers et al., 2005), Multiregression Analysis (MRA), Partial Least Squares (PLS) (Capron, Smeyers-Verbeke, & Massart, 2007; Le Moigne et al., 2008), and CAIMAN

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Research Article

Luminescent determination of flavonoids in orange juices by LC with post-column derivatization with aluminum and terbium

A new post-column derivatization system is described and applied to the determination of flavonoids in citric beverages after their separation by LC using a monolithic column. The derivatization involves the formation of the chelates of the analytes with aluminum (III) and terbium (III) in the presence of the surfactant SDS and the measurement of the terbium sensitized luminescence at λ_{ex} 360 and λ_{em} 545 nm. Naringin, hesperidin, quercetin, naringenin, and kaempferol have been chosen as analyte models. The large Stokes shift and the relatively long wavelength emission of terbium(III) can minimize interferences from background sample matrix, which usually emit at shorter wavelengths. Calibration graphs were constructed in the intervals 6.0–1700 ng/mL naringin, 9.8–1700 ng/mL hesperidin, 2.1–2000 ng/mL quercetin, 5.2–1500 ng/mL naringenin and 2.5–2000 ng/mL kaempferol, with regression coefficients higher than 0.9935 in all instances. The precision of the method, expressed as RSD%, was established at two concentration levels, with values of 1.3 and 4.7%, which corresponded to the minimal and maximal error zones of the calibration graphs. The practical usefulness of the method is demonstrated by the analysis of orange juices, which were diluted and directly injected into the chromatographic system, obtaining recoveries between 86.9 and 108.2%.

Keywords: Aluminum and terbium complexes / Flavonoid compounds / Orange juices / Post-column derivatization
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1 Introduction

Flavonoids are a group of polyphenolic compounds widely distributed in vegetables and fruits [1]. The determination of these compounds is of great interest owing to their multiple biological effects, including antioxidant activity, antitumor, antimutagenic, antibacterial and angioprotective properties [2, 3]. They also contribute to different plant properties such as colour, flavour, fragrance, nutrition, stability and therapeutic properties. Flavonoids such as 2-phenyl-benzo-α-pyrones are classified according to the multitude of substitution patterns in the two benzene rings of their basic structure. Variation in their heterocyclic rings gives rise to flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavones [4]. The flavonoid content in orange juices has special interest as it contributes to the quality of these samples [5].

The main separation technique applied to the determination of flavonoids in orange juices is LC [6–11], using reversed-phase columns, although gas chromatography [12], involving the formation of trimethylsilyl derivatives, has been also described for this purpose. The usefulness of a monolithic column, which can operate at higher flow rates with lower backpressures than conventional columns, has been previously described for the LC separation of some flavonoids in wine samples [13]. However, to the best of our knowledge, this type of column has not been used up to date for the determination of these compounds in orange juices.

Photometric [6–10], coulometric [10] and MS [6, 11] detection systems have been reported in the LC methods described for the analysis of these samples. Some flavonoids, such as quercetin and kaempferol, have been fluorimetrically determined in body fluids using aluminum(III) as post-column derivatization reagent to obtain fluorescent chelates [14]. Another post-column derivatization system previously described for the determination of these compounds in wine samples involves the formation of the corresponding terbium(III) chelates and the measurement of the sensitized luminescence [13]. This phenomenon is an intermolecular energy transfer process, in which the ligand acts as donor and terbium(III) acts as acceptor and emits luminescence, that allows very sensitive and selective determinations, although its use as detection system in LC has been relatively limited [15].

This article reports for the first time the use of a monolithic column for the direct separation of flavonoids in orange juice samples and a new post-column derivatization

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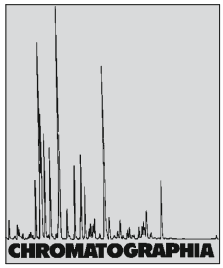
Long-Wavelength Fluorescence Detection of Flavonoids in Orange Juices by LC



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Abstract

A new post-column liquid chromatographic reaction system for the determination of flavonoids in orange juices is based on the use of the long wavelength fluorophor cresyl violet and cerium(IV) in a cetyl trimethylammonium bromide micellar medium. Two flavone aglycones (quercetin and kaempferol), a flavonone aglycone (naringenin), one flavone-*O*-glycoside (rutin) and two flavanone-*O*-glycosides (hesperidin and naringin) have been used as analyte models. The reaction process involves the interaction between the analyte, cerium(IV) and cresyl violet giving rise to a decrease in the fluorescence, measured at λ_{ex} 585, λ_{em} 625 nm, which is proportional to the analyte concentration. Dynamic ranges of the calibration graphs and detection limits, obtained with standard solutions of the analytes are (ng mL^{-1}): quercetin (12.2–4,000, 3.7), kaempferol (3.5–1,000, 1.0), naringenin (6.7–1,000, 2.0), rutin (5.0–800, 1.5), hesperidin (10.1–1,000, 3.0), and naringin (17.8–800, 1.8). The determination coefficients were higher than 0.993 in all instances. The precision of the method, expressed as RSD%, was established at two concentration levels, with values ranging between 2.8 and 6.2%. The practical usefulness of the developed method is demonstrated by the analysis of natural and commercial orange juices, which were filtered, diluted and directly injected into the chromatographic system, with apparent recoveries between 86.9 and 107.0%.

Keywords

Column liquid chromatography
Long wavelength fluorescence detection
Flavonoid compounds
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Introduction

The use of long-wavelength fluorophores (LWF) as analytical reagents is an attractive option to improve the selectivity of fluorimetric analysis and avoid or minimize the sample treatment step of the analytical process. These dyes allowing measurements are obtained in a region of the electromagnetic spectrum (>600 nm) in which the potential absorption or emission associated to sample matrix is minimized. Also, Raman interference is greatly diminished and the probability of non-radiative quenching processes is decreased due to the usually short fluorescence lifetime of these fluorophores. LWFs have been described as enzymatic substrates, immunoassay labels, sensing systems and derivatizing reagents in liquid chromatography (LC) and capillary electrophoresis [1]. Although the lack of sufficient reactive groups for targeting of analytes is a limitation of these fluorophores, their analytical usefulness has been expanded using electrostatic and redox interactions [2–6].

This article describes for the first time the use of the oxazine LWF cresyl violet (CV) in a post-column LC reaction system for the indirect determination of

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Determination of antioxidant additives in foodstuffs by direct measurement of gold nanoparticle formation using resonance light scattering detection

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ABSTRACT

The capability of antioxidant compounds to reduce gold(III) to gold nanoparticles has been kinetically studied in the presence of cetyltrimethylammonium bromide using stopped-flow mixing technique and resonance light scattering as detection system. This study has given rise to a simple and rapid method for the determination of several synthetic and natural antioxidants used as additives in foodstuff samples. The formation of AuNPs was monitored by measuring the initial reaction-rate of the system in about 5 s, using an integration time of 0.1 s. Dynamic ranges of the calibration graphs and detection limits, obtained with standard solutions of the analytes, were (μmol L^{-1}): gallic acid (0.04–0.59, 0.01), propyl gallate (0.04–1.41, 0.01), octyl gallate (0.03–0.35, 0.08), dodecyl gallate (0.02–0.30, 0.007), butylated hydroxyanisol (0.07–0.39, 0.009), butylated hydroxytoluene (0.04–0.32, 0.01), ascorbic acid (0.11–1.72, 0.03) and sodium citrate (0.07–1.29, 0.02). The regression coefficients were higher than 0.994 in all instances. The precision of the method, expressed as RSD%, was established at two concentration levels of each analyte, with values ranging between 0.6 and 4.8%. The practical usefulness of the developed method was demonstrated by the determination of several antioxidant additives in foodstuff samples, which were extracted, appropriately diluted and assayed, obtaining recoveries between 95.4 and 99.5%. The results obtained were validated using two reference methods.

1. Introduction

Natural and synthetic antioxidants are a group of compounds used as additives to prevent or retard oxidation reactions in food products. There is a trend to limit the use of synthetic antioxidants, owing to their potential toxic effects, but they are still found in a relatively wide range of foodstuffs, although their use is subject to very strict safety regulations [1–6]. Phenolic compounds such as propyl (PG), octyl (OG) and dodecyl (DG) esters of gallic acid (GA), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are the main synthetic antioxidants still authorized, although they are being replaced by natural antioxidants, such as ascorbic (AA) and citric (CA) acids. CA is also used as a food additive for other purposes, such as acidifier and flavouring agent.

The determination of synthetic antioxidants in food samples is mainly carried out by using reverse-phase liquid chromatography (LC) with photometry or mass spectrometry (MS) as detection systems [7,8]. Methods based on gas chromatography (GC), with flame ionization detector (FID) or MS, and capillary electrophoresis (CE) with photometric or electrochemical detection have been also described [7]. The limits of detection (LODs) reported for most of these methods are at the level of μmol L^{-1}. These methods are very useful for the identification of the analytes, but they are time-consuming for screening purposes.

The use of gold nanoparticles (AuNPs) as analytical reagents has allowed the development of very sensitive methods based on their special optical and electrochemical properties. For instance, they have been used as labels in immunoassays and hybridization assays and as nanoscaffolds to develop chemical sensors [9]. These methods require the previous synthesis of the NPs, using generally tetrachloroauric acid and a reducing reagent, usually citrate [10,11]. Other reagents such as cysteine [12], ascorbic acid [10,13,14], sodium borohydride [15,16] and gallic acid [17,18] have also shown their usefulness for this purpose. The experimental conditions chosen to obtain AuNPs are critical factors that affect the size, shape and potential aggregation of the NPs and, consequently, their properties. Some of these methods [14,15] involve the use of cetyltrimethylammonium bromide (CTAB) to favour the synthesis of the NPs. The formation of ion pairs between $AuCl_4^-$ and cationic surfactants prior to the formation of AuNPs was described [19,20].

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Determination of polyphenolic content in beverages using laccase, gold nanoparticles and long wavelength fluorimetry

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ABSTRACT

An enzymatic fluorimetric method for the determination of polyphenol compounds in beverages is described, which is based on the temporal inhibition caused by these compounds on the oxidation of the long wavelength fluorophor indocyanine green (λ_{ex} 764 nm, λ_{em} 806 nm), in the presence of the enzyme laccase and positive charged gold nanoparticles (AuNPs). The oxidation of the dye gives rise to a fast decrease in its fluorescence, but it is delayed by the polyphenol, obtaining a time period directly proportional to its concentration, which has been used as the analytical parameter. The behaviour of several benzenediols and benzenetriols in the system and the modification of the activity of the enzyme by its interaction with AuNPs have been studied.

The system has been optimized using gallic acid as a polyphenol model, but the dynamic ranges of the calibration graphs and the detection limits for several of the polyphenols assayed were obtained (μmol L^{-1}): gallic acid (0.13-5, 0.04), catechol (0.08-5, 0.01), hydroquinone (0.05-2, 0.01), hydroxyhydroquinone (0.09-5, 0.03), pyrogallol (0.17-5, 0.04). Most of the values of the regression coefficients were 0.999 and the precision of the method, expressed as RSD% and checked at two concentration levels of each analyte, ranged between 1.8 and 5.6%. The method has been applied to the determination of polyphenol content in several foodstuff samples and the results compared with those obtained with the standard Folin-Ciocalteu method.

KEYWORDS: Polyphenols; laccase; gold nanoparticles; stopped-flow mixing technique; long wavelength fluorimetric detection; beverage samples

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   Chromatographic determination of nine flavonoids using fluorescence resonance energy transfer as post-column derivatization system.
   Álvaro Andreu-Navarro, Juan M. Fernández-Romero, Agustina Gómez-Hens.

2. II Encuentro sobre Nanociencia y Nanotecnología de investigadores y tecnólogos de la universidad de Córdoba (NANOUCO).
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   Kinetic study of the formation of coloidal gold nanoparticles by gallic acid using stopped-flow mixing device and light scattering detection.
   Álvaro Andreu-Navarro, Juan M. Fernández-Romero, Agustina Gómez-Hens.

3. XII Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica (GRASECA).
   Córdoba, 10 y 11 de Junio de 2010.
   Determinación directa de flavonoides en zumos de naranja mediante cromatografía de líquidos y detección fluorescente a larga longitud de onda.
   Álvaro Andreu-Navarro, Juan M. Fernández-Romero, Agustina Gómez-Hens.

4. III Encuentro sobre Nanociencia y Nanotecnología de investigadores y tecnólogos de la universidad de Córdoba (NANOUCO).
   Córdoba, 10 y 11 de Febrero de 2011.
   Determination of antioxidant additives in foodstuffs by direct measurement of gold nanoparticle formation using resonance light scattering detection.
   Álvaro Andreu-Navarro, Juan M. Fernández-Romero, Agustina Gómez-Hens.

5. 13as Jornadas de Análisis Instrumental.
   Barcelona, 14-18 Noviembre de 2011
   Fluorimetric determination of polyphenols using a long-wavelenght-fluorophor and laccase immobilised on gold nanoparticles.
   Álvaro Andreu-Navarro, Juan M. Fernández-Romero, Agustina Gómez-Hens

12as JORNADAS DE ANÁLISIS INSTRUMENTAL

LIBRO DE RESÚMENES

Barcelona
21-23 de Octubre de 2008

PO-CSA-53

CHROMATOGRAPHIC DETERMINATION OF NINE FLAVONOIDS USING FLUORESCENCE RESONANCE ENERGY TRANSFER AS POST-COLUMN DERIVATIZATION SYSTEM

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A liquid chromatographic method with on-line luminescent detection for the determination of nine antioxidative phenolic (galic, caffeic and p-coumaric acids) and flavonoid (naringenin, naringuin, hesperidin, quercetin, rutin and kaempferol) compounds is reported. The post-column derivatization system is based on the formation of the fluorescent chelates of these compounds with aluminium(III), which act as energy donors, and the energy transfer process from these chelates to terbium(III), which acts as an energy acceptor. A micellar medium provided by the surfactant sodium dodecylsulfate was used for the luminescence detection at λ_{ex} 412, λ_{em} 545 nm. The long wavelength emission minimizes potential interferences from background sample matrix, which usually emit at shorter wavelengths. The analytical features of the fluorimetric methods, such as dynamic ranges of the calibration graphs, detection limits and precision data have been obtained. The practical usefulness of the developed method is demonstrated by the analysis of citric juices.

Universidad de
Córdoba

Instituto Andaluz
de Química Fina y Nanoquímica

Consejería de Innovación,
Ciencia y Empresa

II ENCUENTRO SOBRE NANOCIENCIA Y NANOTECNOLOGÍA DE INVESTIGADORES Y TECNÓLOGOS DE LA UNIVERSIDAD DE CÓRDOBA

Córdoba, 14 de enero de 2010

P-16

KINETIC STUDY OF THE FORMATION OF COLLOIDAL GOLD NANOPARTICLES BY GALLIC ACID USING STOPPED-FLOW MIXING DEVICE AND LIGHT SCATTERING DETECTION

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The capability of gallic acid to reduce gold (III) chloride trihydrate to colloidal gold nanoparticles and the aggregation process in the presence of the cationic surfactant cetyltrimethyl ammonium bromide (CTAB) have been kinetically studied using a stopped-flow mixing device and light scattering as detection system. This study has given rise to a simple and rapid method for the determination of gallic acid in natural samples and can be extended to other antioxidant compounds. The aggregation process has been monitored by measuring the initial reaction-rate (v_0) and the light scattering signal at a prefixed-time (ΔIF_{40}), using stopped-flow mixing technique, which makes the method applicable to automate routine analysis. Each measurement was obtained in about 60 s, using an integration time of 0.1 s. The dynamic range of the calibration graph obtained for gallic acid, using the initial rate method, was 0.06 – 0.6 μM, and the detection limit was 0.01 μM. The precision of the method, expressed as relative standard deviation, ranged between 0.9 and 4.8 %.

REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA
SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

XII GRASEQA 2010

Córdoba, 10 y 11 de junio

LIBRO DE RESÚMENES

UNIVERSIDAD D CÓRDOBA

SC-36

Determinación directa de flavonoides en zumos de naranja mediante cromatografía de líquidos y detección fluorescente a larga longitud de onda

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Se describe un nuevo método para la determinación de flavonoides en zumos de naranja mediante separación cromatográfica y derivatización post-columna con detección fluorimétrica a larga longitud de onda. El sistema propuesto se basa en el uso de violeta de cresilo y cerio(IV) en un medio micelar de bromuro de cetiltrimetil amonio. Los flavonoides utilizados como analitos modelo y representativos de los existentes en zumo de naranja, son: dos flavonas agliconas (quercetina y kaempferol), una flavanona aglicona (naringenina), una flavona- O-glicosido (rutina) y dos flavanonas–O-glicosidos (hesperidina y naringina). El sistema derivatizante presenta una intensa señal fluorescente, la cual se monitoriza a las longitudes de onda de excitación y emisión de 585 y 625 nm, respectivamente. Esta señal disminuye al interaccionar el sistema con los analitos, siendo la disminución proporcional a la concentración de los mismos. Los intervalos dinámicos de las calibraciones y los límites de detección, obtenidos con disoluciones estándar de los flavonoides y expresados en ng mL^{-1} son: 12,2 – 4000, 3,7 (quercetina), 3,5 – 1000, 1,0 (kaempferol), 6,7 – 1000, 2,0 (naringenina), 5,0 – 800, 1,5 (rutina), 10,1 – 1000, 3,0 (hesperidina), y 17,8 – 800, 1,8 (naringina). Los coeficientes de regresión lineal fueron mayores de 0.993 en todos los casos. La precisión de método, expresada como desviación estándar relativa (%DER), fue estudiada a dos niveles de concentración para cada analito, obteniendo valores comprendidos entre 2,8 y 6,2 %. La utilidad práctica de método propuesto se ha demostrado mediante su aplicación al análisis de muestras de zumos de naranja, las cuales fueron diluidas y directamente inyectadas en el sistema cromatográfico. Los porcentajes de recuperación obtenidos están comprendidos entre 86,9 y 107,0 %.

NANO UCO

LIBRO DE RESÚMENES

NANOUCO III

Encuentro sobre Nanociencia y Nanotecnología de Investigadores y Tecnólogos Andaluces

Córdoba, 10 y 11 de febrero de 2011

EDICIONES DON FOLIO

P-1

DETERMINATION OF ANTIOXIDANT ADDITIVES IN FOODSTUFFS BY DIRECT MEASUREMENT OF GOLD NANOPARTICLE FORMATION USING RESONANCE LIGHT SCATTERING DETECTION

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The capability of antioxidant compounds to reduce gold(III) to gold nanoparticles (AuNPs) has been kinetically studied in the presence of cetyltrimethylammonium bromide using stopped-flow mixing technique and resonance light scattering as detection system. This study has given rise to a simple and rapid method for the determination of several synthetic and natural antioxidant used as additives in foodstuff samples. The formation of AuNPs was monitored by measuring the initial reaction-rate of the system in about 5 s, using an integration time of 0.1 s. Dynamic ranges of the calibration graphs and detection limits, obtained with standard solutions of the analytes, were (μmol L^{-1}): gallic acid (0.04 – 0.59, 0.01), propyl gallate (0.04 – 1.41, 0.01), octyl gallate (0.03 – 0.35, 0.08), dodecyl gallate (0.02 – 0.30, 0.007), butylated hydroxyanisol (0.07 – 0.39, 0.009), butylated hydroxytoluene (0.04 – 0.32, 0.01), ascorbic acid (0.11 – 1.72, 0.03) and sodium citrate (0.07 – 1.29, 0.02). The regression coefficients were higher than 0.994 in all instances. The precision of the method, expressed as RSD%, was established at two concentration levels of each analyte, with values ranging between 0.6 and 4.8 %. The practical usefulness of the developed method was demonstrated by the determination of several antioxidant additives in foodstuff samples, which were extracted, appropriately diluted and assayed, obtaining recoveries between 95.4 and 99.5 %. The results obtained were validated using two reference methods.

30 SECYTA

SECYTA

13as JORNADAS DE ANÁLISIS INSTRUMENTAL

RECINTO GRAN VIA. 14-18 NOVIEMBRE 2011

EXPOQUIMIA
Salón Internacional de la Química

Fira Barcelona

FLUORIMETRIC DETERMINATION OF POLYPHENOLS USING A LONG- WAVELENGTH-FLUOROPHOR AND LACCASE IMMOBILISED ON GOLD NANOPARTICLES

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A new method for the determination of polyphenols based on their competitive redox interaction with a long-wavelength-fluorophor (LWF) in the presence of the enzyme lacasse immobilised on gold nanoparticles (AuNPs) is presented. The biocatalyst causes a fluorescence inhibition of the LWF, but their effect is delayed in the presence of polyphenols. The competitive redox reaction has been kinetically studied using stopped-flow mixing technique and fluorimetry as detection system. The behaviour of several polyphenols (phenol, gallic acid, catechol, hydroquinone, resorcinol, pyrogallol and phloroglucinol) on the system has been comparatively studied and a simple and rapid method for the determination of these compounds has been developed. Initial-rate and induction time measurements have been used as analytical parameter using Cardio Green (λex= 764 nm, λem= 806 nm) as LWF. Dynamic ranges of the calibration graphs and detection.