



FACULTAD DE CIENCIAS DE LA SALUD

Departamento de Tecnología de la
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Evaluación de parámetros de Calidad y Seguridad
en Zumos de Mandarina

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Art 1*

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*“Lo que sabemos es una gota, lo que ignoramos
un inmenso océano”*

Isaac Newton

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ÍNDICE ABREVIATURAS

- $A_{1\%1\text{cm}}$: Coeficiente de absorción específico
- AAS: Espectroscopía de absorción atómica
- Ab: Anticuerpo
- ADN: Ácido desoxirribonucleico
- AES: Auger electron spectroscopy
- Ag*: Antígeno marcado
- Ag: Antígeno
- AI: anticuerpo inmovilizado
- AP: Fosfatasa alcalina
- B: Señal debida a los estándares
- B₀: señal en ausencia de analito
- BOCG: Boletín Oficial de las Cortes Generales
- BOE: Boletín Oficial del Estado
- CAECV: Comité de Agricultura Ecológica de la Comunidad Valenciana
- CCAA: Comunidades Autónomas
- Cds: Discos compactos de audio-video
- CDs: Ciclodextrinas
- CE: Comunidad Europea
- CE: Electroforesis capilar
- CEE: Comunidad Económica Europea
- CGTasa: Ciclodextrina glucanotransferasa
- CI: Conjugado inmovilizado
- DG SANCO: Dirección General de Salud y Consumidores
- DIME- β -CDs: (2, 6-dimetil)- β -CDs
- D_{L50}: Dosis letal, 50%
- DVDs: Disco óptico de almacenamiento de datos

EEUU: Estados Unidos
EIA: Inmunoensayo enzimático
ELISA: Enzyme-Linked ImmunoSorbent Assay
EPA: Agencia de Protección Ambiental
ER: Equivalentes de Retinol
FAO: Organización de Naciones Unidas para la Alimentación y la Agricultura
GC: Cromatografía de Gases
GRAS: En General Reconocido como Aditivo Seguro
HP- β -CDs: Hidroxipropil- β -CDs
HPE- β -CDs: Hidroxietil- β -CDs
HPLC: Cromatografía Líquida de Alta Eficacia
I₅₀: Concentración de analito que reduce el 50% de los sitios específicos de unión del anticuerpo
IAS: Técnicas Inmunoquímicas
IDA: Ingesta Diaria Admisible
IR: infrarrojo
JECFA: Comité de Expertos en Aditivos Alimentarios
JMPR: Joint FAO/WHO Meeting on Pesticide Residues
LC: Cromatografía Líquida
LMRs: Límites máximos de residuos
LOAC: Lab-on a chip
LOD: Límite de detección
mAbs: Anticuerpos monoclonales
MAGRAMA: Ministerio de Agricultura, Alimentación y Medio Ambiente
MARM: Ministerio de Agricultura, Alimentación y Medio Ambiente
MEMs : Sistemas microelectromecánicos
MS: Espectrometría de Masas
OFs: Plaguicidas Organofosforados
OMS: Organización Mundial de la Salud
pAbs: Anticuerpos policlonales

PC: Policarbonato

PCP: Pentaclorofenol

PMMA: Polimetilmetracrilato

QqQ: Espectrometría de Masas de Triple Cuadrúpulo

rAb: Anticuerpos recombinantes

RC: Reactividad cruzada

RD: Intervalo de trabajo

RIA: Radioinmunoensayo

SFE: Extracción con fluidos supercríticos

Tm: Tonelada

TRIME- β -CDs: (2, 3, 6-trimetil)- β -CDs

UE: Unión Europea

USDA: United States Department of Agriculture

UV-Vis: Ultravioleta Visible

ϵ : Coeficiente de Absorción Molar

λ_{max} : Longitud de Onda Máxima

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CAPÍTULO I. INTRODUCCIÓN

1. LOS CÍTRICOS

1.1. HISTORIA

El origen de los cítricos se localiza en el sureste de Asia, en una zona que abarca desde Arabia Oriental hasta Filipinas y desde el Himalaya hasta Indonesia. Dentro de esta extensa región, el noreste de la India, el norte de Birmania y la provincia de Yunnan -en el centro sur de China-, se consideran los tres centros de origen más importante (Gmitter y Hu, 1990).

En España, los cítricos se adaptaron muy bien al clima levantino, sin embargo, no hubo plantaciones regulares hasta finales del siglo XVIII. Durante la primera mitad del siglo XIX el crecimiento de la citricultura fue lento, posteriormente los resultados comerciales empezaron a ser positivos, sobre todo en el campo valenciano por las condiciones climatológicas, la calidad de los suelos y la facilidad del riego entre otros factores. Aumentó el cultivo y la producción y entre los años 1860-1870, los cítricos empezaron a alcanzar una significación económica y exportadora importante, que supuso el inicio de la gran expansión actual. En poco tiempo la naranja pasó a ser uno de los principales productos de exportación española.

El cultivo de la mandarina se introduce en España en el año 1845 y unos años más tarde la variedad de naranja sanguina, procedente de la isla de Malta. Finalmente el pomelo, de introducción reciente, fue importado de Estados Unidos. Actualmente el cultivo de cítricos se extiende por la mayor parte de las regiones tropicales y subtropicales comprendidas entre las latitudes 44º N y 41º S (Agustí, 2000).

Los tres géneros de cítricos cultivados en la actualidad son : *i*) *Fortunella* (kumquat), *ii*) *Poncirus*, formado por especies de hoja trifoliada caduca, de gran resistencia al frío y utilizados como portainjertos y *iii*) *Citrus*, que incluye las especies más importantes desde el punto de vista agronómico como las naranjas dulces (*Citrus sinensis* [L.] Osb.), mandarinas (*Citrus reticulata* Blanco y *Citrus unshiu* Marc.), pomelos (*Citrus paradisi* Macf.), limones (*Citrus limon* Burm. f.) y limas (*Citrus aurantifolia* L.) (Agustí, 2003).

1.2. IMPORTANCIA ECONÓMICA DEL SECTOR CITRÍCOLA

Los cítricos se cultivan en más de un centenar de países, abarcando una superficie total de 6,7 millones de hectáreas, que producen 108 millones de toneladas, cifra muy superior a la de otras frutas como los plátanos (70 millones de toneladas) o las uvas (69 millones).

España, con una producción de poco más de 6 millones de toneladas, ocupa el quinto lugar en la producción mundial de cítricos, precedido de Brasil (20 millones de toneladas), China (supera los 17 millones de toneladas), EEUU (11 millones de toneladas) y México (6.5 millones de toneladas). La producción de naranjas es la mayoritaria a nivel mundial (60%), seguida de las mandarinas (24%), limones (12%) y pomelos (4%). Las exportaciones mundiales de cítricos superan los 12 millones de toneladas y España es el país con el mayor volumen de exportación de cítricos para su consumo en fresco -3 millones de toneladas-, seguido por Suráfrica y EEUU, con 2 y 1 millón de toneladas, respectivamente. Esto datos evidencian que España puede canalizar en torno al 25% del comercio mundial de cítricos.

Además, España es el segundo productor mundial de mandarina -10% de la producción total-, siendo la UE su mercado más importante, principalmente Alemania y Francia, países a los que destina el 50% de la exportación.

La producción de cítricos en España se localiza en cuatro comunidades autónomas: la Comunidad Valenciana, que constituye la zona de producción más importante (62% de la producción nacional), Andalucía (22%), Región de Murcia (13,5%) y Cataluña (2,5%). Los cítricos de mayor producción en España son las naranjas (53%), seguidas de las mandarinas (33%) y los limones (14%) y liderando la Comunidad Valenciana la producción de naranjas y mandarinas. En el grupo de las mandarinas destaca la producción de clementinas (82%), especialmente de la variedad Clementino de Nules (*Citrus clementina* Hort. Ex Tan.), (FAO, 2013; MAPA, 2013).

Los cítricos constituyen con diferencia el principal grupo de frutales en España en cuanto a superficie cultivada. De hecho, el 60,9% de la superficie cultivada de frutales corresponde a los cítricos (MARM, 2013), seguidos de los frutales de hueso (27,4%) y de pepita (11,7%).

En el año 2012, casi un 0,7% de la superficie geográfica de España se destinó al cultivo de cítricos, correspondiendo ésta mayoritariamente al cultivo de naranja

y mandarina, que representan respectivamente el 50% y 36 % de la superficie cítrica total, y en menor medida al limonero, con un peso relativo del 13%. La principal área cítrica continúa localizándose en la Comunidad Valenciana (**Figura I.1**), con aproximadamente el 60% de la superficie total, seguida de Andalucía (25%) y la Región de Murcia (14%).

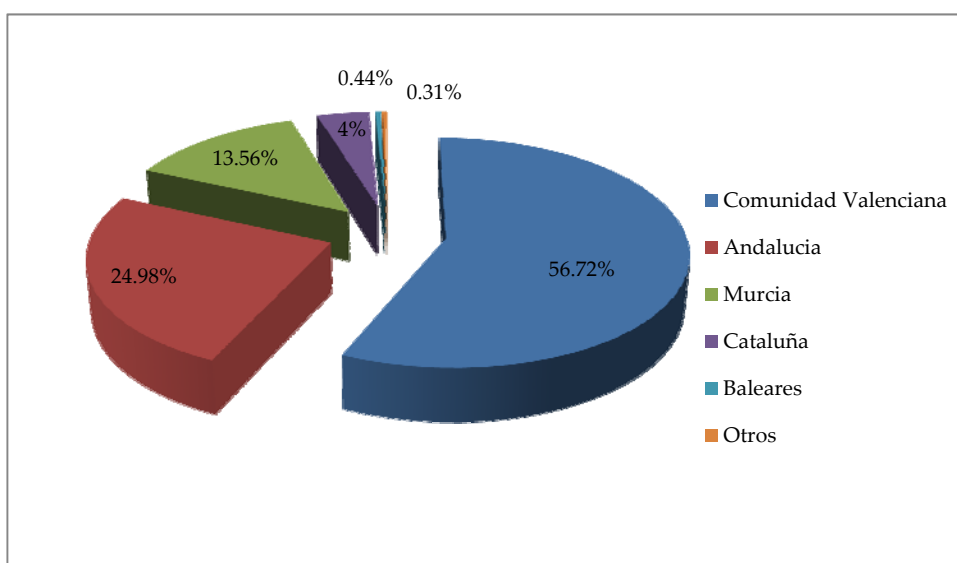


Figura I.1: Distribución de la superficie cítrica española por CCAA (comunidades autónomas) (Fuente: MARM, 2013).

La Región de Murcia, tercera zona productora de España, ha registrado una evolución espectacular en cuanto a superficie cítrica cultivada, con incrementos del 150%. Por otra parte, según las estimaciones del Servicio de Estudios Agrarios y Comunitarios de la Consejería de Agricultura, Pesca y Alimentación de la Generalitat Valenciana y partiendo del supuesto de que no se produzcan eventos extraordinarios que alteren de forma significativamente la evolución española de cítricos, se estima que la campaña 2012/2013 será de 6,1 millones de toneladas, el 5,74% menos que en la cosecha anterior. El descenso estimado será de 372.000 toneladas para el conjunto de cítricos, aunque la situación varía por productos.

La mayor caída corresponderá a las mandarinas, con cerca de 2,1 millones de toneladas, el 13,1% menos que en 2011/2012. Esta disminución se debe principalmente a las clementinas, cuya producción se reduce un 17,6% hasta 1.430

10³ toneladas, lo que contrasta con el crecimiento de un 46,56% para las satsumas, que llegarán a las 192.000 toneladas.

Por el contrario, la cosecha de naranja dulce será el 1,97%, más voluminosa que la del año pasado, con 3,1 millones de toneladas. Por variedades, se prevé una cosecha un 1,74% mayor de navel, con 2,34 millones de toneladas, y las variedades tardías crecerán el 8,5%, hasta las 548.000 toneladas, mientras que las blancas caerán el 9,58%, hasta las 217.000 toneladas.

La cosecha de cítricos de la Comunidad Valenciana en la campaña 2012-2013 será de 3.013.856 toneladas, lo que representa un descenso del 4,9% en el volumen de producción respecto a la campaña anterior. Mientras la primera zona productora de España prevé para esta campaña una disminución de la producción, Andalucía espera producir durante esta temporada más de 1,6 toneladas de cítricos, una cifra que supone un aumento del 4,2% respecto a 2011-2012 y un 8,5% más que la media de las cuatro últimas campañas (**Tabla I.1**).

Tabla I.1: Producción citrícola estimada en el periodo 2012-2013 tanto a nivel nacional como por Comunidades Autónomas (Fuente: Freshfel, 2013).

ZONA	Previsión 2012/13 (Tm)	Diferencia 2011/12 (Tm)%
Andalucía	1.683.635	+4,2%
Valencia	3.013.856	-4,9%
Murcia	557.000	-14,9%
ESPAÑA	6.109.000	-5,7%

Según las estimaciones realizadas por los servicios técnicos de la Consejería de Agricultura, Pesca, Alimentación y Agua de la Generalitat Valenciana, los datos revelan una caída del 12% en la producción de mandarinas, que se cifra en 1.383.867 toneladas, con descensos significativos en el subgrupo clementinas y en el de mandarinos e híbridos (**Tabla I.2**).

En este grupo, las clementinas presentan una merma del 10,18% que se reparte de forma equilibrada entre las tempranas, las de media temporada y las tardías. La variedad clemenules presenta en esta campaña una bajada del 13,49%. El descenso más acusado se da en el subgrupo híbridos, con un 27,73%, motivado por una importante reducción de la producción de ortanique, clemenvilla y fortune. Por el contrario, la cosecha de satsumas se estima que crecerá un 21%.

En el caso de las naranjas se prevé una cosecha de 1.422.679 toneladas, lo que supone un moderado incremento del 3,68% respecto al volumen obtenido

durante la campaña 2011-2012. Dentro de este grupo, cabe destacar el incremento de un 4,12% del subgrupo navel, debido al aumento de las navelinas, y del 1,97% de las blancas motivado por la subida de la valencia late un 9,7%.

Tabla I.2: Producción cítrica estimada en el periodo 2012-2013 por cultivo (Fuente: MAGRAMA, 2013).

Cultivo*	2010	2011	Avance 2012/2013
Naranja	3.114,8	2.801,3	2.884,1
Satsuma	167,2	150,1	177,1
Clementina	1.508,4	1.413,6	1.263,4
Hibrido	521,3	552,2	441,0
Mandarina Total	2.196,9	2.115,9	1.881,5

A pesar de esta bajada en la producción cítrica la campaña ofrece fruta de excelente calidad y calibre, en parte, gracias al Código de Buenas Prácticas Comerciales que desde febrero del 2013, al que todos los operadores del sector se han adherido para asegurar un marco de relaciones de mercado transparentes que favorezcan el equilibrio de la cadena agroalimentaria, ofreciendo productos seguros y de calidad (BOCG, 2013).

Para la campaña 2012-2013, Murcia seguirá siendo la tercera comunidad autónoma productora de cítricos en España, que en el año en curso representaron el 13% del valor de todos los productos vegetales de la Región, y un 9% de la producción de la rama agraria, incluyendo la ganadería. Además, Murcia es la principal productora de limón y pomelo. En 2011 los cítricos de la Región de Murcia representaron el 8% de la producción nacional. En 2010, las producciones de limón y pomelo supusieron el 56 y el 68%, respectivamente, de la cosecha nacional.

La Consejería de Agricultura y Agua de la Región de Murcia prevé una bajada moderada en la cosecha de cítricos a consecuencia de las bajas temperaturas registradas durante los primeros meses del año 2013. Según las estimaciones, la cosecha total de cítricos asciende a 557.000 toneladas, lo que supone un descenso del 14,9 por ciento en relación al volumen de la pasada campaña, que alcanzó 654.983 toneladas. Las bajas temperaturas durante el invierno afectaron principalmente al limón, y en menor medida a la naranja (5,9 %) y mandarina (2,4 %).

La importancia de la citricultura en el mundo, y en particular en España, está fuera de toda duda. Sin embargo, no se está exenta de inconvenientes a consecuencia de los techos en el consumo y producciones crecientes, que conducen a una caída de precios. El avance de la citricultura implica una mejora de la rentabilidad de las exportaciones, que pasa por la reducción de los costes de cultivo, análisis de rutina más rápidos y baratos que no retrasen la salida de los cítricos al mercado, la reducción de la estacionalidad de la oferta, la mejora de la calidad del fruto o dar salida a los excedentes a través de zumos y productos derivados de mayor calidad, capaces de satisfacer las exigencias del mercado (Agustí, 2000). Éste puede constituir uno de los mercados de mayor crecimiento, complementando así al de la producción de fruta para consumo en fresco.

1.3. AGRICULTURA ECOLÓGICA

La Agricultura Ecológica es un sistema agrario basado en la obtención de alimentos de máxima calidad organoléptica, sanitaria y en el proceso productivo, respetando el medio ambiente y conservando al mismo tiempo la fertilidad de la tierra de cultivo mediante la utilización óptima de los recursos naturales, sin el empleo de productos de síntesis química, procurando así un desarrollo agrario perdurable (CAECV, 2013).

La agricultura ecológica se encuentra regulada legalmente en España desde 1989, al amparo del Reglamento de Denominación Genérica "Agricultura Ecológica", que fue de aplicación hasta la entrada en vigor del Reglamento (CEE) 2092/91 sobre la producción agrícola ecológica y su indicación en los productos agrarios y alimenticios.

Desde el 1 de enero de 2009 -fecha en que comenzó a aplicarse la producción ecológica-, se encuentra regulada por el Reglamento (CE) 834/2007 del Consejo relativo a la producción y etiquetado (MAGRAMA, 2013). Dentro del crecimiento favorable que presenta la producción ecológica, especialmente en términos de exportación, destaca la producción de cítricos gracias al crecimiento de la demanda exterior de productos como la naranja.

Según un informe publicado por la Consejería de Agricultura y Pesca de la Junta de Andalucía en 2006, los principales beneficios directos del proceso de conversión a la citricultura ecológica son: *i*) el incremento de los ingresos debido a los mejores precios de venta y *ii*) una incidencia de plagas similar -o inferior-, a las

detectadas en explotaciones convencionales. Entre los aspectos negativos, encontramos el incremento de los costes de abonado y control de hierbas, la disminución de los calibres o el menor volumen de producción.

Además, hay que valorar las cualidades que ofrecen estos productos y que contribuyen a su comercialización y a mejorar la percepción por parte de los consumidores. En este sentido, destacan aspectos como la calidad organoléptica, la ausencia de residuos químicos en la fruta y una contribución positiva al mantenimiento de la biodiversidad y el ecosistema.

Según los datos publicados por el MAGRAMA, la superficie inscrita de cítricos ecológicos en España en el año 2011, alcanzó las 5.855 hectáreas. La mayor parte de esta superficie corresponde al cultivo de naranja ecológica con 3.041 hectáreas, seguida del limón con 1.417 hectáreas y 1.165 hectáreas para mandarina.

Por Comunidades Autónomas, Andalucía presenta la mayor superficie inscrita con 3.921 hectáreas, seguida por la Comunidad Valenciana con 1.008 hectáreas y por la Región de Murcia con 718 hectáreas, quedando el resto de comunidades muy por debajo de estas cifras.

Por su parte, la producción total de cítricos ecológicos a nivel nacional alcanzó en dicho periodo las 108.615 toneladas, de las que más de la mitad (57.969 hectáreas) fueron de naranjas ecológicas (MAGRAMA, 2013).

1.4. LAS MANDARINAS (*Citrus reticulata*)

1.4.1. Requerimientos edafoclimáticos

Generalmente, los mandarinos son árboles más resistentes al frío que los naranjos, sin embargo, sus frutos son más sensibles al frío. Los árboles también son más tolerantes a la sequía. En general, las mandarinas pueden sufrir más fácilmente lesiones por frío que las naranjas y pomelos.

El mandarino es muy fácil de adaptar a diversos climas. Puede cultivarse en condiciones climáticas desérticas, semitropicales y subtropicales. A pesar de esta adaptabilidad, para que el fruto llegue a tener una buena calidad y la producción sea copiosa, cada variedad necesita unas condiciones climáticas muy concretas. Por ejemplo, una de las variedades más exigentes en cuanto a las condiciones climáticas

es la "Clementina"; actualmente su distribución se limita a las zonas costeras de Marruecos, España y Córcega (Amorós, 1999).

1.4.2. Características generales de las mandarinas

Sin lugar a duda, la mandarina es una fruta atractiva al consumidor gracias a su sabor, color, aroma y facilidad de pelado. Además, es un producto poco perecedero y soporta relativamente bien el transporte y comercialización posterior.

Las mandarinas se producen en gran parte del mundo y se comercializan en fresco y transformadas, ya sean zumos, gajos en conserva o mermelada.

Los frutos del mandarino son bayas de forma redondeada denominadas hesperidios, en los que se distinguen tres partes: *i*) el epicarpio o flavedo, corresponde a la porción coloreada de la piel y contiene la mayor parte de los pigmentos y aceites esenciales de la mandarina. Inmediatamente debajo del epicarpio, se localiza el *ii*) mesocarpio o albedo, que es una capa blanca y esponjosa que contiene flavonoides, sustancias pécticas y hemicelulasas y envuelve completamente el *iii*) endocarpio, que es la porción comestible de los frutos (**Figura I.2**). La capa correspondiente al albedo y flavedo -la piel del fruto-, se denomina pericarpio.

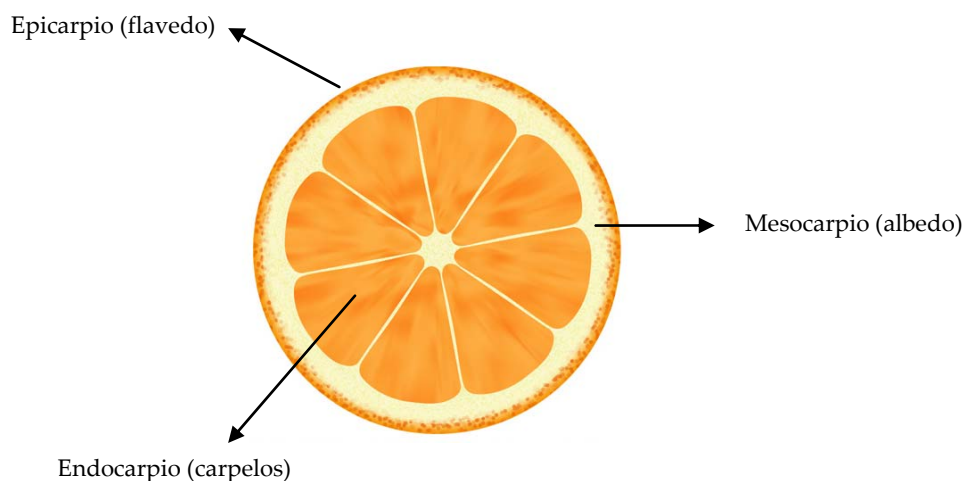


Figura I.2: Sección transversal de una mandarina (Fuente: Ting y Rouseff, 1986)

El endocarpio está formado por entre 9 y 13 carpelos (“gajos”), dentro de los cuales se encuentran vesículas en forma de huso, que contienen el zumo. En el endocarpio se localizan las semillas, que contienen una importante cantidad de grasa (Ting y Rouseff, 1986).

Los componentes químicos de los frutos se distribuyen entre estas tres estructuras, aunque en algunos casos suelen encontrarse en mayor concentración en alguna de ellas. Así, tanto los azúcares como los ácidos se localizan fundamentalmente en las vesículas que contienen el zumo (Ting y Rouseff, 1986).

Al igual que el resto de los agrios, las mandarinas pertenecen al género *citrus*. Sin embargo, dentro de este género su clasificación posterior es problemática.

1.5. PARÁMETROS FÍSICO-QUÍMICOS EN ZUMOS DE MANDARINA

Por sus características organolépticas y nutritivas especiales, los zumos de mandarina son bebidas muy particulares. La caracterización y análisis de los zumos cítricos es un aspecto de capital importancia en el procesado de los zumos. Desde un punto de vista nutricional los cítricos son significativos. Ya que son una fuente importante de ácido ascórbico, compuesto con actividad vitamínica C (Rojas-Hidalgo, 1998; Yanishlieva-Maslarova y Heinonen, 2001). La acidez que confieren estos compuestos constituye un importante factor en relación con la aceptabilidad de los zumos cítricos. Así, el zumo de mandarina se suele considerar aceptable con un 1% de ácidos y un pH en torno a 3,5 (Varnan y Sutherlan, 1994). De acuerdo con las normas generales establecidas en la Directiva 2012/12/UE del Parlamento Europeo y del Consejo, de 19 de abril de 2012, por la que se modifica la Directiva 2001/112/CE del Consejo, relativa a los zumos de frutas y otros productos similares destinados a la alimentación humana (BOE, 2012), se considera como zumo aquel obtenido por procedimiento mecánico del endocarpio de mandarinas (*Citrus reticulata*), maduras y en buen estado, conservado exclusivamente por medios físicos, sin fermentar, pero fermentable, destinado al consumo directo (**Tabla I.3**). El producto deberá tener el color y sabor característicos del zumo de mandarina.

Se permite la restitución de los componentes volátiles naturales del zumo a cualquier zumo de mandarina del que se hayan extraído dichos componentes volátiles naturales. El zumo podrá haber sido concentrado previamente y reconstituido posteriormente con agua para conservar los factores esenciales de

composición y calidad del zumo. Según la nueva directiva 2012/12/UE, a partir del 28 de octubre de 2015, ningún zumo de frutas contendrá azúcares añadidos.

Tabla I.3: *Parámetros de calidad en zumos de mandarina (Fuente: BOE, 2012).*

Parámetros	Unidad de medida	Valor	Observaciones
Grado Brix		min. 10,5	Zumo directo
Grado Brix		min. 11,2	Zumo a base de concentrado
Acidez	meq/L	90 - 300	Los valores indicados corresponden a 5,8 - 19,2 g/L, calculados como ácido cítrico anhidro a pH 8,1
Acido cítrico	g/L	6 - 22	
Acido D-isocítrico	mg/L	65 - 200	El valor inferior a 65 mg/L se obtiene en productos de alta ratio. En clementinas se han obtenido hasta 40 mg/L.
Acido cítrico: Acido D-isocítrico	mg/L	máx. 130	En clementinas se han obtenido valores superiores a 200
Acido L-ascórbico	mg/L	min. 100	La media natural de contenido de ácido L-ascórbico del zumo recién exprimido está entre 250 y 350 mg/L. Deben garantizarse los 100 mg/L de ácido L-ascórbico a la fecha de consumo preferente-
Sacarosa	g/L	20-60	Algunas variedades de mandarina presentan contenidos altos de sacarosa, especialmente en productos recién exprimidos. Su participación porcentual en el total de azúcares puede ser superior al 50% y llegar hasta el 70%.
Maltosa		ausencia	
Isomaltosa		ausencia	
Índice de Formol mL NaOH 0,1M/100 ml		15 - 26	Cuando el valor es inferior al valor mínimo establecido, debe examinarse el origen.
Cenizas	g/L	2,5 - 5,0	
Fósforo total	mg/L	90 - 210	
Potasio	mg/L	1000 - 2300	

1.6. COMPUESTOS BIOACTIVOS EN ZUMOS DE MANDARINA

Los frutos cítricos y sus zumos son una fuente importante de compuestos bioactivos en la dieta, (carotenoides, flavonoides, vitamina C, vitamina A y elementos minerales, entre otros) con efectos beneficiosos para la salud (Gorinstein y col., 2001; Mahan y col., 2000). Los efectos biológicos de estos compuestos dependen de la fracción de los mismos que puede asimilar el organismo para ejercer un efecto funcional. Los efectos protectores frente al daño oxidativo en células y tejidos, y la modulación del ciclo celular por un determinado alimento, se deben principalmente a las sinergias entre los diferentes compuestos bioactivos del mismo.

El reconocimiento de los componentes fisiológicamente activos en los frutos cítricos como la naranja y mandarina, y su contribución a la salud humana, se ha convertido en un área de investigación emergente. El género *Citrus* se ha caracterizado por una acumulación sustancial de glicósidos de flavonona, los cuales no se encuentran en otras frutas. La vitamina C y los carotenoides, por ejemplo, parecen jugar un papel importante en la prevención de ciertos tipos de cáncer, enfermedades cardiovasculares y cataratas, a través de la neutralización de procesos oxidativos (Rodríguez-Amaya, 2012).

Entre los principales compuestos bioactivos que podemos encontrar en el zumo de mandarina destacan los siguientes:

1.6.1. Pigmentos Carotenoides

El sistema de determinación de la calidad de los cítricos del departamento de agricultura de Estados Unidos (USDA), reconoce la importancia del color en la calidad de los productos comerciales de cítricos y adjudica la misma importancia al color que al sabor. El color de los zumos de mandarina se debe fundamentalmente a los pigmentos carotenoides, aunque en algunas variedades tienen bastante influencia los antocianos. En los frutos no maduros el color se debe a las clorofilas, a medida que maduran sus niveles disminuyen, aumentan la concentración de carotenoides. Los principales carotenoides responsables del color del zumo de mandarina son α -caroteno y β -caroteno, zeta-anteraxantina (amarillento), violaxantina (amarillento), β -citraurina (naranja rojizo) y β -criptoxantina (naranja) (Lin y Chen, 1995). Las mandarinas son el fruto cítrico de color más intenso y la

coloración rojo-anaranjada de su endocarpio se atribuye a la alta concentración de β -criptoxantina y β -caroteno (Farin y col., 1983).

Como ya se ha comentado, el color de los zumos de naranja y mandarina se debe generalmente a los pigmentos carotenoides. En las naranjas y mandarinas, estos pigmentos se encuentran fundamentalmente en el epicarpio o flavedo (corteza) y en el endocarpio (pulpa y zumo), que constituye la principal porción comestible del fruto (Pascual y col., 1993).

Las primeras publicaciones en las que se hace referencia a los pigmentos conocidos actualmente como carotenoides datan de principios del siglo XIX. En ellas se evidenció la presencia de unos compuestos lipofílicos y bastantes lábiles en pimientos maduros, hojas otoñales y zanahorias, derivando de su nombre científico *-Daucus carota-*, el término "caroteno". Al tratarse de compuestos poco estables, la investigación relativa a estos pigmentos fue avanzando lentamente durante el resto del siglo (Eugster, 1995).

No fue hasta principios del siglo XX (1907), cuando Willstätter determinó la fórmula molecular del "caroteno" ($C_{40}H_{56}$) y de la "xantofila" de las hojas ($C_{10}H_{56}O_2$). Un año antes, el botánico ruso Tswett logró separar las clorofilas a y b de los carotenoides de un extracto de hojas verdes, mediante una columna de carbonato cálcico (Tswett, 1906). Precisamente el término "cromatografía" se debe a que en este ensayo se separaron bandas de distinta coloración.

La naturaleza altamente insaturada de los carotenoides, se puso de manifiesto en 1928 mediante hidrogenación catalítica, comprobándose algunos años más tarde la relación existente entre el número de dobles enlaces conjugados y el color de los pigmentos. La formulación correcta de los grupos terminales de licopeno, α -caroteno, β -caroteno, luteína, zeaxantina y de otros carotenoides se debió a Karrer y col. (1930). En este período también se evidenció la relación existente entre los carotenoides y la vitamina A (Moore, 2001), despertando así el interés en el estudio de estos pigmentos. Años más tarde, Zechmeister (1934) elucidó correctamente 11 carotenoides y Karrer y Jucker (1948) llegaron a 31. Los nuevos descubrimientos se fueron sucediendo progresivamente, y en los años 90 se habían descrito más de 600 carotenoides (Rodríguez-Amaya, 1999).

Los carotenoides son sustancias hidrofóbicas, lipofílicas y en consecuencia insolubles en agua. Se disuelven en disolventes orgánicos como acetona, alcohol, éter dietílico, tetrahidrofurano y cloroformo. Los carotenos son fácilmente solubles

en éter de petróleo y hexano. Las xantofilas se disuelven mejor en etanol y metanol (Rodríguez-Amaya, 1995).

Atendiendo a su estructura se incluyen dentro de los terpenos, denominados también isoprenoides -moléculas muy abundantes en los vegetales y su clasificación viene determinada por el número de isoprenos que contienen-, se consideran biosintéticamente derivados del ácido mevalónico, intermediario metabólico que aporta la unidad básica estructural. Los carotenoides son los únicos tetraterpenoides conocidos (Háda y col, 2012).

Están formados básicamente por ocho unidades de isopreno, de modo que la unión de cada unidad se invierte en el centro de la molécula. Como resultado de ello, los dos grupos metilo centrales de la cadena polieno están separados por seis átomos de carbono, mientras que el resto lo están por cinco. La **Figura I.3** muestra la estructura de algunos carotenoides a los que se atribuye actividad antioxidante (Schroeder y col., 2006).

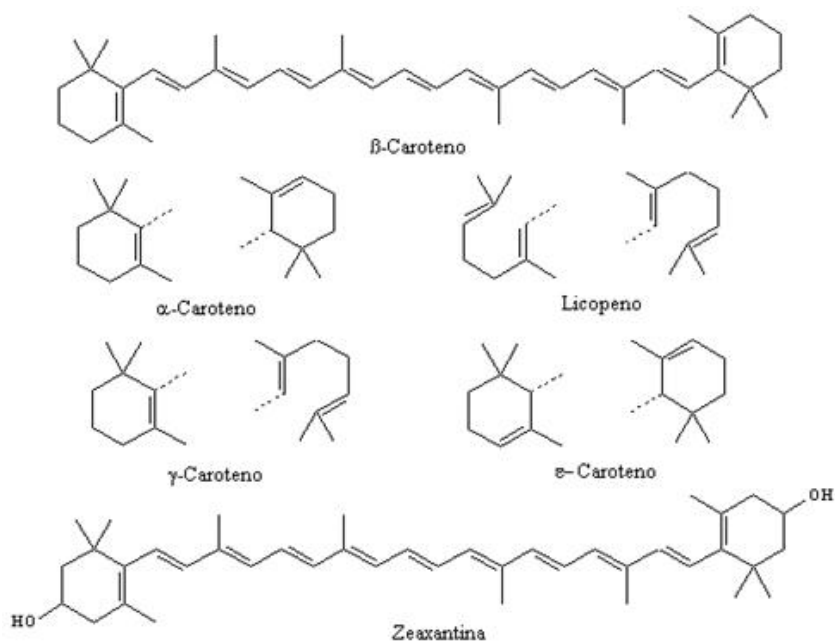


Figura I.3: Estructura de algunos carotenoides característicos de naranja y mandarina (Fuente: Meléndez-Martínez y col., 2007).

Pueden dividirse en dos grandes grupos: los carotenos, que son estrictamente carotenos y las xantofilas, que contienen oxígeno (Mínguez-Mosquera, 1997). Los cítricos pueden llegar a tener más de 115 compuestos carotenoides distintos. Los principales carotenoides encontrados en naranja y mandarina son: criptoxantina, violaxantina, neoxantina, luteína, anteraxantina, α -caroteno, y β -caroteno (Meléndez-Martínez y col., 2007).

1.6.1.1. Biosíntesis de los carotenoides

Los carotenoides son sintetizados por plantas superiores, algas (especies de *Dunaliella*, *Haematococcus*, etc.), hongos (*Blakeslea trispora*, *Phycomyces blakesleanus*, *Puccinia distincta*, etc.) y bacterias (*Rhodospirillum rubrum*, *Rhodospirillum rubrum*, *Rhodospirillum rubrum*, especies de *Brevibacterium*) (Yuan y col., 2002; Davoli y Weber, 2002).

Como se comentó anteriormente, la biosíntesis de los pigmentos carotenoides se engloba dentro de la ruta biosintética de los isoprenoides, compuestos que derivan del ácido mevalónico. El precursor inmediato de los pigmentos carotenoides es el geranyl pirofosfato, compuesto de 20 átomos de carbono, formado por cinco unidades de isopreno, que por dimerización da lugar al fitoeno, caroteno de 40 átomos de carbono (Fraser y Bramley, 2004). A partir de este caroteno incoloro -con tres dobles enlaces conjugados-, se forman sucesivamente una serie de carotenos mediante reacciones de desaturación: fitoflueno -cinco dobles enlaces conjugados-, ζ -caroteno -siete dobles enlaces conjugados-, neurosporeno -nueve dobles enlaces conjugados-, y licopeno (once dobles enlaces conjugados). A través de reacciones de ciclación, se obtienen los primeros carotenoides cíclicos a partir de neurosporeno y licopeno.

Las xantofilas se forman posteriormente por introducción de funciones oxigenadas (Rodríguez-Amaya, 2001; Horneo-Méndez y Britton, 2002).

Las etapas finales en la biosíntesis de carotenoides se esquematizan en la **Figura I.4**, aunque debe tenerse en cuenta que en muchos casos, existen pocas evidencias bioquímicas o simplemente ni las hay, en relación con los mecanismos propuestos.

La información relativa a las diferentes enzimas que intervienen en la síntesis de carotenoides puede consultarse en Fraser y Bramley, 2004 y Sandmann, 2005.

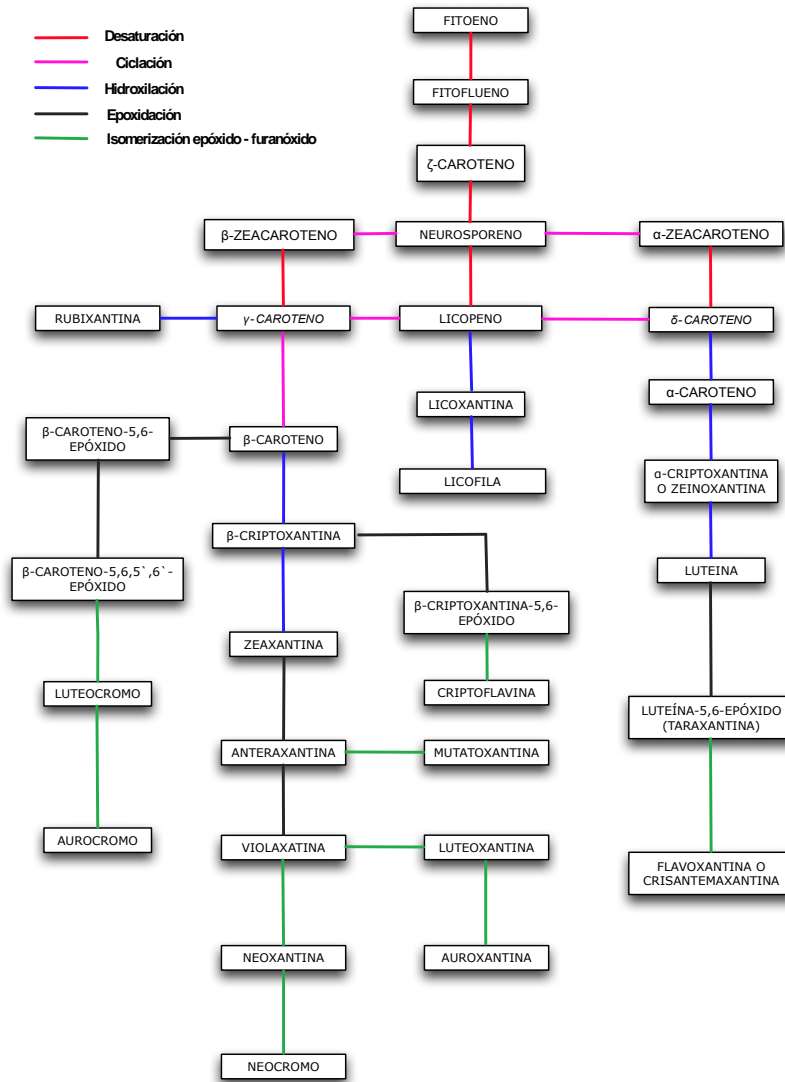


Figura I.4: Etapas finales en la biosíntesis de carotenoides (Fuente: Fraser y Bramley, 2004 y Sandmann, 2005).

1.6.1.2. Factores que afectan al contenido en carotenoides

El perfil de carotenoides en los productos vegetales depende de una serie de factores, como la variedad, parte de la planta, estado de maduración, clima y

zona geográfica de producción, procesado industrial y condiciones de almacenamiento, entre otros. La variedad afecta a la composición de carotenoides en términos cuantitativos, ya que habitualmente en todas las variedades de una misma especie se suelen encontrar los mismos carotenoides mayoritarios (Rodríguez-Amaya, 2001).

No cabe duda que el perfil de carotenoides varía considerablemente en función de la parte de la planta que se considere. Así, en el caso de los frutos se ha comprobado que la concentración y el tipo, varían entre la piel o corteza y la pulpa (Gandul-Rojas y col., 1999). La maduración de los frutos favorece la carotenogénesis, debido a la transformación de los cloroplastos en cromoplastos. Como consecuencia de ello, el patrón típico de carotenoides de los cloroplastos da paso a un perfil mucho más complejo, evidenciándose un aumento importante de estos pigmentos, en términos cuantitativos (Rodríguez-Amaya, 2001).

Las zonas geográficas de producción también influyen sensiblemente en el contenido de carotenoides, probablemente debido a las diferencias climáticas y nutrientes del suelo de cultivo.

Respecto a la estabilidad, los carotenoides son pigmentos estables en su ambiente natural, pero cuando los alimentos se someten a tratamiento térmico, o son extraídos en disolución en aceites o en disolventes orgánicos, se vuelven mucho más lábiles. Así, se ha comprobado que los procesos de oxidación son más acusados cuando se pierde la integridad celular, de forma que en alimentos vegetales triturados la pérdida de compartimentación celular pone en contacto sustancias que pueden modificar estructuras e incluso destruir los pigmentos. No todos los tipos de cocinado afectan en la misma medida a los carotenoides, de forma que la pérdida de estos pigmentos aumenta en el siguiente orden: cocinado con microondas < cocinado al vapor < hervido < salteado (Rodríguez-Amaya, 1999).

Generalmente, los carotenoides son insolubles en agua y por ello las pérdidas por lixiviación durante el lavado y procesado de frutos son mínimas. Otros tratamientos empleados en la industria alimentaria, como por ejemplo el tratamiento a alta presión, no afectan significativamente a los niveles de carotenoides en diversos productos vegetales. El escaldado industrial de los alimentos puede originar pérdidas de carotenoides, si bien la inactivación enzimática que produce, previene mermas posteriores durante el procesado y almacenamiento. En cambio, la congelación, la adición de antioxidantes y la

exclusión del oxígeno -vacío, envases impermeables al oxígeno, atmósfera inerte-, disminuyen las pérdidas durante el procesado y almacenamiento de los alimentos (Rodríguez-Amaya, 1999).

La destrucción de estos pigmentos reduce el valor nutritivo de los alimentos e induce una decoloración y una pérdida de sus características organolépticas. El grado de decoloración va a depender fundamentalmente de la presencia de agentes oxidantes en el medio -sobre todo oxígeno molecular-, y de que se comunique suficiente energía - en forma de luz o calor-, para que la reacción de degradación tenga lugar. La reacción de decoloración supone la pérdida de conjugación de la molécula y, en principio, no tiene por qué implicar la rotura del esqueleto hidrocarbonado, por lo que cualquier factor capaz de interrumpir la deslocalización electrónica existente -resonancia-, podría provocar la pérdida de color. Si las condiciones oxidantes son débiles y la energía suministrada no es suficiente, se vuelve a restaurar el orbital molecular, con la posibilidad de que la estructura adopte la configuración *cis* o *trans*, en función de que haya habido rotación en el enlace. Si las condiciones son muy severas, el grado de degradación progresa, fragmentándose entonces el pigmento (Meléndez-Martínez y col., 2007).

En resumen, se puede afirmar que son varios los factores que influyen en la degradación de carotenoides en sistemas modelo, como por ejemplo la estructura del carotenoide, el grado de exposición a la luz, la actividad de agua, temperatura, presencia de oxidantes o antioxidantes en el medio, presencia de sulfitos, etc. Sin embargo, los estudios de estabilidad son más complejos en matrices reales como los alimentos, debido a sus diferencias estructurales, composición, tipo de procesado industriales, etc.

1.6.1.3. Propiedades físico-químicas

Los carotenoides son compuestos lipofílicos, aunque existen algunas excepciones. Debido a ello son insolubles en agua y solubles en disolventes orgánicos como acetona, etanol, metanol, acetato de etilo, éter dietílico, hexano, éter de petróleo, cloroformo y piridina, entre muchos otros.

Los puntos de fusión son elevados, generalmente comprendidos en el rango 130-220 °C. Los carotenoides cristalizan formando estructuras diferentes y los cristales son muy susceptibles a la oxidación, por lo que deben conservarse en atmósfera inerte o al vacío. La solubilidad de estos cristales es pequeña, siendo más

solubles en disolventes orgánicos clorados, como cloroformo y diclorometano y en benceno (Britton, 1992; Schiedt y Liaaen-Jensen, 1995; Rodríguez-Amaya, 2001). El sistema de dobles enlaces conjugados presente en los carotenoides es responsable de la intensa coloración de estos compuestos. Para que estos pigmentos tengan coloración perceptible, deben poseer al menos siete dobles enlaces conjugados. Así, por ejemplo, el ζ -caroteno es amarillo pálido, mientras que otros carotenoides más saturados y de estructura similar son incoloros, como es el caso del fitoeno -3 dobles enlaces conjugados-, y fitoflueno -5 dobles enlaces conjugados- (Rodríguez-Amaya, 2001).

El color de los pigmentos carotenoides se debe concretamente a la deslocalización de los electrones a lo largo de la cadena hidrocarbonada insaturada. Así, desde un punto de vista analítico, el color de los carotenoides es de gran importancia, ya que un cambio de color durante alguna de las etapas de análisis es indicativo de degradación o de modificación estructural de pigmentos.

El espectro de absorción UV-Vis de los carotenoides se utiliza habitualmente para elucidar su estructura. Para cada compuesto aparecen tres máximos en el espectro, cuyas longitudes de onda dependen del número de dobles enlaces conjugados y del disolvente empleado (**Tabla I.4**).

Tabla I.4: Relación entre $\lambda_{\text{máx}}$ y longitud del cromóforo en carotenoides acíclicos. (Fuente: Rodríguez-Amaya, 2001).

Carotenoide	Número de dobles enlaces conjugados	$\lambda_{\text{máx}}$ en éter de petróleo (nm)
Fitoeno	3	276, 286, 297
Fitoflueno	5	331, 348, 367
ζ -caroteno	7	378, 400, 425
Neurosporeno	9	414, 439, 467
Licopeno	11	444, 470, 502
Espiriloxantina	13	465, 492, 525

En el caso de éter de petróleo, hexano y etanol, los máximos se localizan a longitudes de onda muy próximas, mientras que en acetona los máximos se desplazan entre 2 y 6 nm. El desplazamiento es aún mayor cuando los carotenoides

están disueltos en cloroformo, diclorometano o benceno (10-20 nm) o tolueno (18-24 nm) (Schiedt y Liaaen-Jensen, 1995; Rodríguez-Amaya, 2001).

Con independencia del disolvente empleado, los valores de λ_{\max} aumentan a medida que lo hace la longitud del cromóforo, lo cual se hace patente en carotenoides acíclicos.

Los dobles enlaces no conjugados, como es el caso del doble enlace entre los carbonos 4 y 5 del anillo ϵ , al no contribuir al grupo cromóforo, no afectan significativamente al espectro. Los grupos aldehído o cetona conjugados con la cadena poliénica, aumentan la longitud de onda del cromóforo.

En cuanto a los grupos hidroxilo y metoxilo, no afectan al cromóforo, de ahí que los espectros del β -caroteno y sus hidroxiderivados (β -criptoxantina y zeaxantina), sean prácticamente idénticos (**Figura I.5**) (Britton, 1992).

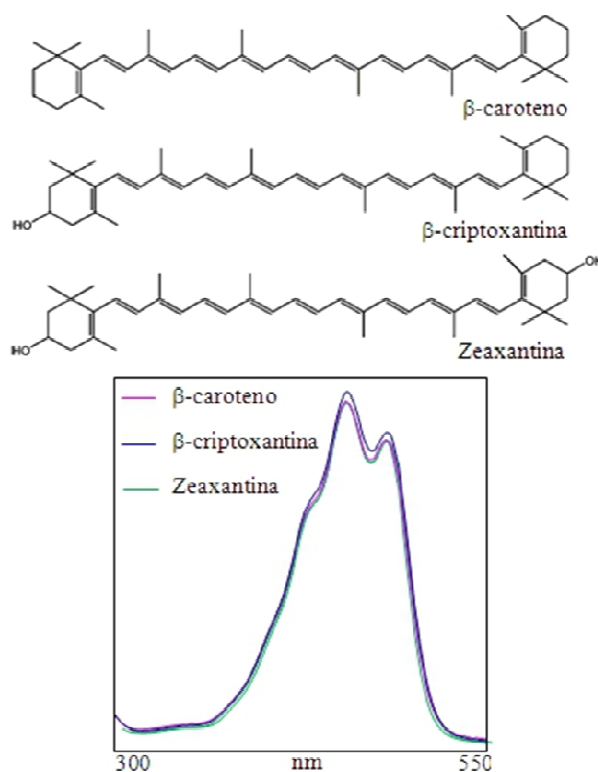


Figura I.5: Espectros de β -caroteno, β -criptoxantina y zeaxantina en acetona

(Fuente: Britton, 1992).

Por otra parte, la forma general del espectro, así como la persistencia de las bandas de absorción (o estructura fina), reflejan el grado de planaridad del cromóforo. El sistema de dobles enlaces conjugados de los carotenoides acíclicos puede adoptar una conformación casi completamente planar, de ahí que sus espectros se caractericen por la presencia de máximos y mínimos perfectamente definidos.

La intensa absorción de luz por parte de los carotenoides es además la base para su determinación cuantitativa (Schiedt y Liaaen-Jensen, 1995). En disolución, los carotenoides se ajustan a la ley de Lambert-Beer, de ahí que se cuantifiquen espectrofotométricamente (Rodríguez-Amaya, 2001), relacionando la absorbancia de dicha disolución a una determinada longitud de onda, con un valor estándar expresado como coeficiente de absorción (Britton y col., 2004). Para ello se recomienda que la absorbancia de la disolución -a la longitud de onda de medida- esté comprendida entre 0,2 y 0.8 (Mínguez-Mosquera, 1997).

El coeficiente de absorción específico ($A^{1\%_{1cm}}$), se define como la absorbancia teórica de una disolución de concentración 1% (P/V), en una cubeta de 1 cm de paso de luz, mientras que el coeficiente de absorción molar (ϵ), se define como la absorbancia teórica de una disolución de concentración 1 molar. Ambos coeficientes están relacionados mediante la siguiente expresión (Britton y col., 2004):

$$\epsilon = (A^{1\%_{1cm}} \times \text{peso molecular}) / 10$$

El coeficiente de absorción molar es característico del cromóforo e independiente del peso molecular del carotenoide, por lo que podría considerado el mismo valor para carotenoides distintos que presenten un cromóforo idéntico, como ocurre por ejemplo con β -caroteno y zeaxantina.

En cambio, los valores de ($A^{1\%_{1cm}}$) no serían los mismos para ambos compuestos, si bien están relacionados por sus pesos moleculares mediante la expresión:

$$A^{1\%_{1cm}}(\text{zeaxantina}) = A^{1\%_{1cm}}(\beta\text{-caroteno}) \times (536/568)$$

Así, la exactitud en la cuantificación de carotenoides va a depender de los coeficientes de absorción. Para la determinación de estos coeficientes se recomienda

pesar entre 1 y 2 mg del pigmento puro con una precisión de $\pm 0,001$ mg y disolverlos completamente en un disolvente apropiado (Britton, 1995). Este procedimiento suele ser bastante complejo, por lo que el contenido en carotenoides suele subestimarse con frecuencia (Britton, 1992). La determinación cuantitativa de carotenoides lleva asociada cierta inexactitud.

Para calcular la concentración de un determinado carotenoide se aplica la siguiente fórmula (Britton, 1991):

$$x = Ay / A^{1\%_{1\text{cm}}} \times 100$$

donde (x) es el peso del carotenoide en gramos, (y) el volumen de la disolución en mililitros, (A) la absorbancia medida experimentalmente y ($A^{1\%_{1\text{cm}}}$) el coeficiente de absorción específico.

1.6.1.4. Importancia nutricional

La importancia de los carotenoides en los alimentos va más allá de su rol como meros pigmentos naturales. De los más de 600 carotenoides conocidos actualmente, aproximadamente 50 de ellos serían precursores de la vitamina A, basándose en consideraciones estructurales. La provitamina A más importante es el β -caroteno, considerando factores como asiduidad y bio-actividad. Además, el γ -caroteno, la β -criptoxantina y α -criptoxantina también presentan actividad de vitamina A. No obstante, a pesar de tener menor bio-actividad que el β -caroteno, la β -criptoxantina también merece atención, dado que es el principal carotenoide de muchas frutas como mandarinas, melocotones, nectarinas, papayas, caquis y tomates. La **Figura I.6** resume las propiedades físicas y químicas más importantes de los carotenoides.

Diferentes estudios evidencian el efecto beneficioso de la ingesta de carotenoides sobre el sistema inmune, así como la reducción del riesgo de padecer ciertas enfermedades cardiovasculares, cáncer o el desarrollo de cataratas (Krinsky, 1994; Byers y Perry, 1992) (**Figura I.7**).



Figura I.6: Propiedades físicas y química más importantes de los carotenoides

(Fuente: Krinsky, 1994).

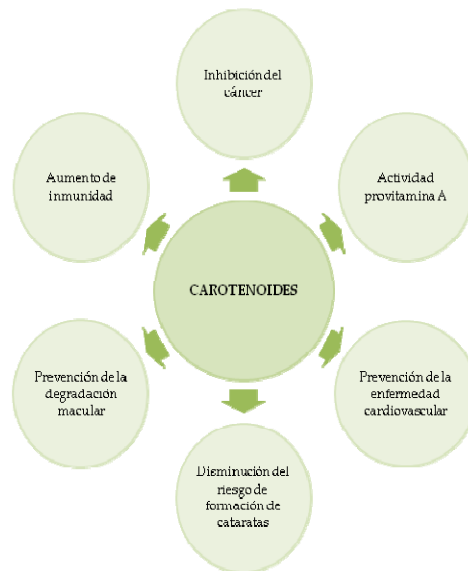


Figura I.7: Propiedades saludables atribuidas a los carotenoides (Fuente: Krinsky,

1994).

Estos efectos biológicos son independientes de la actividad pro-vitamina A, y se han atribuido a la capacidad antioxidante de los carotenoides, ya que pueden ralentizar la actividad los radicales libres -átomos o grupos de átomos que poseen un electrón no compartido- a través de estructuras resonantes o mediante captura de oxígeno singlete (Palozza y Krinsky, 1992; Eichholzer y col., 2001; Rodríguez-Amaya, 2010).

La capacidad de los carotenoides para capturar el oxígeno singlete se relacionó con el sistema de dobles enlaces conjugados (Dutta y col., 2005). La cadena poliénica, irónicamente también es la causa de la inestabilidad de los carotenoides, incluyendo su susceptibilidad a la oxidación e isomerización geométrica (Rodríguez-Amaya, 1999).

1.6.1.5. Niveles de ingesta y recomendaciones

La actividad provitamina A de algunos carotenoides como α -caroteno, β -caroten y, β -criptoxantina, está ampliamente demostrada, como ya se ha comentado con anterioridad. Debido a este hecho y a su amplia distribución en los alimentos, son los carotenoides provitamínicos de referencia al establecer las ingestas recomendadas de vitamina A, las cuales se expresan como equivalentes de retinol (ER) (1ER= 1 μ g de retinol = 12 μ g de β -caroteno = 24 μ g de α -caroteno = 24 μ g de β -criptoxantina). Se estima que el consumo medio de vitamina A oscila entre los 744 y 811 equivalentes de retinol por día en hombres, y entre 530 y 716 equivalentes de retinol por día en mujeres. Se ha evidenciando que entre el 26% y el 34% de la vitamina A consumida por hombres y mujeres, respectivamente, es proporcionada por los carotenoides provitamínicos (Food and Nutrition Board, 2002).

El requerimiento mínimo de vitamina C para evitar el síndrome conocido como escorbuto es de 10 mg, mientras que la recomendación se eleva a 60 mg al día (FNB, 2000).

1.6.2. Vitamina C

El ácido ascórbico o vitamina C, es una vitamina hidrosoluble presente en frutas y vegetales tales como los cítricos y las verduras frescas. No es sintetizable por el organismo, por lo que se debe ingerir a través de los alimentos destacando las verduras verdes, frutas cítricas y patatas. Debido a que esta vitamina es un

ácido, es razonablemente estable en soluciones ácidas, pero en soluciones básicas o neutrales se oxida rápidamente por el oxígeno disuelto. Un pH alcalino (mayor a 7), el cobre y el hierro, también aceleran su oxidación. La oxidación del ácido ascórbico se debe a la pérdida de dos átomos de hidrógeno, convirtiéndose en ácido dehidroascórbico (**Figura I.8**), que puede transformarse en ácido 2,3-dicetogulónico.

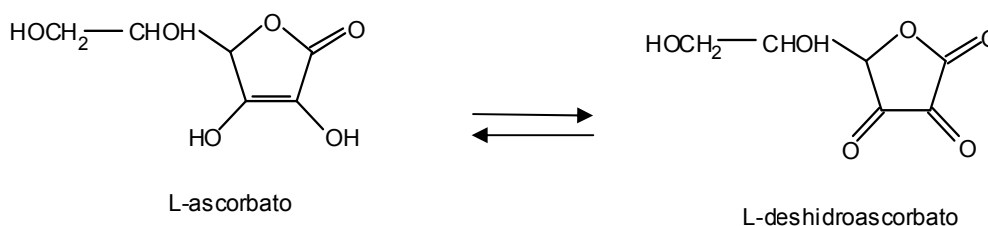


Figura I.8: Molécula de ácido ascórbico oxidada y reducida

(Fuente: Fennemma, 1996).

El ácido dehidroascórbico también posee actividad biológica, debido a que en el organismo se reduce para formar ácido ascórbico, mientras que el ácido 2,3-dicetogulónico es biológicamente inactivo.

1.6.2.1. Características funcionales de la vitamina C

Las funciones de la vitamina C se deben al equilibrio redox (forma oxidada y reducida), participando en algunas reacciones de hidroxilación, donde mantiene una actividad enzimática óptima por donación de electrones. La vitamina C incrementa la absorción de hierro no hemo, y participa en la inactivación de radicales -sumamente reactivos-, en las células de los tejidos. Además, retarda la formación de nitrosaminas -posibles carcinógenos-, en el organismo. La evidencia acumulada vincula el ácido ascórbico a diferentes unidades del sistema inmune (Mahan y Arlín, 1992).

Se ha evidenciado que el ácido ascórbico desempeña diferentes funciones en el proceso metabólico, tanto de los animales como del hombre. Tiene relación con el metabolismo del tejido conectivo, en particular con el colágeno. Es esencial o al menos interviene en la hidroxilación de la prolina en hidroxiprolina, que es una

etapa de la síntesis del colágeno. También participa en otras reacciones de hidroxilación (Simon y Hudes, 2000).

El ácido ascórbico parece servir de coenzima o cofactor cuando la tasa de reacción es crítica, como ejemplo las reacciones de hidroxilación, donde el cobre o el hierro deben mantenerse en forma reducida.

Participa también en la formación de ciertos neurotransmisores como la serotonina, en la conversión de dopamina a noradrenalina y en otras reacciones de hidroxilación, que incluyen a los aminoácidos aromáticos y a los corticoides.

Su concentración disminuye bajo situaciones de estrés, cuando hay una intensa actividad de las hormonas de la corteza suprarrenal. La vitamina C cumple una función importante en el sistema inmunológico, ayudándolo a combatir las infecciones y las células cancerígenas (Morillas-Ruiz, 2010).

1.7. ANÁLISIS SENSORIAL EN LA INDUSTRIA ALIMENTARIA

La industria agroalimentaria española ha tenido que adaptarse el reto que supuso la creación en 1993 de un mercado único europeo. La calidad de sus productos es la mejor arma, y su control debe ser uno de sus objetivos más importantes. La noción global de calidad de un producto agroalimentario presenta dos aspectos diferentes. El primero es puramente técnico y comprende los aspectos químicos y físicos, bioquímicos, microbiológicos y dietéticos que quedan perfectamente establecidos por la legislación; sin embargo, no basta para determinar la calidad tal como es entendida por el consumidor. Para éste interviene un segundo aspecto totalmente decisivo: la apreciación hedónica (Barcina, 2000).

El análisis sensorial puede requerir individuos no entrenados, entrenados (cualificados) y expertos. En todos los casos el tratamiento estadístico de los datos permite obtener resultados fiables. El análisis sensorial de los alimentos puede realizarse a través de diferentes pruebas, según la finalidad para la que estén diseñados (Carpenter y col., 2000).

1.7.1. Análisis sensorial en zumos de mandarina

A pesar de que en España se producen más de 2 toneladas de mandarinas al año y que la producción va en aumento, el procesado de zumo de mandarina es bajo en comparación con el de zumo de naranja, aunque se prevé un incremento

debido a la saturación de productos frescos. Existen pocos estudios sobre la mandarina y la escasa bibliografía existente se centra en su composición (Moshonas y Shaw, 1997; Trifiro y col., 1999; Pérez y col., 2005), la detección de la adición de zumo de mandarina al zumo de naranja (Knight, 2000) o en los efectos del tratamiento térmico sobre la aceptabilidad de los zumos (Sentandreu y col., 2005; Pérez-López y col., 2006), pero poco se sabe acerca de qué variedades de mandarinas son más idóneas para la producción de zumo.

En este sentido, Carbonell y col. (2007) desarrollaron una lista de atributos que pueden ser utilizados de forma fiable en el análisis sensorial de zumos de mandarina frescos y procesados. De los 29 atributos que finalmente se incluyeron en la lista destacan el color (tono), turbidez, intensidad de olor, olor a mandarina, olor a naranja, sin olor, intensidad de sabor, gusto a mandarina, gusto a naranja, astringencia, acidez y dulzor. La evaluación realizada por el panel de catadores, constituido por 11 jueces entrenados, dio resultados lo suficientemente homogéneos, fiables y reproducibles para poder ser aplicado en futuras investigaciones en pruebas de clasificación.

1.8. CONSERVACIÓN DEL ZUMO

Durante la pasteurización, el zumo es sometido durante 6-8 segundos a temperaturas entre 90-92 °C. El tratamiento térmico tiene dos objetivos, por un lado conseguir la inactivación de enzimas para evitar la aparición de turbidez en el zumo, y por otro la eliminación de microorganismos.

El tratamiento térmico, además de inactivar microorganismos, destruye en mayor o menor medida constituyentes deseables como nutrientes, compuestos bioactivos, aromas, color y textura (Manso y col., 2001; Lee y Coates, 2003; Polydera y col., 2004) que redundan en una pérdida de la calidad del producto (Meléndez y col., 2007). Para solucionar este problema, la industria alimentaria estudia alternativas que puedan paliar estos inconvenientes. Entre otros, destacamos el empleo de ciclodextrinas como medio de encapsulación de estos compuestos bioactivos (Szejtli, 1982), o bien el empleo de tratamientos de conservación menos agresivos como la ultracongelación (Stinco y col., 2013).

2. CICLODEXTRINAS (CDs)

2.1. HISTORIA Y DEFINICIÓN

Las ciclodextrinas (CDs) son azúcares cíclicos naturales de seis (α -CDs), siete (β -CDs) u ocho (γ -CDs) unidades de glucosa, unidas por enlaces α (1-4) (**Figura I.9**). También son conocidas como cicloamilosa, ciclomaltosas o dextrinas de Schardinger (Villiers, 1891; Eastburn y Tao, 1994). Se obtienen como resultado de una reacción de transglucosilación intramolecular en la degradación del almidón, llevada a cabo por la enzima ciclodextrina glucanotransferasa (CGTasa) de *Bacillus macerans* (Szejtli, 1998).

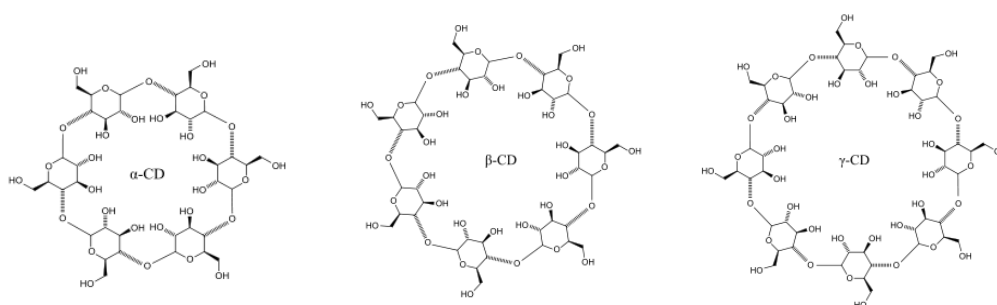


Figura I.9: Estructura de α -, β - y γ -CDs.

Todos los tipos de CDs tienen estructura troncocónica, debido a la conformación en forma de silla de las unidades de glucopiranosas. Orientados hacia la boca ancha de la estructura troncocónica se encuentran los -OH secundarios de los carbonos C-2 y C-3 de cada unidad de glucosa; y hacia la boca estrecha de la estructura se encuentran los -OH primarios de los C-6, quedando los grupos -CH₂OH orientados hacia la superficie externa del cono. Los hidrógenos de los carbonos más apolares de los azúcares (C-3 y C-5) y el oxígeno de los enlaces glucosídicos entre las unidades de glucosa, quedan orientados hacia el interior de la estructura troncocónica (**Figura I.10**).

La orientación específica de todos estos grupos, da lugar a una molécula cuya superficie externa es hidrofílica, por lo que es soluble en agua, y cuya cavidad interna es hidrofóbica (Szejtli, 1982).

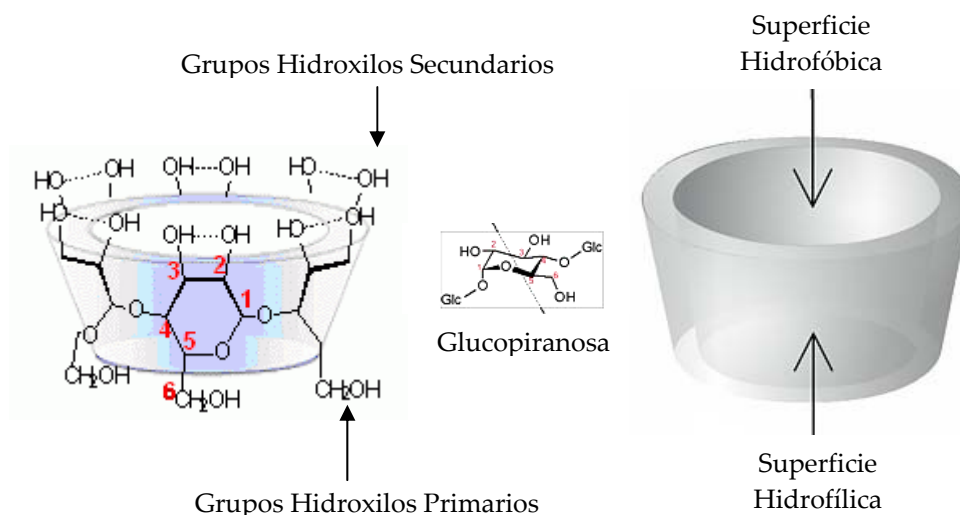


Figura I.10: Estructura troncocónica y orientación de los grupos en las ciclodextrinas.

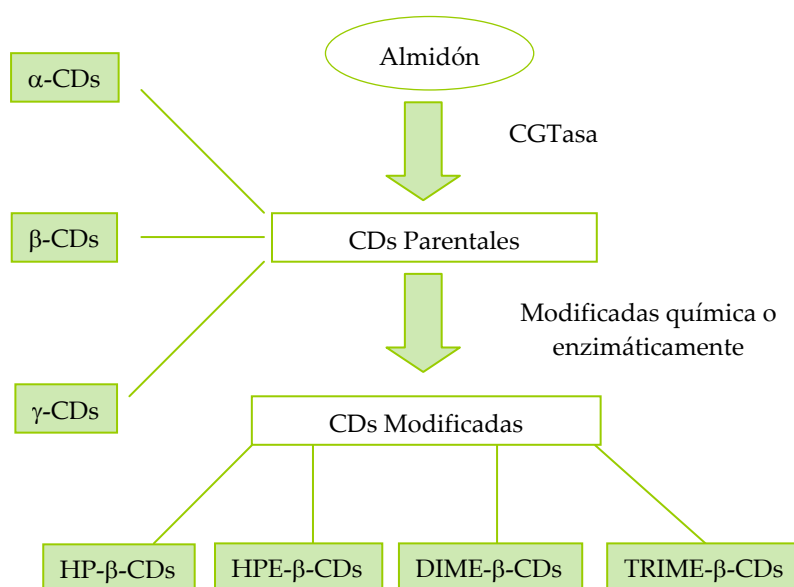
2.2. PROPIEDADES

Hay tres tipos de CDs denominadas de primera generación, parentales o naturales: α -, β - y γ -CDs. β -CDs es el tipo más utilizado debido a su bajo coste. Las principales características de las CDs naturales se describen en la **Tabla I.5**.

Tabla I.5: Propiedades de las CDs.

Propiedades	α -CDs	β -CDs	γ -CDs
Número de glucopiranosas	6	7	8
Peso molecular	972	1135	1297
Solubilidad en agua a 25 °C	14,5	1,85	23,2
Diámetro exterior (Å)	14,6	15,4	17,5
Diámetro de la cavidad (Å)	4,7-5,3	6,0-6,5	7,5-8,3
Altura de la cavidad (Å)	7,9	7,9	7,9
Volumen de la cavidad	174	262	427

A partir de las CDs naturales se han sintetizado otros tipos de CDs llamadas derivadas o modificadas, que son obtenidas por acilaciones, esterificaciones o eterificaciones de los OH de los carbonos primarios y secundarios de las CDs naturales. Dependiendo del sustituyente adicionado, la solubilidad de las CDs modificadas es diferente a las de su parental. Prácticamente, todas las CDs modificadas tienen cambios en el volumen de su cavidad hidrofóbica y, estas modificaciones pueden mejorar su solubilidad acuosa. La síntesis de CDs modificadas requiere la selección de reactivos, optimización de las condiciones de reacción y una buena separación de los productos. Las β -CDs han sido modificadas con más de 20 sustituyentes diferentes en una región específica de manera selectiva (**Figura I.11**).



- HP- β -CDs: hidroxipropil- β -CDs.
- HPE- β -CDs: Hidroxietil- β -CDs.
- DIME- β -CDs: (2, 6-dimetil)- β -CDs.
- TRIME- β -CDs: (2, 3, 6-trimetil)- β -CDs.

Figura I.11: Tipos de CDs modificadas.

2.3. FORMACIÓN DE COMPLEJOS DE INCLUSIÓN

La característica más notable de las CDs es su capacidad para formar complejos de inclusión con numerosos compuestos tanto orgánicos como inorgánicos en sus diferentes estados de agregación, siempre que el compuesto que se vaya a incluir tenga un tamaño compatible con las dimensiones de la cavidad hidrofóbica de la CDs (Villiers, 1981). Además, la formación del complejo se ve favorecida por las características de polaridad de la molécula huésped, aunque son muchos los autores que especulan que los factores decisivos en la formación del complejo son más bien geométricos que químicos (Muñoz-Botella y col., 1995). Puede afirmarse que el proceso de inclusión transcurre gracias a la interacción -energéticamente favorable- entre la molécula huésped, relativamente apolar, y la cavidad hidrofóbica -imperfectamente solvatada-, de las CDs.

Para que se forme el complejo de inclusión, ha de producirse una interacción entre la molécula huésped y la CD, que generalmente se califica como "interacción hidrofóbica". La unión resultante entre la CD y la molécula huésped no es fija o permanente, sino que es un equilibrio dinámico gobernado por una constante, cuya fuerza depende del tamaño relativo de la molécula complejada y de las interacciones establecidas entre la CDs y la molécula complejada (**Figura I.12**).

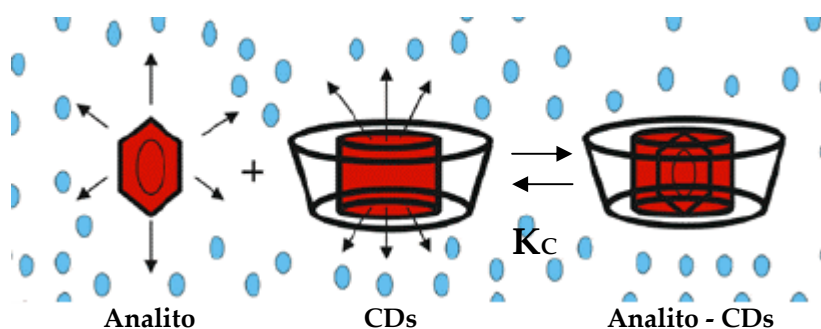


Figura I.12: Formación del complejo huésped-CDs.

Las CDs han sido aplicadas con éxito en diferentes campos: alimentación (Fujishima y col., 2001), farmacia (Bhardwaj y col., 2000), cosmética (Holland y col., 1999), protección del medio ambiente (Bhardwaj y col., 2000), bioconversión (Dufosse y col., 1999), envasado e industria textil (Hedges, 1998).

La lista de moléculas susceptibles de ser encapsuladas en CDs es muy variada e incluye compuestos de diferente naturaleza: hidrocarburos alifáticos de cadena lineal o ramificada, aldehídos, cetonas, alcoholes, ácidos orgánicos, ácidos grasos, compuestos aromáticos, gases y compuestos polares tales como halogenados, oxiácidos y aminas (Schmid, 1989).

Mientras que la altura de la cavidad hidrofóbica es la misma para los tres tipos de CDs nativos, el número de unidades de glucosas de cada una de ellas determina el diámetro interior de la cavidad y su volumen (**Figura I.13**).

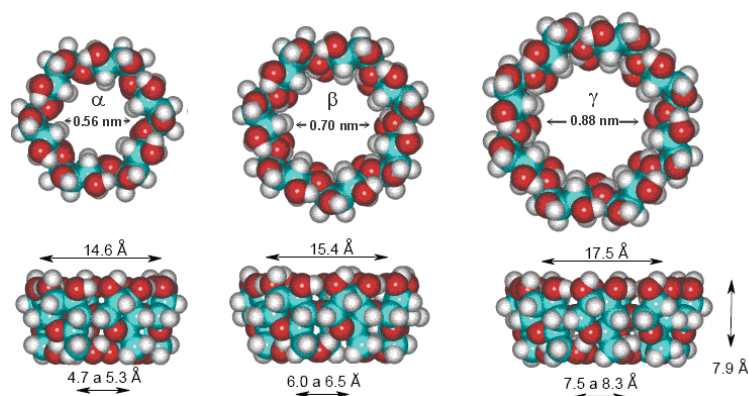


Figura I.13: Diámetro externo e interno de las diferentes CDs.

En base a estas dimensiones, las α -CDs pueden complejar moléculas de bajo peso molecular o compuestos con cadenas laterales de hidrocarburos alifáticos; las β -CDs pueden complejar moléculas aromáticas y heterociclos; y las γ -CDs pueden acomodar moléculas más grandes, tales como macrociclos y esteroides (Martín del Valle, 2004).

El equilibrio inicial para formar el complejo es muy rápido (a menudo ocurre en pocos minutos), sin embargo, el equilibrio final puede dilatarse más en el tiempo. Por otra parte, la disociación del complejo de inclusión es un proceso relativamente rápido, generalmente impulsado por un gran aumento en el número de moléculas de agua en el medio ambiente circundante. En disoluciones poco concentradas resulta difícil que la molécula huésped encuentre la CD, quedando libre en el medio acuoso.

2.4. APLICACIONES DE LAS CD_s

Cada molécula de analito es complejada por una molécula de CD_s, produciendo -desde un punto de vista microscópico-, una microencapsulación. Esto puede provocar cambios en las propiedades químicas y físicas de la molécula huésped, como pueden ser:

- Estabilización de moléculas sensibles a la oxidación en presencia de oxígeno y luz.
- Modificación de la reactividad química.
- Fijación de moléculas altamente volátiles.
- Aumento de la solubilidad.
- Transformación de sustancias líquidas en polvo.
- Protección frente a la degradación por microorganismos.
- Enmascaramiento de malos olores y sabores.
- Enmascaramiento de pigmentos o colores.

Estas características de las CD_s, hacen que sean adecuadas para su aplicación en diferentes campos como: química analítica, agricultura, farmacia, industria alimentaria y cosmética (Singh y col., 2002).

2.4.1. Industria alimentaria

En la industria alimentaria, las CD_s se han utilizado como agentes complejantes de vitaminas, colorantes alimentarios y aromas (Loftsson y Brewster, 1996; Buschmann y Schollmayer, 2002; Szejtli, 1998). Pueden modificar y/o eliminar sabores y aromas desagradables en alimentos y bebidas. Así, se han empleado para minimizar la astringencia debida a la naringina presente en zumos cítricos (Szente y Szejtli, 2004). De hecho, el sabor amargo de zumos de mandarina disminuye sensiblemente al añadir el 0,3 % de β -CD_s antes del tratamiento térmico. Esto es debido a que naringina y limonina (compuestos responsables del sabor amargo) forman complejos estables con β -CD_s, lo que reduce el amargor del zumo.

También se han aplicado en la protección del flavor en alimentos y bebidas durante el procesado y almacenamiento, para disminuir la degradación o pérdidas de componentes aromáticos, ya que mejoran *i*) la estabilidad molecular y física, provocando un retraso en el crecimiento de cristales y también *ii*) la estabilidad

química, ya que disminuye la reactividad, volatilidad o descomposición térmica, fotodegradación, hidrólisis, oxidación y evita la formación de isómeros que pueden dar lugar -en reacciones secundarias-, a compuestos no deseados en el producto final (Cabral, 2010).

Determinados componentes de los alimentos son sensibles a la luz, como es el caso del citral -componente responsable del olor a cítricos frescos-, que en presencia de luz UV se transforma en fotocitral A y fotocitral B. Estos dos compuestos le confieren al alimento un aroma y sabor no deseados. Cuando el citral es complejado con β -CDs, no se observan productos de descomposición en las 6 primeras horas de exposición del alimento a la luz (Szejtli y col., 1979).

En resumen, las CDs forman complejos de inclusión con grasas, sabores y aditivos alimentarios, protegiéndolos de su degradación u oxidación. Los sabores pueden ser volátiles, aceites o líquidos y su complejación con CDs constituye una alternativa a la tecnología convencional de encapsulación utilizada para la protección de sabores (Reineccius y col., 2002, 2003, 2005; Reineccius y Risch, 1986).

2.5. REGULACIÓN DE LAS CDs

En la actualidad, no existe armonización con respecto al empleo de CDs en los alimentos, ya que varía entre distintos países. En EEUU, α -, β - y γ -CDs están consideradas como GRAS (lista de la FDA de los aditivos alimentarios que son "generalmente reconocidos como seguros") y pueden ser comercializadas como tal. En Japón, α -, β - y γ -CDs son reconocidos como productos naturales y su comercialización en el sector de la alimentación está limitada por consideraciones de pureza. En Australia y Nueva Zelanda α - y γ -CDs están clasificados como nuevos alimentos a partir de 2004 y 2003, respectivamente (Cravotto y col., 2006).

FAO/OMS a través del Comité de Expertos en Aditivos Alimentarios (JECFA) recomienda un consumo máximo de β -CDs en alimentos de 500 mg/kg/día. Para α -y γ -CDs no se ha establecido la ingesta diaria admisible (IDA). Por otra parte, en julio de 2005 la Agencia de Protección Ambiental (EPA) de EEUU, acabó con la necesidad de establecer un nivel máximo admisible de residuos de α -, β - y γ -CDs en diversos productos alimenticios (EE.UU. Federal Register, 2005) (Astray y col., 2009). En Europa, están aprobadas α -, β - y γ -CDs como ingredientes alimentarios, y por lo tanto, deberán figurar en la lista de ingredientes alimentarios en los alimentos que las contengan (Diario Oficial de la Unión Europea, 2008).

3. SEGURIDAD E INOCUIDAD DE LOS ZUMOS CÍTRICOS

3.1. PRODUCTOS FITOSANITARIOS

En la actualidad, una de las prioridades en el mundo y especialmente en los países en vías de desarrollo, es cubrir las necesidades alimentarias de una población que va en aumento. En este sentido, el tratamiento de los cultivos con productos fitosanitarios de origen natural o sintético -plaguicidas-, garantiza el aporte de alimentos, ya que se utilizan como biocidas para combatir los parásitos de los cultivos, del ganado, de los animales domésticos y del hombre y su ambiente. Sin embargo, los plaguicidas son sustancias tóxicas y peligrosas, que pueden provocar efectos adversos en el hombre y su entorno al entrar en contacto con ellos.

Esta peligrosidad presenta varios aspectos: peligrosidad en su fabricación y formulación, peligrosidad para el aplicador que puede entrar en contacto con él, peligrosidad ecológica, es decir, su acción sobre el medioambiente con efectos secundarios indeseables (desequilibrios biológicos, aparición de nuevas plagas, resistencias, fitotoxicidades, acción sobre la fauna silvestre terrestre y acuícola, toxicidad para abejas, etc.) y por último, peligrosidad para el consumidor de productos tratados a causa de los residuos tóxicos que estos alimentos pudieran contener (Ballesteros, 2004; Soil Association, 2010). Aunque todos los aspectos comentados son importantes, éste último es el que más preocupa a la opinión pública.

La aparición de los plaguicidas de síntesis cambió totalmente el sistema de control de plagas y enfermedades de los cultivos, fundamentalmente a causa de su efectividad y facilidad de aplicación. Sin embargo, su empleo excesivo los ha convertido en contaminantes persistentes en el medio ambiente, en el cual se encuentran ampliamente distribuidos.

Dado que tanto los residuos de plaguicidas como sus metabolitos suponen un riesgo para la salud y el medio ambiente, las autoridades de la mayoría de los países han dictado normas con el fin de proteger la salud de los consumidores.

Estas normas son muy variadas y recogen, desde obligaciones formales de homologación y registro -incluyendo la fijación de límites máximos de residuos (LMR)-, a la prohibición pura y simple del empleo de ciertos plaguicidas, pasando por restricciones y limitaciones de uso (Codex Alimentarius Commission, 1996; European Commission, 2001; PAN, 2010; DG SANCO, 2013). Con estas normas se

pretende, en general, hacer compatible la protección de la salud pública con la defensa sanitaria de los cultivos frente a plagas y enfermedades.

Los zumos de fruta –mayoritariamente cítricos-, han experimentado un acusado incremento en el consumo en los últimos años, ya que además de una buena fuente de nutrientes, son una opción saludable para mantener hidratado el organismo. De hecho, más del 50% de la población toma un zumo en el desayuno, alcanzando cifras similares en almuerzo y merienda en niños en edad escolar.

Así, teniendo en cuenta que los niños consumen zumos de fruta con relativa frecuencia y que son más susceptibles a los productos fitosanitarios, al encontrarse en una etapa temprana de desarrollo, determinar los residuos de plaguicidas en zumos de fruta es absolutamente necesario, siendo los insecticidas organofosforados clorpirifos, fentión, malatión y diazinón, los que con mayor frecuencia se emplean en agricultura citrícola.

3.1.1. Plaguicidas organofosforados

Los plaguicidas organofosforados (OFs) son ésteres, amidas o tioderivados del ácido fosfórico, fosfónico, fosforotioico o fosfonotioico, que controlan una gran variedad de plagas. La fórmula estructural de estos compuestos -caracterizada por la presencia de tres grupos funcionales éster-, se muestra en la **Figura I.14**, en la que R₁ y R₂ son radicales alquilo, generalmente metilo o etilo.

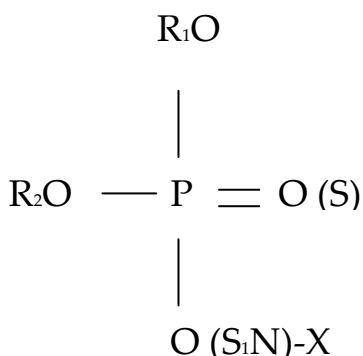


Figura I.14: Estructura genérica de los compuestos organofosforados.

El grupo X es característico de cada especie química; habitualmente un radical arilo y suele contribuir de forma importante a las propiedades físicas,

químicas y biológicas de este tipo de compuestos. Por otra parte, el número de grupos químicos se ve incrementado por la gran variedad de radicales que pueden presentar, ya que si bien dos de ellos son siempre metilo o etilo, el tercer radical es diferente en cada tipo de plaguicida perteneciente a esta familia (UNEP, 1991).

3.1.2. Analitos objeto de estudio

Para evaluar la presencia de residuos de productos fitosanitarios en zumo de mandarina, se seleccionaron dos plaguicidas organofosforados –clorpirifos y fentión-, cuyo empleo es frecuente en cultivos cítricos. La elección está justificada por su elevado consumo –sólo en actividades agrícolas supera en Europa los 500.000 kg/año (Barceló, 1993; Azizi y Hadian, 2008) , y el considerable impacto que provocan en los ecosistemas acuáticos, circunstancia que motiva su inclusión en la lista de plaguicidas prioritarios de la UE (Oliver y col., 2000; Kumari y col., 2006) .

3.1.2.1. Clorpirifos

El clorpirifos [tiofosfato de *O,O*-dietilo y de *O*-(3,5,6-tricloro-2-piridilo)], perteneciente a la familia de los organofosforados, presenta acción insecticida y acaricida, así como actividad por contacto, ingestión e inhalación. Fue introducido en el mercado en 1.966 por la compañía Dow Chemical, que registró las marcas Dursban y Lorsban. Produce fosforilación irreversible de la acetilcolinesterasa de los tejidos y alteraciones en el sistema nervioso central.

El clorpirifos (**Figura I.15**) se aplica especialmente en cultivos hortícolas y frutales, así como en el tratamiento de semillas de algunas hortalizas. También se utiliza en el tratamiento de la madera, aplicaciones domésticas y como ectoparasiticida del ganado ovino y vacuno.

Es un sólido cristalino de color blanco, moderadamente volátil (tensión de vapor $0,0187 \times 10^{-3}$ mmHg a 20 °C) y fácilmente soluble en disolventes orgánicos (a 25 °C, 7,9 kg/kg benceno, 6,5 kg/kg acetona, 6,3 kg/kg cloroformo y 450 g/kg metanol). En cambio, es muy poco soluble en agua (2 mg/L a 25 °C). Se degrada lentamente en el suelo a 3,5,6-tricloro 2-piridinol (TCP), que posteriormente da lugar a otros metabolitos y CO₂.

Presenta una toxicidad aguda (DL_{50}) por vía oral de 102 mg/kg en ratón, 135 mg/kg en rata, 504 mg/kg en cobaya y entre 1.000-2.000 mg/kg en conejo. No es carcinógeno ni teratógeno en ratas.

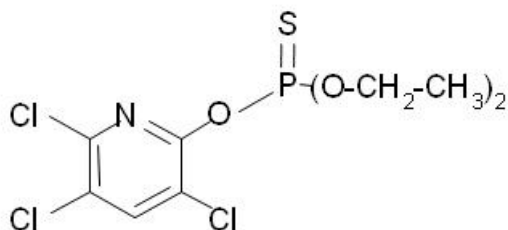


Figura I.15: Estructura del clorpirifos.

Es considerado de toxicidad moderada, no presenta propiedades de bioacumulación en los organismos vivos y se metaboliza y excreta con relativa rapidez. La UE establece un LMRs para clorpirifos en mandarina de 2 mg/kg, 0,3 mg/kg en naranjas y 0,2 mg/kg en limones (DG SANCO, 2013).

3.1.2.2. Fentión

El fentión (tiofosfato de *O,O*-dimetilo y de *O*-(4-metiltio-*m*-tolilo) actúa por contacto e ingestión afectando a la respiración, siendo efectivo para el tratamiento de dípteros y otros insectos domésticos (**Figura I.16**). Presentado en 1.960 por Bayer AG, que registró las marcas Lebaycid y Baytex.

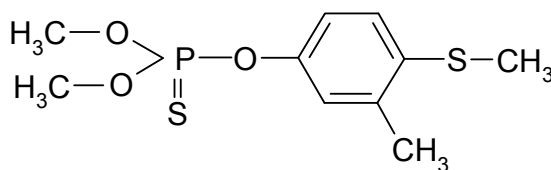


Figura I.16: Estructura del fentión.

Es un insecticida que posee un efecto inicial rápido y una acción persistente en el tiempo, que se combina con una notable acción a los álcalis. Presenta baja volatilidad e interfiere en la transmisión de los impulsos nerviosos por inhibición

de la colinesterasa. Se ha evidenciado su eficacia como larvicida en la lucha contra mosquitos. A temperatura ambiente, es un líquido oleoso incoloro con una tensión de vapor de $2,776 \times 10^{-6}$ mmHg a 20 °C. Es poco soluble en agua (54-56 mg/L a 20 °C), siendo fácilmente soluble en la mayoría de disolventes orgánicos (P_{ow} : logP 4,84).

Es estable a la luz y a elevadas temperaturas (≤ 210 °C). Relativamente estable en medios ácidos y moderadamente estable en alcalinos. En plantas se metaboliza oxidándose a sulfóxido y sulfona, que también presentan propiedades insecticidas, pasando finalmente a fosfato de sulfona que se hidroliza.

En ratas presenta una dosis letal (DL_{50}) por vía oral de 190 mg/kg y entre 150-175 mg/kg en conejo. Para frutos cítricos la UE estableció en el año 2008 un LMR de 3 mg/kg, rebajando ese valor tras una revisión reciente a 0,01 mg/kg (DG SANCO, 2013).

3.2. DETERMINACIÓN DE RESIDUOS DE PLAGUICIDAS

El control de residuos de plaguicidas en alimentos se aborda desde dos enfoques diferentes, aunque complementarios: medida de los niveles de residuos en los productos agrícolas de consumo directo y estudio de la dieta total, en el que se controla el nivel de residuos de plaguicidas en 234 alimentos cinco veces por año (Barceló, 1993; WHO, 2008; EFSA, 2008). Los resultados de este estudio se utilizan como referencia para proponer medidas reguladoras de tolerancia y determinar los valores aceptables de ingesta diaria de residuos de plaguicidas por persona. En este sentido, tanto la agencia norteamericana de protección medioambiental (EPA) como el grupo experto en residuos de plaguicidas de la Organización Mundial de la Salud (JMPR), han recomendado y estimado las dosis de referencia para una serie de plaguicidas según su uso e importancia económica. El nivel de tolerancia permitido depende del plaguicida, aunque en general, oscila en el intervalo de 0,1 a 50 mg/kg (sustancia activa/alimento).

La metodología utilizada actualmente para la determinación de plaguicidas y sus metabolitos a niveles traza es muy variada, e incluye una serie de etapas: extracción, purificación, separación, detección y confirmación; con el fin de detectar y cuantificar específicamente una serie de compuestos en matrices de composición más o menos compleja.

En general, las etapas de preparación de muestra son el paso limitante en ensayos de plaguicidas, debido a su complejidad, diversidad de problemas y, en

ocasiones, a su escaso desarrollo. No ocurre lo mismo con las etapas de detección, que han experimentado un importante crecimiento y evolución en los últimos 25 años, distinguiendo dos grandes grupos de técnicas: los métodos de barrido o cribado (*screening en versión anglosajona*) y los métodos de conformación (Toldrá y Reig, 2006).

Antes de los años 60, la identificación y cuantificación de plaguicidas en matrices ambientales y alimentos era difícil y laboriosa, realizándose por métodos espectroscópicos tradicionales (infrarrojos o UV-visible) o métodos biológicos, tales como las técnicas de inhibición enzimática o los bioensayos. El desarrollo de la cromatografía de gases (GC) y la cromatografía líquida de alta eficacia (HPLC) revolucionó el análisis de residuos de plaguicidas, permitiendo la determinación simultánea de varias clases de plaguicidas. Además, se ampliaron las posibilidades analíticas, pudiendo determinar compuestos termolábiles, poco volátiles y/o polares. Su robustez y sensibilidad las ha convertido en herramientas básicas de análisis, y la mayoría de los métodos oficiales están basados en ellas (Ferrer y col., 2011).

Los laboratorios oficiales que determinan residuos de plaguicidas utilizan generalmente métodos multiresiduo bien establecidos como el de Luke (Luke y Doose, 1975) o el de Mills (Mills y col., 1993), diseñados para cuantificar de modo rutinario unos 65 compuestos diferentes. Sin embargo, a pesar de ser muy útiles, en muchas ocasiones no pueden ser aplicados, bien por no detectar la especie de interés o por no ajustarse a las necesidades del problema. En cualquier caso, tienen en común un alto consumo de disolventes orgánicos y exhaustivos procedimientos de purificación y limpieza previos al análisis cromatográfico.

La cromatografía de gases (GC) se utiliza habitualmente en la detección de plaguicidas organofosforados y organoclorados (Fenoll y col., 2007; Ravelo-Pérez y col., 2008), mientras que se recomienda la cromatografía líquida (LC) para plaguicidas polares y termolábiles (Sagrati y col., 2007; Nguyen y col., 2009).

En los últimos años se han introducido numerosos avances en el análisis de residuos de plaguicidas. Así, se ha prestado especial atención a los límites de detección, reducción de costes y mejora de la precisión, desarrollando varias combinaciones GC y HPLC con espectrómetros de masas (MS) de trampa de iones o triple cuadrupolo (QQQ), e incluso equipos de masas en tándem (MS/MS) para un

gran número de aplicaciones analíticas. (Ferrer y Thurman, 2007; Radisic y col., 2009; Purcaro y col., 2012; Luonga y col., 2013).

Aunque las técnicas cromatográficas son extraordinariamente útiles para la determinación de residuos de plaguicidas, su aplicación a programas de control medioambiental y de seguridad alimentaria no está exenta de ciertos inconvenientes, entre los que cabe destacar los derivados de la elevada inversión en equipos y el alto precio de los análisis (ver **Tabla I.6**).

Tabla I.6: Precios públicos por la realización de análisis de muestras agroalimentarias. Identificación y cuantificación de residuos fitosanitarios y otros productos similares.

Actividad a realizar	Precio/muestra (€)
Preparación de muestras para el análisis (extracción, destilación, mineralización, etc.).	19
Identificación o cuantificación de una sustancia, o grupo de sustancias, por cromatografía en capa fina.	25
Identificación o cuantificación de una sustancia mediante técnicas espectrofotométricas (UV/V, IR, AES, AAS, etc.).	25
Identificación o cuantificación de una sustancia, o grupo de sustancias, mediante técnicas separativas (GC, LC, SFE, CE, etc.).	49
Determinación de una sustancia mediante kits comerciales (análisis enzimáticos, inmunoensayo, etc.).	49
Identificación o cuantificación de una sustancia, o grupo de sustancias, mediante GC-MS.	61
Identificación o cuantificación de una sustancia, o grupo de sustancias, mediante LC-MS.	240
Identificación o cuantificación de una sustancia mediante técnicas cromatográficas combinadas con técnicas inmunoquímicas de purificación.	73

Fuente: Orden SCO/47/2004, de 14 de enero, por la que se fijan los precios públicos por la realización de actividades del Centro Nacional de Alimentación. Agencia Española de Seguridad Alimentaria y Nutrición (AESAN).

Además, requieren personal altamente cualificado y no son adecuados para analizar grandes volúmenes de muestras en tiempos reducidos, indispensable para llevar a cabo programas de vigilancia y control efectivos. Son muy laboriosos en su desarrollo y requieren etapas previas de concentración y purificación de la muestra que pueden provocar pérdida de analitos y falta de reproducibilidad (Pico y col., 2007; Aramendia y col., 2007; Zhang y col., 2011).

Para llevar a cabo un riguroso control de residuos se requieren métodos sensibles, selectivos, rápidos, automatizables, económicos y fáciles de usar. Además, en la medida de lo posible, éstos deben prescindir de disolventes orgánicos, lo que supone un menor coste y un mayor respeto medioambiental. Todo ello ha generado la necesidad de desarrollar métodos alternativos -principalmente de *screening*- para la determinación de residuos de plaguicidas.

El interés de estos métodos analíticos es doble. Primero, por la necesidad de reducir los costes de los programas de vigilancia, y segundo, por la necesidad de conocer las fuentes, distribución, destino y efectos de los contaminantes que pueden ser perjudiciales para la salud y el medioambiente.

Es muy común disponer de grandes series de muestras en las que una elevada proporción están libres de residuos o los tienen en cantidades no detectables, por lo que una estrategia de *screening* para identificar las muestras negativas y priorizar la positivas, reduce costes y mejora los servicios analíticos (Muñoz-Olivas, 2004). Estos métodos de *screening* pueden diseñarse para trabajar en el laboratorio o fuera del mismo.

El desarrollo de estas nuevas metodologías es importante por varias razones, entre las que se incluyen: aspectos económicos, aumento de la frecuencia de muestreo, eficiencia y sensibilidad. Sin embargo, hay otra razón importante, la seguridad alimentaria y el grado en el que estas metodologías pueden evitar la contaminación. Esta es una consideración relativamente nueva, lo que hace que se estén desarrollando o implementando métodos dirigidos a cumplir los criterios establecidos en materia de seguridad y control alimentario. Por todo ello, son muchas las investigaciones que se están realizando con este fin, entre las que destacan: el análisis en campo o línea de fabricación, las técnicas de *screening* y los nuevos métodos de extracción.

Entre las técnicas analíticas desarrolladas para estos propósitos se encuentran las inmunoquímicas (IA), que ya han comenzado a jugar un papel

relevante en el *screening* de contaminantes orgánicos, como lo demuestran las diferentes revisiones aparecidas (Suri y col., 2009; Kim y col., 2011; Conzuelo y col., 2013).

Las principales razones de este auge se deben a la existencia de una extensa variedad de formatos, mayor número de reactivos y sobre todo, a su mayor distribución y acceso comercial. La determinación de residuos de plaguicidas mediante técnicas inmunoquímicas es una alternativa que complementa, y en algunos aspectos supera, a las técnicas cromatográficas. Sin embargo, los inmunoensayos no quedan exentos de ciertos inconvenientes relacionados con la dificultad de su desarrollo, la estabilidad de los inmunorreactivos y la reproducibilidad e interferencia de compuestos con estructura similar. Además, son métodos unianálito, lo que dificulta el análisis simultáneo de una familia de compuestos o sustancias de diferentes familias. Actualmente, muchas investigaciones se dirigen hacia la resolución de todas estas limitaciones y los resultados que se van obteniendo auguran soluciones viables a corto plazo (Lee y Kennedy, 2001; Kim y col., 2011).

3.3. MÉTODOS INMUNOQUÍMICOS

Lo que hoy se conoce como técnicas inmunoquímicas o inmunoensayos tienen su origen en el área de la química clínica, y engloban todas aquellas herramientas analíticas que utilizan como principio básico la interacción anticuerpo-antígeno (Ab-Ag). En muchos casos, esta interacción es reversible, y está gobernada por la ley de acción de masas; en ella intervienen fuerzas electrostáticas, puentes de hidrógeno e interacciones hidrofóbicas y de Van der Waals.

El primer IA cuantitativo, un radioinmunoensayo (RIA) para el análisis de insulina humana a niveles de picogramos, fue descrito por Yalow y Berson en 1961. El desarrollo de los RIA abrió la posibilidad de que la reacción Ag-Ab pudiera ser utilizada como base para el desarrollo de nuevos métodos analíticos de alta sensibilidad y selectividad. Así, en la década de los 70, Ercegovich (1971) sugirió el empleo de métodos inmunoquímicos para el *screening* de residuos de plaguicidas, que sirvió de base para el desarrollo del primer radioinmunoensayo para los insecticidas aldrín y dieldrín (Langone y Van Vunakis, 1975), al que le siguieron otros basados en el mismo principio.

Sin embargo, la peligrosidad inherente al uso de isótopos radioactivos hacía necesario que los ensayos se realizaran en laboratorios especialmente equipados, lo que suponía un gran inconveniente. La solución definitiva a la intensa búsqueda de IA no radioactivos vino propiciada por la demostración de que las enzimas podían ser acopladas a Ab y Ag (Avrameas y Uriel, 1966), y por la observación de que los Ab y Ag podían ser fácilmente inmovilizados sobre soportes plásticos (Engvall y Perlmann, 1971), lo que propició el desarrollo de una nueva técnica conocida como ELISA (*Enzyme-Linked ImmunoSorbent Assay*).

Esta aportación, junto a la realizada por Hammock y Mumma en 1980, supuso la génesis del desarrollo de numerosos inmunoensayos enzimáticos (EIA) para una gran variedad de plaguicidas, aumentando espectacularmente su aceptación a partir de 1988 (Zulin y col., 2002; Viñas y col., 2002; Tadeo y col., 2002; Caballo-López y Luque de Castro, 2003, Goto y col., 2003; Piao y col., 2009; Kim y col., 2011; Wang y col., 2013).

3.3.1. Componentes clave de los inmunoensayos

Las técnicas inmunoquímicas utilizan como reactivo principal los anticuerpos, que son proteínas que se producen en el sistema inmunológico de los animales vertebrados, en respuesta a la presencia en el organismo de moléculas o cuerpos extraños, uniéndose a estos agentes invasores neutralizando su actividad. Los anticuerpos se caracterizan por presentar sitios de reconocimiento que les permiten establecer interacciones altamente específicas con los antígenos, cuya extensión depende de la intensidad de la interacción antígeno/anticuerpo. La producción de Abs se realiza fundamentalmente inmunizando animales de sangre caliente, como por ejemplo conejos, cabras, ratones o pollos (Hock y col., 1995a ; Liu y col., 2009; Xu y col., 2009).

Los anticuerpos aislados de muestras de sangre de animales inmunizados son por naturaleza policlonales (pAbs); consisten en una población heterogénea de inmunoglobulinas, algunas de las cuales reconocen los sitios antigénicos o epítopos del inmunógeno.

Por otro lado, los anticuerpos monoclonales (mAbs) son poblaciones homogéneas de inmunoglobulinas secretadas por clones de una célula de hibridoma, resultado de la fusión de una célula de mieloma inmortal con un linfocito B procedente de un animal inmunizado (Kölher y Milstein, 1975),

garantizando la tecnología del hibridoma la producción ilimitada de mAbs con propiedades constantes (Hock y col., 1995b). Sin embargo, la producción de mAbs necesita un equipamiento especial, material y medios de cultivo celulares bastante caros, además de personal altamente cualificado, por lo que en la mayoría de IAs se emplean pAbs.

Una tercera posibilidad surge de las aportaciones de la Biología Molecular y los avances en las técnicas de Ingeniería Genética, que han propiciado el desarrollo de anticuerpos recombinantes (rAb). Esta tecnología permite la expresión de regiones funcionales de los Abs en sistemas huésped -relativamente simples y baratos-, así como la modificación de las propiedades de unión del Ab por manipulación genética a nivel del ADN. Así, se pueden obtener nuevos Abs con diferentes propiedades de unión mediante nuevas inmunizaciones.

Aunque para producir rAbs se han utilizado como sistemas huésped levaduras, plantas y células de insectos, la técnica más extendida se basa en el empleo de la bacteria *Escherichia coli* (Hall y col., 1997; Kim y col., 2003; Brun y col., 2004; Choia y col., 2006), por lo que a los rAbs también se les llama Abs coliclonales.

En resumen, la producción de anticuerpos es, en la mayoría de los casos compleja, y comprende principalmente las siguientes etapas: *i)* selección de la molécula de interés analítico, *ii)* diseño y síntesis del hapteno, que consiste en la preparación de un derivado de la molécula objeto de análisis, que contenga un grupo funcional apropiado para su unión a proteínas, *iii)* unión covalente del hapteno a la proteína deseada y, finalmente, *iv)* la inmunización del animal en función del anticuerpo deseado. Básicamente, son dos los elementos esenciales a tener en cuenta en el desarrollo de técnicas inmunoquímicas. Por una parte, los reactivos, que incluyen a los anticuerpos, antígenos y a cualquier otra especie biológica o química utilizada para la visualización de la reacción Ab-Ag. Por otra, los formatos, cuya elección estará condicionada al papel, lugar de utilización y uso final que tenga que desempeñar el método, es decir, de alta o baja capacidad de ensayo, método de campo o de laboratorio, cualitativo o cuantitativo.

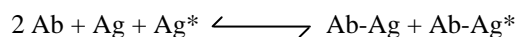
3.3.2. Tipos de inmunoensayos

En general, existen varios criterios de clasificación de los inmunoensayos: *i)* en función del modo de operación, inmunoensayos competitivos y no competitivos; *ii)* según las fases en que se realiza la interacción Ag-Ab, inmunoensayos

homogéneos y heterogéneos; *iii*) en función del marcador y método de detección, inmunoensayos enzimáticos, radioinmunoensayos, fluoroinmunoensayos, etc. A continuación se describen los principales formatos de inmunoensayo utilizados en diferentes campos de aplicación: clínico, medioambiental y agroalimentario.

3.3.2.1. Inmunoensayos competitivos y no-competitivos

Un ensayo competitivo puede ser descrito por la siguiente reacción inmunoquímica:



Donde el antígeno libre (Ag) compite con un antígeno marcado (Ag*) por un número fijo y limitado de sitios de unión del anticuerpo. La extensión de la unión Ab-Ag* depende de la concentración de Ag libre. A mayor concentración de analito (Ag libre), menos Ag* se une al Ab, permitiendo la cuantificación de la concentración del analito (Ag libre). Basados en este concepto general, existen dos modos de operar:

a) Ensayo directo: donde el anticuerpo compite con el analito y un hapteno marcado con una especie fácilmente detectable (por ejemplo una enzima).

b) Ensayo indirecto: donde la extensión de la unión Ag-Ab se mide habitualmente utilizando un segundo anticuerpo específico (anticuerpo secundario), marcado con una especie fácilmente detectable.

Los formatos de ensayo no-competitivos, son habitualmente utilizados en las determinaciones de moléculas de gran tamaño que poseen más de un epitopo o determinante antigénico (por ejemplo una bacteria). En estos ensayos se emplea un sistema de dos anticuerpos específicos, de modo que uno de ellos sirve para "fijar" el antígeno al soporte sólido. Tras un proceso de lavado y eliminación de las fracciones no unidas, se adiciona el segundo anticuerpo específico que sirve para cuantificar la extensión de la reacción.

Los ensayos no-competitivos son generalmente más sensibles que los correspondientes ensayos competitivos, sin embargo, este tipo de ensayo no es adecuado para moléculas de bajo peso molecular, como es el caso de los productos fitosanitarios.

3.3.2.2. *Inmunoensayos homogéneos y heterogéneos*

Los inmunoensayos homogéneos no requieren la separación entre las fracciones de inmunorreactivos libres y unidas. Utilizan el cambio de señal producido cuando el antígeno marcado se une al anticuerpo. Esa variación en la señal se debe a un cambio de la actividad del marcador y puede ser utilizada para cuantificar la cantidad de analito. Este tipo de ensayos presenta la ventaja de requerir tiempos de análisis más cortos, ya que no son necesarias etapas de lavado. En contrapartida, hay que señalar que la sensibilidad es menor y el efecto matriz de las muestras más elevado.

En los ensayos heterogéneos, existe una separación física entre las fracciones unidas y libres. Aunque este formato de ensayo incluye un mayor número de etapas, debido a los procesos secuenciales de lavado que deben realizarse para eliminar los componentes que no han reaccionado, el efecto matriz es menor. Por lo general, mejoran la sensibilidad y los límites de detección. Los ensayos inmunoenzimáticos, especialmente en formatos ELISA (*enzyme-linked immunosorbent assay*), son un ejemplo de este tipo de ensayos, cuyos fundamentos se comentan a continuación.

3.3.3. **Técnica ELISA**

La mayoría de los inmunoensayos para plaguicidas utilizan la técnica ELISA (Ballesteros y col., 2006; Shelver y col., 2007; Qian y col., 2009 Xu y col., 2012). Se trata de un ensayo heterogéneo en fase sólida que utiliza una enzima como especie marcadora, y donde uno de los inmunorreactivos está fijado (generalmente adsorbido) en la superficie de un tubo de ensayo o en los pocillos de una placa ELISA.

Las configuraciones más usuales en este tipo de ensayos son las siguientes:

3.3.3.1. *ELISA competitivo directo*

i) Formato de anticuerpo inmovilizado (AI): en este caso se establece un equilibrio entre el anticuerpo unido a la fase sólida (por adsorción directa o mediante reactivos orientadores), el analito y un conjugado analito-enzima (trazador enzimático) que están en disolución. Tras una etapa de incubación, los

reactivos no unidos son eliminados por lavado, midiendo por último la señal emitida por el trazador enzimático unido al Ab. La disminución de la actividad enzimática es directamente proporcional a la cantidad de analito presente en la muestra.

ii) Formato de antígeno inmovilizado: este formato está basado en la competición entre el antígeno inmovilizado (usualmente derivatizado y unido a una proteína, denominado conjugado de tapizado) y el analito, por una cantidad fija de anticuerpo marcado.

3.3.3.2. *ELISA competitivo indirecto (CI)*

El fundamento es el mismo que el del formato de antígeno inmovilizado, pero en este caso la concentración del analito se mide indirectamente, utilizando un segundo anticuerpo unido covalentemente a una enzima.

3.3.3.3. *ELISA sandwich*

En este ensayo se utiliza un segundo anticuerpo marcado para detectar la interacción entre el analito y el anticuerpo unido a la fase sólida. Sin embargo, esta configuración es restrictiva para analitos con más de un sitio de unión de anticuerpo, por lo que no es utilizada en el caso de los productos fitosanitarios.

3.3.4. Características de los inmunoensayos competitivos

En los IAs competitivos en fase heterogénea, la cantidad de marca unida a la fase sólida (señal) es inversamente proporcional a la del analito objeto de análisis (**Figura I.17**). Cuando se representa la señal obtenida frente al logaritmo de la concentración de analito, se obtiene una curva de calibrado típica dosis-respuesta que presenta una pauta sigmoide.

En este caso, el ajuste matemático de la curva de calibrado obtenida para diferentes concentraciones de analito, responde a la siguiente ecuación (Raab, 1983):

$$\text{Señal} = \frac{A - D}{1 + (x/C)^B} + D$$

donde A representa la asíntota máxima, que corresponde a la señal en ausencia de analito; D es la asíntota mínima y corresponde a la señal en exceso de analito; B es la pendiente de la curva en el punto de inflexión; x es la concentración de analito; y C , el valor de la concentración de analito en el punto de inflexión. Este punto es utilizado para estimar la sensibilidad del inmunoensayo (I_{50}) (Grojtan y Keel, 1996).

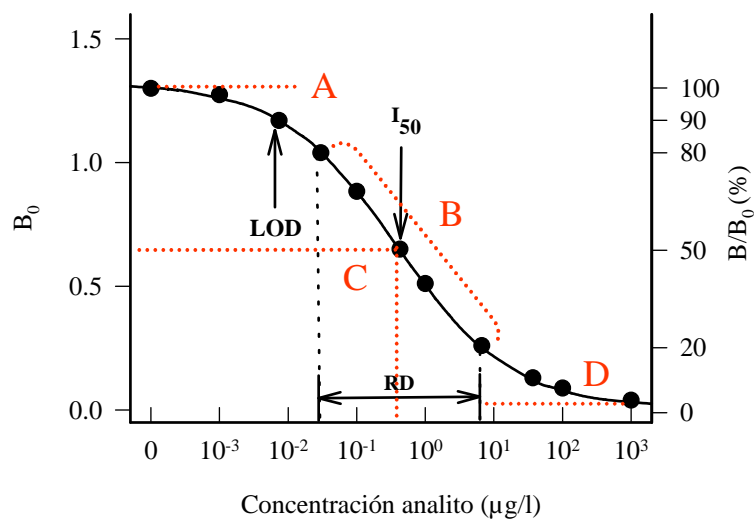


Figura I.17: Curva sigmoide típica dosis-respuesta.

Los parámetros más característicos de la curva de calibrado son los siguientes: señal en ausencia de analito (B_0); señal debida a los estándares (B); límite de detección (LOD), que corresponde a la menor concentración de analito que produce una señal distinguible de la correspondiente a B_0 para una determinada muestra, y con cierto grado de confianza. Con frecuencia se define como la concentración de analito que reduce el 10-20% de los sitios específicos de unión de anticuerpo y se calcula restando al valor de B_0 tres veces la desviación estándar (Hennion y Barceló, 1998). La I_{50} es la concentración de analito que reduce el 50% de los sitios específicos de unión del anticuerpo y el intervalo de trabajo (RD) el rango de concentración de analito que reduce los sitios de unión específicos del anticuerpo entre el 20-80%. Dentro de este intervalo, las variaciones de señal se

relacionan linealmente con la concentración de analito y pueden obtenerse resultados cuantitativos en muestras reales con relativa precisión (Brady, 1995)

Por otro lado, los IAs también se caracterizan por su alta selectividad. Así, la selectividad de un anticuerpo se define como la capacidad para reconocer exclusivamente un determinante antigénico. En este sentido, un anticuerpo es específico cuando sustancias con estructura y composición semejante al analito no son reconocidas. La selectividad o reactividad cruzada (RC) se determina, habitualmente, comparando la I_{50} dada por el analito de interés con las obtenidas con otros compuestos de estructura similar.

Este parámetro se calcula mediante la siguiente ecuación:

$$RC = \frac{I_{50} \text{ (analito)}}{I_{50} \text{ (interferente)}} \times 100$$

Cuando el valor RC es menor del 10% se considera que el anticuerpo es específico, y si es menor del 1% muy específico.

El hecho de que un Ab muestre un valor elevado de RC (baja selectividad) hacia compuestos relacionados estructuralmente con el analito, es considerado como una desventaja. Sin embargo, ese Ab puede ser utilizado para propósitos de *screening*, obteniendo así un valor global para una familia de compuestos.

3.3.5. Tratamiento de la muestra para ensayos inmunoquímicos

La determinación de residuos de plaguicidas mediante técnicas inmunoquímicas debe resolver un aspecto clave: el tratamiento de muestra. La mayoría de los tratamientos de muestra utilizan disolventes orgánicos y procedimientos desarrollados para aplicar en métodos instrumentales, cromatográficos especialmente. Por ello, la aplicación de las técnicas inmunoanalíticas a residuos, utilizando los métodos oficiales y de referencia que han sido validados para muestras medioambientales y alimentarias, requieren de un estudio profundo. Como se ha demostrado previamente (Gabaldón y col., 2007) es posible determinar niveles de residuos de plaguicidas realmente bajos utilizando métodos de extracción convencionales en medios orgánicos.

En general, las muestras acuosas son analizadas mediante IA sin pretratamiento, excepto la eliminación de material particulado y el ajuste del pH y fuerza iónica, en caso necesario. Sin embargo, algunas veces los iones presentes en la muestra pueden provocar interferencias o inducir la formación de precipitados por reacción con los componentes del tampón de ensayo (Jourdan y col., 1995; Glass y col., 2004). Asimismo, las sustancias húmicas presentes en extractos de agua y suelo pueden unirse inespecíficamente al Ab e interferir en las uniones específicas del analito (Dankwardt y col., 2000; Bjerner y col., 2002). En consecuencia, estas reacciones pueden generar falsos positivos.

En el caso de alimentos líquidos, como por ejemplo zumos, generalmente no es necesaria una excesiva preparación de muestra, basta con diluirla y ajustar el pH. Algunos ejemplos los constituyen los trabajos de Wittmann y Hock, 1993 que determinan atrazina en leche y zumo de manzana, González-Martínez y col., 1997 que analizan muestras de zumos, aguas y mieles reforzadas con carbaril, Watanabe y col., 2007 que determinan directamente imidacloprid en muestras de zumo de manzana y uva o los trabajos de Plana y col., 2014 que determinan tetraconazol en zumos de frutas.

3.3.6. Formatos inmunoquímicos aplicados al análisis de plaguicidas

Desde hace algunos años se vienen comercializando kits para la determinación de residuos de plaguicidas mediante inmunoensayo.

Los kits comerciales para análisis de residuos de plaguicidas están basados en los principios de la técnica ELISA, generalmente en formato de Ab inmovilizado; pueden ser cualitativos, semicuantitativos o cuantitativos y difieren en los formatos de presentación -tubos de ensayo, placas de 96 pocillos, tiras reactivas, etc-. Además, permiten la determinación rápida y simultánea de un elevado número de muestras, lo que unido a su fácil transporte, precisión y sensibilidad, hacen que su uso sea ideal para análisis en campo o en línea de fabricación (González-Martínez y col., 1997; Vass y col., 2008; Zhou y col., 2012).

Los kits comerciales ofrecen rapidez cuando se emplean en la determinación de residuos de plaguicidas en matrices acuosas; sin embargo, estos métodos presentan dificultades cuando se aplican a matrices más complejas, como suelos y alimentos, debido a la aparición de efectos matriz, por lo que la mayoría de las muestras no pueden analizarse sin tratamiento previo. Generalmente, las

muestras de alimentos presentan una amplia variabilidad en su composición, contenido en azúcares y lípidos, etc. Hay que tener en cuenta que los componentes proteínicos, así como los taninos o polifenoles, pueden unirse o interferir en las reacciones entre el Ab y el trazador enzimático.

Las principales características de los kits de inmunoensayo y su aplicación a la determinación de plaguicidas en diferentes matrices han sido ampliamente discutidas (Gabaldón y col., 1999; Lee y Kennedy, 2001; Piao y col., 2009; Zhou y col., 2012) presentando como principales limitaciones el tiempo requerido para el ensayo (por lo general superior a dos horas) y su especificidad (métodos unianálisis).

Basados en técnicas inmunoquímicas y en respuesta a la creciente necesidad de disponer de métodos analíticos portátiles y que permitan el análisis simultáneo de una familia de compuestos o sustancias de diferentes familias, se están desarrollando otras metodologías centradas en la puesta a punto de microinmunoensayos multianálisis (microarrays).

3.3.7. Microinmunoanálisis sobre soportes sólidos

Desde hace unos años se han producido grandes avances en la miniaturización y automatización de métodos analíticos. Uno de los ejemplos más prominentes es el que incorpora la metodología de *microarrays* (micromatrices), que actualmente es una herramienta muy popular y presenta numerosas aplicaciones en el sector farmacéutico, agroalimentario y las ciencias de la vida (Lamartine, 2006; Scarselli y col., 2005; Hoeberl, y col., 2006).

La técnica de micromatrices permite depositar miles de puntos sobre un portaobjetos (soporte) para su estudio en paralelo. Así, un *microarray* consiste en una matriz bidimensional de depósitos ordenados sobre un substrato sólido, en posiciones determinadas (López y col., 2002). Como puede verse en la **Figura I.18**, se trata de un sistema de análisis masivo que ofrece una superficie de reacción generalmente plana, mejorando así las etapas de lavado e incubación, a la vez que proporciona una mayor densidad de muestra (Uttamchandani y col., 2006).

El soporte y el tipo de unión del reactivo inmovilizado están íntimamente relacionados, ya que la metodología y el modo de anclaje dependen de las características de la superficie sobre la cual se van a realizar los ensayos. Se pueden distinguir dos grandes grupos: soportes porosos y soportes no porosos.

Los chips “porosos” son chips en los que las interacciones entre el material a inmovilizar y el soporte sólido de inmovilización no tienen por lo general carácter covalente (Hoheisel y col., 2001). Los soportes más comúnmente empleados son pequeñas porciones de geles o membranas porosas de nylon o nitrocelulosa presentes en porta objetos de cristal.



Figura 1.18: Imagen de diferentes microarrays comerciales.

Los chips lisos o “no porosos” son aquellos en los que el material se encuentra habitualmente inmovilizado covalentemente a la superficie sólida, que le sirve de soporte y puede ser de cristal o cualquier otro material como silicio, carbono, vidrio, materiales mixtos (composites), plásticos o metales.

Este tipo de soportes son inertes y presentan ventajas con respecto a los porosos: mínimo volumen de hibridación, gran capacidad de miniaturización y alta resolución (López y col., 2002).

Existen un buen número de alternativas a estos soportes como el empleo de materiales poliméricos sintéticos que ofrecen propiedades mecánicas y químicas atractivas, son más baratos y su fabricación es más sencilla y flexible. Las aplicaciones analíticas que actualmente utilizan discos de plástico como soporte pueden dividirse en dos grupos: aquellas que utilizan la propulsión fluidica y las que utilizan la fuerza centrífuga.

3.3.7.1. Micromatrices basadas en la propulsión fluidica

Las plataformas microfluídicas, son elementos integrantes de sistemas “lab-on a chip” o LOAC, capaces de realizar ensayos de laboratorio en una red de canales y cámaras miniaturizados. Debido a sus dimensiones reducidas ofrecen numerosas ventajas. Por ejemplo, ya que los canales sirven como vía para el

movimiento de las muestras y reactivos así como de cámaras de reacción, su pequeño volumen redundante en un menor coste por el ahorro en reactivos y en la necesidad de menores cantidades de muestra. Esta tecnología permite desarrollar métodos automatizados, miniaturizables (escalables) y sencillos.

Se presentan con distintas formas, tamaños y materiales (cristal, plástico, silicio, cuarzo) (Manz y col., 1991; Shi y col., 1999), pero todas tienen en común una estructura consistente en canales y cámaras de dimensiones microscópicas donde tiene lugar el análisis de la muestra. Reactivos y muestras son introducidos en la microestructura y transportados de una sección a otra mientras se realiza el mezclado, la reacción y la monitorización (Honda y col., 2005). Tras realizar los experimentos los resultados analíticos se obtienen mediante detectores ópticos convencionales tales como densitómetros, fotómetros o fluorímetros, adaptados a la geometría del dispositivo **Figura I.19**).

Estos dispositivos permiten realizar tanto la preparación de la muestra como el análisis y la detección de los reactivos en un mismo dispositivo de fácil manejo y, generalmente, de precio competitivo.

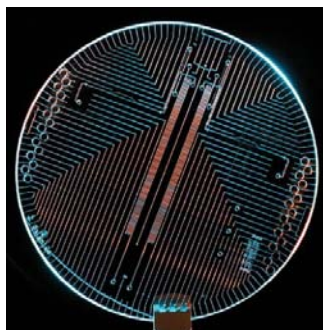


Figura I.19: *Dispositivo Lab-on-a-chip (LOAC).*

3.3.7.2. *Micromatrices basadas en la fuerza centrífuga*

Los discos de plástico que utilizan la fuerza centrífuga son una alternativa muy interesante a los dispositivos LOAC. Dichos sistemas bombean los fluidos utilizando la fuerza centrífuga que se genera cuando el disco gira, eliminándose así la necesidad de partes internas móviles y potenciales de voltaje, como los que

necesitan muchos sistemas LOAC. El control de la fuerza centrífuga es mucho más simple, ya que tan sólo depende de la viscosidad del fluido y de la de velocidad de rotación del disco, con lo que pueden manejar mayores cantidades de muestra. Estos discos están formados por numerosos canales que van desde el centro hasta el exterior del disco, de modo que se consigue la misma fuerza centrífuga en todos los canales, permitiendo la realización de muchos análisis a la vez (Felton, 2003). Actualmente existen otras alternativas mucho más avanzadas que van desde equipos de diseño clásico, utilizados en grandes laboratorios de hospitales a tecnologías innovadoras basadas en sistemas microelectromecánicos (MEMs).

Los Micro Sistemas Electro Mecánicos (MEMs) son dispositivos de pequeño tamaño que bien crean una señal eléctrica, debido al cambio de una propiedad física (sensores) o causan un efecto físico cuando son sometidos a una señal eléctrica (actuadores). Entre las metodologías basadas en el uso de discos de plástico como soporte que emplean la fuerza centrífuga, están las caracterizadas por el uso de la tecnología de discos compactos de audio-video (Cds) para la obtención de resultados analíticos. La tecnología del disco compacto como herramienta para el análisis de muestras ha experimentado un gran avance en los últimos años ya que son un medio globalmente accesible, barato y fiable. (Kido y col., 2000).

3.3.8. Discos compactos como plataformas analíticas para análisis masivo

Un disco compacto (Cd) es un soporte digital óptico en el que se almacena información en un sustrato plástico transparente, habitualmente de policarbonato (PC), sobre el que se aplica una película metálica de aluminio, oro, níquel, etc., recubierta de una laca protectora (que protege de la oxidación y del ambiente), generalmente de Polimetilmetracrilato (PMMA) y opcionalmente incorpora una etiqueta (**Figura I.20-A**).

Tanto el PC como el PMMA son materiales transparentes de radiación visible, presentan baja distorsión óptica, alta resistencia mecánica, buena estabilidad térmica y elevada exactitud dimensional, además de gran hidrofobicidad.

En los Cds, la información digital está almacenada en forma de pits, que se imprimen durante el proceso de fabricación del disco de PC. Cada pit tiene una profundidad de unos 125 nm, una anchura de 500 nm y una longitud que oscila entre 830 y 3500 nm (**Figura I.20-B**). Los pits están ordenados en el disco a lo largo

de una única espiral continua y extremadamente larga que constituye la pista de datos (*data track*), que será barrida por el haz láser desde el interior hasta el exterior del CD, en sentido levógiro (Tamarit-López y col., 2008).

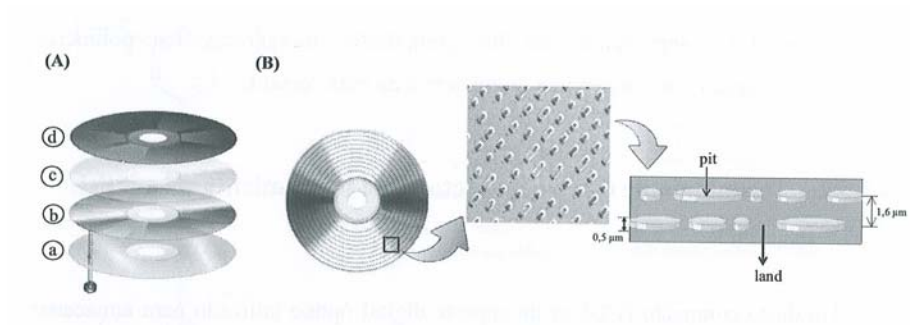


Figura I.20: Dispositivo Lab-on-a-chip (LOAC). (A) Representación esquemática de un CD, (a) PC, (b) película metálica (Al, Au), (c) laca protectora de PMMA, (d) etiqueta. (B) Esquema de la espiral continua que forma la pista de datos.

El espacio entre pistas (distancia radial entre una revolución de la espiral y la siguiente) es de 1600 nm, teniendo aproximadamente 45.000 pistas por cm. Los espacios entre pits a lo largo de la misma pista de datos se conocen como lands (**Figura I.21**).

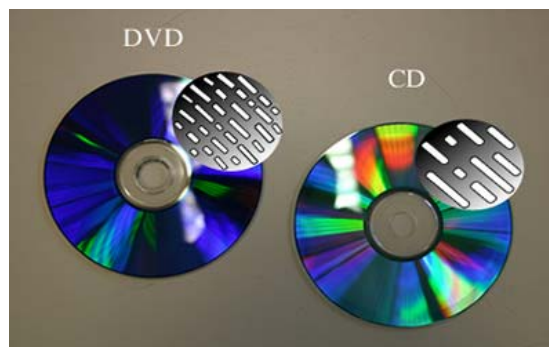


Figura I.21: Diferencias en el tamaño de pits y lands entre DVD's y CD's.

3.3.8.1. Localización y lectura de los datos almacenados en el CD

La información almacenada en forma de pits y lands, es leída empleando un lector de discos compactos. Un lector de Cds (**Figura I.22**), consta de tres piezas: *i*) Motor de arrastre del disco (*Disc drive motor*), motor que hace girar el disco. Se controla para que gire a una velocidad dada ($v_{\max} = 1000 \text{ rpm}$), en función del disco utilizado y de la situación de la pista leída; *ii*) Cabezal óptico (*Laser pickup assembly*), sistema compuesto por un laser que emite a 780 nm (o 640 en caso de discos DVD) y una lente que lo focaliza a fin de ajustar la distancia donde se encuentra la pista de datos.

Lleva incorporado un sensor opto-electrónico capaz de detectar la luz reflejada en la película metálica del disco; *iii*) Mecanismo rastreador (*Tracking mechanism*), sistema compuesto por un motor que mueve el dispositivo láser a lo largo de un eje radial fijo, de modo que el haz de luz puede seguir la pista de datos en espiral mientras el disco gira. Este sistema mueve el láser con una resolución de micrómetros.

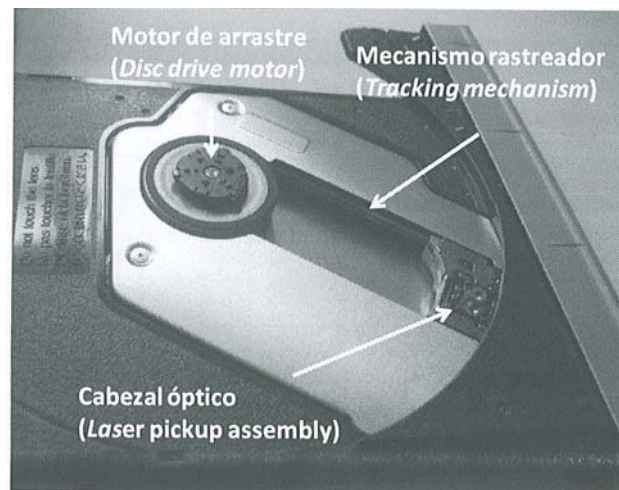


Figura I.22: Imagen de un lector de discos compactos.

La lectura de la información grabada en el disco se efectúa a partir de la modificación de la señal del haz láser reflejado. El lector utiliza el fenómeno de interferencia con la finalidad de proporcionar información binaria. Hay que tener en cuenta que las regiones land reflejan la luz de modo diferente a las pits y que los

sensores ópticos del lector, detectan los cambios en la reflectividad como una señal digital (on/off ó 1/0). La profundidad de los pits es seleccionada de modo que coincide con la cuarta parte de la longitud de onda del láser (780 nm). Por ello, el haz láser reflejado desde una región land estará en oposición de fase respecto al reflejado desde una región pit, tal y como se muestra en la **Figura I.23**. De este modo, cada pit actúa como un micro-interferómetro a través del cual los datos detectados pueden ser digitalizados.

Estudios previos han demostrado que un dispositivo de lectura/grabación óptico (lector/grabador de Cds) integrado en un ordenador personal convencional puede tener un alto potencial analítico, utilizando el láser del lector de Cds como fuente de barrido. Se han utilizado discos de baja reflectividad de PC y una película de oro, de modo que la parte del haz láser del lector de Cds se transmite, siendo detectado mediante un fotodiodo plano colocado en la cara interior opuesta del lector de Cds (**Figura 1.24-A**).

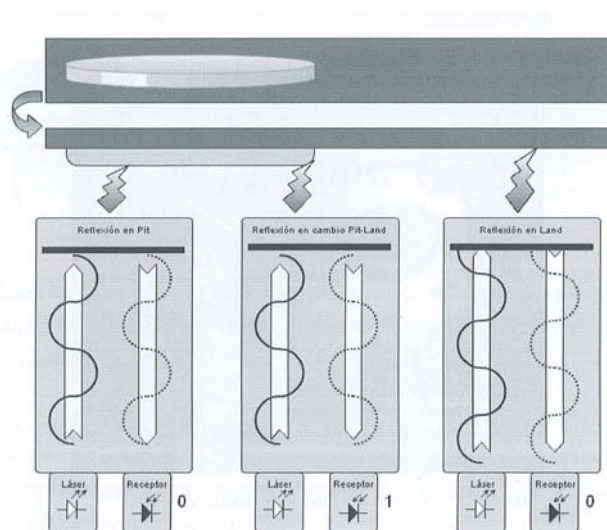


Figura I.23: Esquema del fenómeno de interferencia destructiva en el que se basa el sistema de detección del lector de Cds.

Gracias a la lectura óptica por láser no hay fricción entre el mecanismo de lectura/escritura y la superficie del disco, haciendo posible la convivencia de la información biológica (sonda-muestra) sobre el mismo soporte. Dada la diferencia entre índices de refracción entre el aire y policarbonato la luz incidente del

dispositivo óptico se refracta en la superficie del PC con un ángulo mayor, y por tanto, el punto de luz incidente original (unos $800\ \mu\text{m}$ sobre PC) se focaliza hasta un tamaño de $1,7\ \mu\text{m}$ sobre la superficie metalizada (**Figura 1.24-B**).

Una mejor resolución óptica permite que el detector resuelva spots de menor tamaño y, por tanto, trabajar con microarrays de mayor densidad, permitiendo el análisis de un mayor número de muestras por unidad de superficie. También ha dado buen resultado utilizar el propio láser y el pick-up del lector de Cds como guía y detector, respectivamente, relacionando la atenuación de la señal con la extensión de la reacción analítica (**Figura 1.24-C**).

La salida del láser (dispositivo que contiene tanto el diodo semiconductor que genera el haz, como el *beamsplitter*) es una señal analógica que básicamente transporta la información de reflectividad de la superficie del soporte (incluyendo la superficie ocupada por el ensayo).

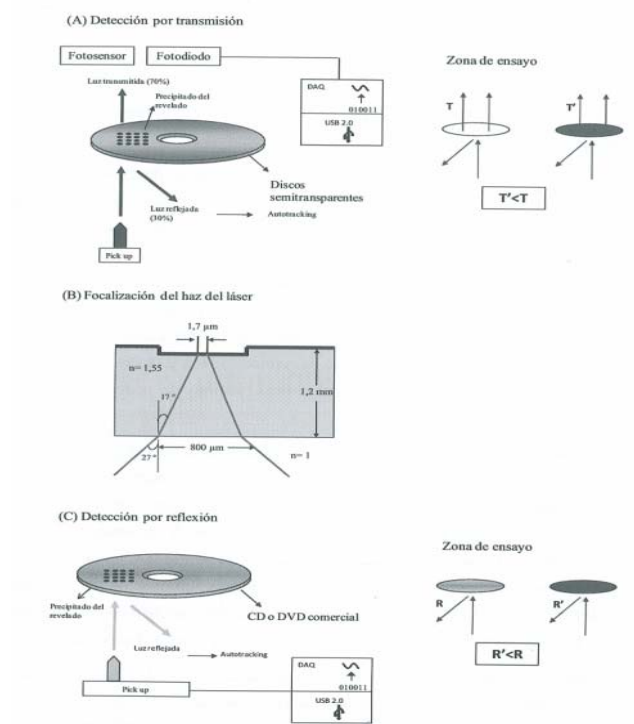


Figura 1.24: Modos de medida empleados en la tecnología de Cds.

Posteriormente la señal es acondicionada y decodificada, proporcionando a su salida la señal digital almacenada sobre el soporte, es decir, la luz absorbida se transformará en una señal eléctrica, digitalizándola mediante una tarjeta de adquisición de datos integrada en el ordenador. Es importante resaltar que la señal eléctrica proporcionada por el cabezal láser es analógica. Si hipotéticamente no fuese así, el acondicionamiento realizado impediría la evaluación de la reactividad de las muestras, ya que esta información se encuentra en la amplitud de la señal analógica resultante de la foto-detección del haz láser. El sistema proporcionará una señal de radiofrecuencia con un ancho de banda de 150 kHz.

Un lector de CD es un aparato electrónico de fabricación a gran escala y consumo masivo, lo que explica su bajo coste, con un precio de mercado que oscila entre los 50 y 300 euros. Esto los hace muy competitivos frente a la técnica habitual de lectura de *microarrays* basada en la utilización de escáneres de fluorescencia (con la superficie de escaneo estándar de 18,75 cm²), cuyo coste se encuentra entre 30.000 y los 100.000 euros. Además, el lector de CD proporciona mayor portabilidad y frecuencia de muestreo a tiempo real.

3.3.9. Antecedentes y estado del arte del empleo de discos compactos como plataformas analíticas

El empleo de los discos compactos como plataformas analíticas ha suscitado el interés de la comunidad científica y las empresas biotecnológicas, dado el gran volumen de mercado. Presentan propiedades químicas y mecánicas atractivas: bajo coste, alta flexibilidad y biocompatibilidad y fácil fabricación. Además, son químicamente resistentes, no aparecen interacciones específicas, tal como demostraron Kido y col., 2000, poniendo a punto el primer inmunoensayo cuantitativo para la determinación de residuos de plaguicidas en aguas, utilizando como soporte la cara de policarbonato de discos compactos convencionales.

Tras este pionero trabajo, se han publicado diferentes patentes y artículos científicos sobre el empleo de Cds para el análisis de muestras químicas y biológicas (Virtanen, 2000; Hammock y col., 2002; Iimura y Ogawa, 2003). En 2009, Lee y col. desarrollaron un ELISA en disco para la determinación de biomarcadores del virus de la hepatitis B (Lee y col., 2009). Ninguno de ellos utiliza el principio de lectura de los escáneres de Cds, sino que el resultado del ensayo se midió

utilizando detectores convencionales como densímetros, fotómetros, fluorímetros, etc., adaptados a una geometría circular.

Existen otros planteamientos, como el propuesto por Lange y col., que desarrollaron un sistema para la detección indirecta de proteína C-reactiva utilizando el cabezal *pickup* de un lector de Cds y una lente de aumento para la adquisición de imágenes de microscopía de alta resolución (Lange y col., 2006).

El grupo de Remacle (Alexandre y col., 2002) ha llevado a cabo análisis de matrices de ADN sobre soportes similares a los Cds, pero en los que la mitad anular más externa es transparente, desprovista de la película metálica. Esta parte externa se reserva para llevar a cabo el ensayo (por tanto, el área analítica es limitada) y la detección se realiza mediante un segundo haz de láser adicional. En estos ensayos, el sistema óptico estándar del lector de Cds se utiliza para trabajar con información numérica en la parte interna de los discos. Otros investigadores utilizaron la rutina de detección de errores del propio mecanismo de lectura de Cds para detectar la presencia de biomoléculas y bacterias sobre la superficie de los discos (Jones y Thigpen, 2005; Jones, 2005). Sin embargo, cada fabricante de lectores/grabadores tienen sus propias microinstrucciones (*firmware*) para la corrección de errores, afectando a la sensibilidad del ensayo (Reber y Perttunen, 2000; Reber y Perttunen, 2004).

Estudios previos han demostrado que un dispositivo de lectura/grabación óptico (lector/grabador de Cds) integrado en un ordenador personal convencional puede tener un alto potencial analítico, utilizando el diodo de luz del lector de Cds como fuente láser de barrido (Morais y col., 2007; Morais y col., 2008). Además, se ha demostrado la utilidad de la tecnología de Cds para la generación de señales inmunoquímicas *in situ* sin interrumpir la lectura normal del disco.

Más recientemente, se ha desarrollado el primer microinmunoensayo multiplexado (es decir, capaz de determinar varias especies simultáneamente de una misma muestra) sobre DVDs, comparándose dos estrategias de lectura: la primera diseñada para la adquisición de datos de señales analógicas atenuadas y la segunda basada en el análisis de los errores de lectura. Los resultados indicaron que la primera alcanza mayor sensibilidad (Morais y col., 2009).

La superficie de un disco estándar (94 cm²), puede contener miles de puntos. En lo que respecta a la capacidad de procesado de muestras, un disco estándar tiene 12 cm de diámetro. Teniendo en cuenta que se trabaja en formato

micromatriz y con microgotas de un diámetro medio de 300 μm y un espaciado de 100 μm , se pueden imprimir 70.800 puntos (biorreceptores) por disco. Estos datos revelan que las metodologías basadas en disco compacto (o sistemas Lab-on-a-CD) son una tecnología de vanguardia y foco de una intensa investigación, como demuestran la gran cantidad de artículos científicos publicados al respecto en los últimos años (Gorkin y col., 2010; Alphonsus y col., 2010; Mao y col., 2010; Lin y col., 2010).

4. JUSTIFICACIÓN TEMÁTICA DE LA TESIS

En los últimos años, a consecuencia del despliegue de la Organización Mundial del Comercio (OMC), se ha producido una reducción de las barreras arancelarias en el sector agroalimentario, fenómeno que se ha visto acompañado por la disminución en la intensidad de las subvenciones incluidas en la Política Agraria Común (PAC). Esta situación ha expuesto a la competencia internacional un sector como el agroalimentario, históricamente acostumbrado a una competencia internacional limitada.

Esta presión competitiva se centró inicialmente en el sector primario y en los productos no elaborados, pero en los últimos años se está extendiendo al resto del sector agroalimentario, que ahora no sólo se enfrenta a un encarecimiento relativo de las materias primas y a la competencia de las multinacionales agroalimentarias, sino que además debe hacer frente a la nueva competencia de productos procedentes de los nuevos miembros de la UE.

Ante esta situación, las empresas agroalimentarias españolas deben recurrir a la diferenciación, con objeto de mantener las sólidas posiciones de mercado que disfrutaban, al tiempo que aprovechando este nuevo proceso globalizador, puedan completar su proceso de internacionalización, iniciado tímidamente en los años 80 en respuesta a la entrada en España de compañías multinacionales, que tuvo lugar a partir de los años 70 del pasado siglo.

Una vía para hacer frente a estos retos pasa por la innovación, con el desarrollo y comercialización de nuevos productos que incorporaran características adicionales a las incluidas en el producto base, que se convertirían en intangibles y

asociados a la marca del fabricante, darán lugar a la generación de grandes ventajas competitivas.

En este marco, una alternativa fundamental para el refuerzo del sector de zumos cítricos pasa por el desarrollo de nuevos productos basados en tecnologías que incidan en el vector salud, en consonancia con las demandas de los consumidores, ligadas cada vez más a adquirir hábitos saludables siguiendo una dieta equilibrada mediante la ingesta de alimentos -como las frutas y verduras-, frescos o procesados, seguros y sin mermas en el contenido de nutrientes y compuestos bioactivos.

En esta tesis se han abordado diferentes aspectos para la mejora de la calidad y seguridad de los zumos de mandarina. En primer lugar, se ha evaluado la calidad de los zumos de mandarina -en términos de nutrientes y no nutrientes-, que favorecen el mantenimiento de la salud, al igual que otras propiedades de aceptabilidad -atributos sensoriales-, obtenidos con dos variedades distintas (clemenpons y orogrande), procedentes de cultivo tradicional y ecológico.

Puesto que el tratamiento térmico puede provocar mermas en el contenido en nutrientes, compuestos bioactivos, aromas y color, entre otros parámetros, que afectan a la calidad del producto elaborado, en una segunda aproximación se evaluó el efecto del tipo de procesado (pasteurización y ultracongelación) y la aditivación con agentes encapsulantes (ciclodextrinas) sobre ciertos parámetros: ácido ascórbico, carotenoides, color y actividad antioxidante; en zumos de mandarina procedente de cultivo tradicional y ecológico.

Desde hace algunos años, los mensajes publicitarios conectan con una idea basada en alimentación ligada con la salud, pero en la que también la concepción está cambiando. La salud es ahora un estado al que se puede llegar -y al que no se llega por una cuestión de azar-, y se consigue disponiendo de conocimientos para hacerlo, realizando esfuerzos que acompañen las conductas correctas y dando marcada atención a las circunstancias individuales. Para ello, es preciso conocer cuáles son las propiedades saludables de los alimentos, pero además el consumidor debe actuar para obtener este bienestar, aportando al consumo alimentario un carácter de autocontrol.

Esto no ha pasado inadvertido y la industria agroalimentaria ha entendido la necesidad de incrementar el valor añadido del producto de consumo, invirtiendo en investigación para desarrollar nuevos alimentos con nuevas propiedades -más

allá de las alimenticias- evidencias científicamente, mediante la incorporación de alimentos enriquecidos a su gama de productos. En este contexto, se desarrollaron zumos de mandarina de la variedad ortanique enriquecidos con extractos de granada (1%) y goji (2%), a los que se les adicionó un 1% de agentes encapsulantes (ciclodextrinas nativas o modificadas), evaluando -tras una pasteurización suave-, ciertos atributos sensoriales, capacidad antioxidante, color y vitamina C a lo largo de la vida útil del zumo (72 días a 4 °C).

Si en la primera parte de la tesis se han evaluado diferentes estrategias para mejorar la calidad de los zumos de mandarina, con el fin de introducir en el mercado productos saludables diferenciados, en la segunda se aborda el desarrollo de herramientas analíticas -basadas en los principios del inmunoensayo- para garantizar la inocuidad de los mismos.

El empleo en agricultura de productos fitosanitarios está plenamente justificado, pero no debemos olvidar que son sustancias tóxicas y peligrosas que provocan efectos adversos en el hombre y su entorno, por lo que son objeto de seguimiento y control, utilizando métodos cromatográficos convencionales, que presentan ciertas limitaciones -capacidad de trabajo reducida y no aplicables al análisis en campo-, no siendo adecuados para el desarrollo programas de vigilancia y control de residuos cada vez más rigurosos, que requieren el desarrollo de métodos analíticos sensibles, selectivos, simples, rápidos, económicos y fáciles de usar.

De entre las diferentes metodologías alternativas, las basadas en Inmunoensayo (IA) presentan un gran potencial y su interés para la detección de plaguicidas en el campo agroalimentario y medioambiental ha aumentado extraordinariamente en los últimos años. Sin embargo, los inmunoensayos, como cualquier metodología analítica, también presentan inconvenientes ya que en general, son métodos unianálisis y los ELISA disponibles en el mercado requieren aproximadamente tres horas de ensayo.

Para solventar estas limitaciones, se diseñaron dos estrategias. En primer lugar, se ha evaluado la posibilidad de reducir los tiempos de competición y revelado de un ELISA convencional, utilizando como modelo dos productos fitosanitarios (clorpirifos y fentión) aplicados habitualmente a cultivos cítricos, para los que se disponía de inmunorreactivos.

En una segunda aproximación y como vía para el desarrollo de plataformas analíticas para análisis masivo, se aborda el desarrollo de inmunoensayos multianalito en dos tipos de soportes: placas ELISA y discos compactos, utilizando como modelo los sistemas clorpirifos y fentión.

En ambos casos, los sistemas analíticos se aplican a la detección de plaguicidas en zumos de mandarina enriquecidos con clorpirifos y fentión, evaluando además el efecto del tratamiento térmico (tiempo y temperatura) sobre la concentración de ambos insecticidas organofosforados.

CAPÍTULO II. OBJETIVOS

1. OBJETIVOS

El análisis de las tendencias actuales de los consumidores pone de manifiesto diversos factores sociales que se dan simultáneamente y que resultan en la búsqueda de productos de conveniencia ligada cada vez más a adquirir hábitos saludables, entre ellos seguir una dieta equilibrada que incluya diferentes porciones de hortalizas y frutas, frescas o procesadas como la mandarina, rica en compuestos beneficiosos para la salud. Pero para que la cadena alimentaria y el comercio funcionen correctamente, se requiere un denominador común, el factor seguridad de los alimentos, que constituye el condicionante de la demanda económica de todos los mercados y la garantía de continuidad de los productos alimenticios en ellos.

Si bien la concentración de compuestos saludables, como los carotenoides, puede variar significativamente entre especies de cítricos y variedades, las prácticas de cultivo, tipo de suelo, agua de riego, el empleo de fertilizantes o productos fitosanitarios, pueden alterar la composición de nutrientes y no nutrientes en la materia prima con la que posteriormente se obtendrá el zumo. Además, el tipo de procesado puede provocar mermas acusadas en el contenido de compuestos saludables y en las cualidades organolépticas del zumo de mandarina.

Con estas premisas se han plantado los siguientes objetivos:

1. Evaluar diferentes parámetros de calidad en zumos de mandarina procedentes de distintas variedades y tipos de cultivo.
2. Estudiar el efecto del tipo de procesado (pasteurización y ultracongelación), sobre ciertos parámetros nutricionales y sensoriales en zumos de mandarina.
3. Estudiar la evolución de la vitamina C, carotenoides y ciertos atributos sensoriales a lo largo de la vida útil de zumos de mandarina enriquecidos con ciclodextrinas nativas o modificadas.
4. Evaluar la evolución de la vitamina C, carotenoides y ciertos atributos sensoriales a lo largo de la vida útil de una nueva bebida a base de mandarina, granada y zumo de Goji.

5. Desarrollo de una nueva metodología analítica sencilla, efectiva y rápida, aplicable al análisis simultáneo de los plaguicidas clorpirifos y fentión en zumos de mandarina.

Para la consecución de este último objetivo general, se plantean los siguientes objetivos parciales:

- 5.1. Simplificar protocolos de tratamiento de muestra y reducir tiempos de ensayos individuales en formato ELISA.
- 5.2. Desarrollar un inmunoensayo en formato ELISA para la determinación simultánea de clorpirifos y fentión en zumos de mandarina.
- 5.3. Integrar los inmunoensayos para clorpirifos y fentión en un ensayo multianalito sobre la superficie de un disco compacto, que sirva como modelo para el desarrollo de plataformas analíticas para análisis masivo.
- 5.4. Evaluar el efecto del tratamiento térmico sobre la concentración de clorpirifos y fentión en zumos de mandarina.
- 5.5. Validar los inmunoensayos desarrollados comparando los resultados obtenidos con los aportados por técnicas cromatográficas.

CAPÍTULO III. ARTÍCULOS PUBLICADOS

Preliminary communication**QUALITY OF ORGANIC MANDARIN JUICE, CV. CLEMENPONS**A.J. PÉREZ-LÓPEZ^{a*}, P. NAVARRO^a, A. SOLER^a and A.A. CARBONELL-BARRACHINA^b^aDepartment of Food Technology and Nutrition, Catholic University of San Antonio, Campus de los Jerónimos, s/n, 30107-Guadalupe, Murcia, Spain^bDepartment of AgroFood Technology, Miguel Hernández University, Carretera de Beniel, km 3,2, 03312-Orihuela, Alicante, Spain

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Everyday consumption of fruits and vegetables is associated with a lowered risk of cancer, hypertension and heart diseases due to the various forms of antioxidants present in these foods such as carotenoids. Besides, consumers demand organic products because they are believed to be more flavourful, healthier, and more respectful to the environment. The effects of organic farming on CIEL^{a*b*} colour, minerals contents, carotenoids composition and sensory quality of Clemenpons mandarin juices were studied. Organic farming of mandarins resulted in juices with higher mineral and carotenoid contents and of better sensory quality. For instance, organic juice contained a total concentration of carotenoids of 17.3 mg l⁻¹ compared to 13.5 mg l⁻¹ of conventional juice. Finally and in general, mandarin juices are good source of some important elements, such as potassium and antioxidants, e.g. β -cryptoxanthin.

Keywords: β -cryptoxanthin; CIEL^{a*b*}; Citrus; potassium; sensory evaluation.

There are over 100 companies involved in producing organic juices and supplying the European market with them. On this market only companies with sustainable competitive advantage will survive. Thus objective and scientific research is needed to prove the possibly better quality of organic products. This manuscript proves that organic foods have higher essential vitamin and mineral contents compared to the traditional products. Organic mandarin juice contained particularly higher levels of vitamin C and essential minerals, such as potassium, calcium, magnesium and iron, as well as cancer fighting antioxidants, such as carotenoids.

Organic agriculture places itself against the development through synthetic fertilizers and other agrochemicals. It differs from many other agricultural innovations because those involved in its development were not primarily agriculturalists, but represented other relevant parts of society including biologists and consumer groups as well (WATSON et al., 2008).

Interest in organically produced food is increasing throughout the world in response to concerns about conventional agricultural practices, food safety, human health, animal welfare and the environment (GREGORY, 2000). These concerns, along with observed organic consumer behaviour has led, in part, to emergence of various groups of organic consumers, namely environmentalists, food-phobics, healthy eaters, humanists, welfare enthusiasts, and hedonists (DAVIES et al., 1995).

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Recent studies by Consumers Unions showed that organically grown foods have fewer and generally lower pesticide residues than conventionally grown foods (BAKER et al., 2002). A survey of supermarket customers in U.S.A. indicated that over 70% of consumers were concerned about the health effects of pesticide residues and were willing to pay over 10% price for pesticide residue-free products (COLLINS et al., 1993).

Organic farming has developed into one of the most dynamic agricultural sectors in the European Union and the U.S.A. The organic sector has grown around 30% annually since 1998. This growth was triggered by the recent political interest in promoting organic agriculture. The most important initiative on a European level is the EU agri-environmental policies introduced as part of the CAP Reform in 1992. The number of registered organic producers increased from 100×280 in 1998 to 135×191 in 2003, which represents a 6% average annual increase over the 1998–2003 period (SOGAARD, 2001). From 1997 to 2005, the number of certified organic acres in the United States grew by 63% while certified organic farmland increased by 166% over the same period (WILLER & MINOU, 2005).

Spain is the second world producer of mandarins and the main supplier of the international market. One of the most grown and exported mandarin cultivars is Clemenpons (BIELSA & NAVARRO, 2007). Currently, the main two items elaborated with mandarins are canned slices and juice, but they only represent about 7% of total trade of this fruit.

Citrus fruits have long been valued as part of a nutritious and tasty diet. The flavours provided by citrus are among the most preferred in the world, and it is increasingly evident that citrus not only tastes good, but is also good for health. It is well established that citrus and citrus products are rich sources of vitamins, minerals, dietary fibre as well as many phytochemicals including flavonoids, glucarates, coumarins, monoterpenes, limonoids, triterpenes, phenolic acids and carotenoids.

Carotenoids can help to reduce the risk of many chronic diseases. Dietary guidelines and recommendations that encourage the consumption of citrus fruit and their products can lead to widespread nutritional benefits across the population (CLEVELAND et al., 1996). The naturally occurring carotenoids are responsible of citrus juice colours and their composition is influenced by several factors including geographical origin, fruit maturity, and particularly variety (BELTRÁN-GONZÁLEZ et al., 2008). Citrus fruits are great sources of phytochemicals, among which carotenoids are present in the largest number (GOODNER et al., 2001). Approximately 115 different carotenoids have been reported in citrus, although many of them were misidentified or turned out to be artefacts (MELÉNDEZ-MARTÍNEZ et al., 2007). The main carotenoids found in conventionally grown oranges and mandarins are β -cryptoxanthin, violaxanthin, neoxanthin, lutein, antheraxanthin, α -carotene, and β -carotene (MATSUMOTO et al., 2007). Even though there is no information available in the literature on the carotenoid composition of organic and conventional orange mandarin cv. Clemenpons, recent studies have demonstrated that organic farming has positive effects, among other quality parameters, on total carotenoid content of mandarins, cv. Hernandina (10.2 and 14.4 mg kg⁻¹ for traditional and organic juices, respectively) (BELTRÁN-GONZÁLEZ et al., 2008).

As previously mentioned, many people believe that organic foods may have higher nutritional value and lower pesticide contamination than conventional food (PÉREZ-LÓPEZ et al., 2006). However, this consumers' expectation should be properly verified by scientific studies. The main problem in a comparative study of organically- and conventionally-produced foods is the selection of the experimental fields in such a manner that they truly represent the cultivation forms to be compared. The farm selected for this study has been monitored by experts of the Board of Organic Agriculture of the Murcia Region (BOAM, 2008).

The main aim of this study was to determinate the effects of type of agricultural practice (organic and conventional) on CIEL^{*}a^{*}b^{*} colour parameters, mineral contents, total carotenoid content, carotenoid composition, and sensory quality of mandarin orange (cv. Clemenpons) juice.

1. Materials and methods

1.1. Fruit

The Clemenpons fruits are of good size, spherical but smoothed at the poles; it peels quite easily and it is almost seedless. The Clemenpons trees have high yields and are precocious, with harvesting being carried out during September–November. Both conventional and organic mandarins were grown in the same farm and under identical conditions of soil, irrigation, and illumination in eastern Spain (Librilla, Murcia). The rootstock was the same for both mandarin trees, by grafting budwood onto citrange rootstock, and all selected trees were about 12-year old and free of diseases. Fruits were collected in winter (last week of November, 2006) and were sorted on the basis of their diameter, pH, total soluble solids content (SSC, °Brix) and maturity index (total soluble solids content / titratable acidity, SSC / TA) (Table 1).

Table 1. The effect of agricultural practice (conventional or organic) on the main properties of Clemenpons mandarins at the time of harvest

Agricultural practice	Weight (g)	Diameter (mm)	Vitamin C (mg l ⁻¹)	SSC ^a (°Brix)	TA ^a (% citric acid)	Maturity index ^a (SSC/TA)
Conventional	76.5±5.9b ^b	60.5±2.1a	241±18b	11.6±0.2a	0.97±0.02a	12.0±0.2a
Organic	72.2±4.7a	57.2±3.4a	292±24a	11.9±0.5a	0.95±0.03a	12.5±0.3a

^aSSC: soluble solid content; TA: titratable acidity; Maturity index is the ratio between the solid soluble content and titratable acidity. ^bMandarin orange fruits with the same letters, within the same column, were not significantly different at P<0.005 for the evaluated property (Tukey's multiple range test)

Table 2. Materials used in both conventional and organic farming of Clemenpons mandarins

Compound	Agricultural practice	
	Conventional	Organic
Soil fertilizers	Ammonium nitrate, calcium nitrate, ammonium sulphate, phosphoric acid, diammonium phosphate, potassium sulphate, potassium nitrate, potassium chloride	Manure, compost, and fulvic and humic acids
Foliar fertilizers	Potassium phosphate, magnesium nitrate, urea, mixture of oligoelements	Algae extracts, amino acids
Herbicides	Bromacil, diuron, diquat, fluroxypyr, glyphosate, norflurazon, paraquat, sulfosate, simazine.	None; weeds are removed by mechanical methods
Pesticides	Malathion, dicofol, methidathion, clopidol	Neem oil, pheromone traps

Mandarin cultivar Clemenpons was studied on conventional and organic farming. Organic production means that no synthetic chemicals were used in the nurturing of these fruit trees and that only natural substances were used to control pest, weeds, etc. Farming of organic mandarin trees followed all rules established by the Board of Organic Agriculture of the Murcia Region (BoAM, 2008). A complete list of the materials used in both conventional and organic farming is included in Table 2.

1.2. Sample preparation

Mandarins were collected and processed the same day, 27 November 2008. The exact weight of fruits processed were 10 T and 5000 kg of conventional and organic Clemenpons, respectively. Mandarin juices were processed in a commercial plant (Murcia, Spain). The juices were obtained by using a Premium Juice Extractor (FMC Corporation, Florida, U.S.A.) (FMC, 2008). This machinery produces juice with a low essential oil content (KIMBALL, 2002).

Freshly squeezed juices were treated in an Alfa Laval plate heat exchanger (Alfa Laval Iberia S.A., Madrid, Spain) for 20 s at a temperature of 98 °C. After this heat treatment, the juice was first transferred to a pre-cooler, which cooled the juice down to 30 °C using forced air, and then to a cooler, which finally took the temperature down to 2 °C. Heat-treated juices were stored in aseptic metallic deposits of 50 000 l at a temperature of 4 °C until the juice was ready for the market. Finally, juices were packaged in brick cartons, made of aluminium foil and polyethylene, and stored under refrigeration conditions (4 °C).

1.3. Physico-chemical analyses

SSC (°Brix) was determined using a portable refractometer Comecta, S.A., model C3 (Barcelona, Spain). TA (% citric acid) was determined in 10 ml of juice by titration to pH 8.2±0.1 with a 0.1 N NaOH solution. The maturity index (MI) was calculated for each mix and expressed as the percentage of the ratio between the SSC and TA.

Vitamin C was measured following the A.O.A.C. (HORWITZ, 2000). Ascorbic acid was estimated by titration with coloured oxidation-reduction indicator, 2,6-dichloroindophenol. EDTA was added as chelating agent to remove Fe and Cu interferences.

All physico-chemical analyses were carried out with 20 fruits of each agricultural practice.

1.4. Instrumental measurement of colour

Colour determinations were made, at 25±1 °C, using Hunterlab Colorflex® (Hunterlab, Reston, Virginia, U.S.A.) spectrophotometer with an illuminant D65 and a 10° observer as references. A sample cup for reflectance measurements was used (5.9 cm internal diameter×3.8 cm height) with a path length of light of 10 mm. Blank measurements were made with the cup filled with distilled water against a reference white background (PÉREZ-LÓPEZ et al., 2006).

Colour data are provided as CIEL*a*b* coordinates, which define the colour in a three-dimensional space (MINOLTA, 1994). Colour analyses were run in 6 replicates.

1.5. Minerals

A multi-place digestion block, Selecta Block Digest 20 (Barcelona, Spain), was used for

sample mineralization. Fifteen ml of juice were treated with 5 ml of 65% (w/v) HNO₃ in Pyrex tubes, placed in the digestion block, and heated at 60 °C for 60 min and at 130 °C (to avoid losses of volatile substances) for 120 min (CARBONELL-BARRACHINA et al., 2002). Solutions were left to cool to room temperature, transferred to a volumetric flask, and diluted to a final volume of 25 ml with ultrahigh-purity deionised water.

Determination of Ca, Mg, K, Na, Cu, Fe, Mn and Zn in previously-mineralized samples was performed with a Unicam Solaar 969 atomic absorption spectrometer (Unicam Limited, Cambridge, U.K.). Minerals were analysed in 5 replicates.

Instruments were calibrated using certified standards. In each analytical batch, at least two reagent blanks, one international reference material (CRM) and one spike were included to provide precision and accuracy for the chemical analysis. The certified material selected for the current experiment was GBW07603 (bush, branches and leaves); this material was provided by LGC Deselaers S.L. (Barcelona, Spain) and produced by the Institute of Geophysical and Geochemical Exploration of China (GBW07603).

1.6. Carotenoid compounds extraction and quantification

The analysis of the carotenoid composition requires an extraction with organic solvent followed by de-esterification of the carotenoid fraction. Mandarin orange juices were extracted with 50 ml of acetone, by using a Kika Labortechnik, T25 basic homogenizer (Jankel & Kunkel GmbH & Co, Staufen, Germany), until the complete exhaustion of colour (extraction was repeated until filtrates are colourless). All extracts were pooled in a separator and shaken with diethyl ether. A sufficient quantity of 10% NaCl was added at the end to aid the separation of the phases. Subsequently the organic phase was dried over anhydrous Na₂SO₄. This phase containing the pigments, in various stages of esterification with fatty acids, was saponified with 100 ml of 20% KOH-methanol for 1 h at room temperature. The pigments were subsequently extracted with diethyl ether, evaporated in a rotary evaporator, and taken up in a maximum of 10 ml of acetone. Aliquots (1 ml) of this was centrifuged at 12 000 r.p.m and stored at -30 °C until analysed.

The carotenoid profile of the extract was quantified using β-apo-8'-carotenal (Sigma Chemical Co.; St Louis, MO, USA) as internal standard, added at the beginning of the extraction process, 1 ml of 100 µg ml⁻¹. This chemical was used as internal standard because it is absent in orange mandarin and, under the proposed conditions it separates well from other carotenoids (MARÍN et al., 2004).

The high-performance liquid chromatography system consisted of an HP-1100 series unit with a photodiode array detector controlled with HP ChemStation software (Hewlett Packard, Palo Alto, CA). The column used was of 250 mm × 4.6 mm i.d., YMC C30, S-5 µm (YMC, Schermbeck, Germany). The mobile phase for this column was 81:15:4 methanol: methyl tertiary butyl ether (MTBE): H₂O (solvent A) and 91:9 MTBE: methanol (solvent B). The gradient elution was 100% A to 50% A and 50% B in 45 min followed by 100% B in the next 10 min and 100% A in the next 5 min at a flow rate of 0.8 ml min⁻¹ (ANDREU-SEVILLA et al., 2008). Carotenoids were monitored at 450 nm and analyses were carried out in triplicate.

Identification was based on the order of elution, retention time, and spectra of absorbance maxima of a particular peak. Standards of violaxanthin, antheraxanthin, β-cryptoxanthin, and α- and β-carotene for quantification purposes were obtained from CaroteNature (Lupsingen, Switzerland); besides, standards of lutein and zeaxanthin were obtained from Extrasynthase (Genay, France). Average fortification recoveries (for a 10 µg carotenoids spike on 10 ml of

a 20 mg l⁻¹ standard) through the complete method were 95, 93, 90, and 92% for violaxanthin, antheraxanthin, β-cryptoxanthin and β-carotene, respectively.

The absolute concentrations of the main mandarin juices carotenoids (violaxanthin, antheraxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene) were worked out by external calibration, performed in compliance with recommended guidelines from calibration curves constructed with the corresponding standards (RODRÍGUEZ-AMAYA, 2001). On the other hand, the levels of luteoxanthin-like, 9-*cis*-violaxanthin, luteoxanthin b, mutatoxanthin-like, *cis*-antheraxanthin, and Zeinoxanthin were calculated using β-apo-8'-carotenal as internal standard, due to lack of proper commercial standards. Total carotenoid content was assessed as the sum of the content of the 13 individual carotenoids studied in this experiment.

1.7. Sensory evaluation with trained panel

Sensory evaluation with trained panel was used to evaluate the quality of mandarin orange juice. A panel of 10 panellists, ages 20 to 50 years (8 female and 2 male, all members of the Catholic University San Antonio of Murcia), with sensory evaluation experience, was trained in descriptive evaluation of citrus juice (SERRANO-MEGÍAS et al., 2005).

The panel was selected and trained following the ISO standard (AENOR, 1997). Further details on selection, training and validation of the panel can be found in the article of PÉREZ-LÓPEZ and CARBONELL-BARRACHINA (2006).

Measurements were performed in individual booths with controlled illumination (70–90 fc) and temperature (23±2 °C) (PÉREZ-LÓPEZ & CARBONELL-BARRACHINA, 2006).

The individual products were scored for the intensities of colour, sweetness, acidity, fresh mandarin juice aroma, floral aroma, and off-flavour. A 0 to 10 scale was used to quantify the intensity of the different aromas, where: 0 = extremely slight intensity, and 10 = extremely high intensity. For the colour quantification also a 0 to 10 scale was used, with 0 = very pale orange and 10 = very dark orange.

Samples were randomly presented, coded with three-digit numbers, in 50 ml plastic cups with lids. The entire experiment was repeated 3 times at 3 different days (all judges scored 2 juice samples on each session for a total of 3 sessions) and the sensory scores were presented as the overall means.

1.8. Statistical analysis

All data were subjected to analysis of variance (ANOVA) and the Tukey's least significant difference multi-comparison test to determine significant differences among mandarin juices. Significance of differences was represented as P≤0.001. The statistical analyses were done using SPSS 14.0 (SPSS Science, Chicago, USA) and figures using Sigma Plot 9.0 (SPSS Science, Chicago, U.S.A).

2. Results and discussion

No significant differences in quality parameters of the conventional and organic mandarins were found at picking time (Table 1). The main properties of the mandarins selected for this experiment were: 74.4 g, 58.9 mm of diameter, 267 mg l⁻¹ vitamin C, and a maturity index of 12.3; organic mandarins contained a significantly higher content of vitamin C than conventional fruits (292 and 241 mg l⁻¹, respectively).

2.1. Instrumental measurement of colour

Juices obtained from organic Clemenpons mandarins presented significantly higher intensities of red, yellow, and orange colour than juice from conventional fruits (Table 3). A change from traditional agricultural practices to more environmentally friendly organic farming practices resulted, in this particular study, in increases of 4.97, 6.28, and 7.98 units for a^* (red colour), b^* (yellow colour), and C_{ab}^* , respectively. Increases above one unit in these colour coordinates can be associated with significant changes in the colour of the products as affected by the variable under study (e.g. agricultural practice). Therefore, increases of about 3 units in a^* , b^* , and C_{ab}^* clearly proved that organic mandarin orange juices have a more intense orange colour (mixture of red and yellow colours) than traditional juices.

Similar positive effects of organic farming on the colour, mineral contents, and aroma composition of Clemenules mandarin juices were reported previously by (PÉREZ-LÓPEZ et al. 2007). Considering the colour data included in Table 3, Clemenpons mandarins (mean values of L^* 56.71, a^* 26.55, b^* 39.41, and C^* 47.51) provided juices with higher intensities of red, yellow and therefore orange colours than Clemenules mandarins (L^* 53.14, a^* 8.89, b^* 29.67, and C^* 30.97).

Table 3. The effect of agricultural practice (conventional or organic) on the colour coordinates of Clemenpons mandarin juices

Agricultural practice	L^*	a^*	b^*	C_{ab}^*	h_{ab}
Conventional	58.12±0.82a [*]	24.06±0.74b	36.27±0.83b	43.52±0.43b	56.44± 0.61a
Organic	55.29±0.48b	29.03±0.25a	42.55±0.37a	51.50±0.35a	55.69±0.25a

*Mandarin orange fruits with the same letters, within the same column, were not significantly different at $P < 0.005$ for the evaluated property (Tukey's multiple range test)

2.2. Mineral contents

Certified values for Ca (%), Mg (%), K (%), Cu (mg kg^{-1}), Fe (mg kg^{-1}), Mn (mg kg^{-1}) and Zn (mg kg^{-1}) were: 1.81±0.07, 0.65±0.03, 1.38±0.04, 274±10, 9.3±0.5, 45±2 and 37±1, respectively, while measured values for the same elements were: 1.82±0.04, 0.65±0.02, 1.37±0.03, 270±8, 9.3±0.3, 45±1 and 37±1, respectively. These results show the goodness of the digestion and spectroscopy analyses of minerals.

Organic farming had a significant effect on the content of all analysed elements, macro-elements (Ca, Mg, and K) and micro-elements (Fe, Cu, Mn, and Zn). In general, all element contents were higher in the juice from the organic mandarins (Table 4) (increases of 17.5, 24, 465, 0.29, 0.09, 0.13, and 0.15 mg l^{-1} for Ca, Mg, K, Fe, Cu, Mn, and Zn, respectively). It is important to state that mandarin orange juice is a very good source of potassium (mean of 1.66 g l^{-1}).

The concentrations of all elements analysed in this study fall within the range reported in the literature for mandarin juices from different geographical areas, such as Florida, California, México and Brazil, and within the range suggested by the guidelines of AIJN (2008) (TING & ROUSEFF, 1986). Besides and as expected as our work was carried out with natural freshly squeezed juices, the concentrations of Cu, Zn, and Fe were below the maximum levels recommended by the Codex Stan 45-1981 of 5, 5, and 15 mg l^{-1} .

Table 4. The effect of agricultural practice (conventional or organic) on the mineral contents of Clemenpons mandarin juices

Agricultural practice	Minerals (mg l ⁻¹)							
	Ca	Mg	K	Na	Fe	Cu	Mn	Zn
Conventional	45.7±3.7b ^a	132±13b	1426±63b	4.64±0.52b	0.68±0.09b	0.24±0.09a	0.36±0.05a	0.21±0.05b
Organic	63.2±4.9a	156±7a	1891±41a	6.17±0.81a	0.97±0.13a	0.33±0.02a	0.49±0.08a	0.36±0.07a

^aMandarin orange fruits with the same letters, within the same column, were not significantly different at P<0.005 for the evaluated property (Tukey's multiple range test)

2.3. Carotenoid composition

Organic farming had also a positive effect on the total carotenoid content in Clemenpons juices, with values being 13.47 and 17.32 mg l⁻¹ for conventional and organic juices. A change from traditional agricultural practices to organic farming practices resulted in an increase of 3.85 mg l⁻¹ (28.6%) of total carotenoid content, which is a very significant improvement in the nutritional value of this juice. Organic farming significantly increased concentrations of all carotenoids in Clemenpons juice (Table 5). The most remarkable changes were observed for 9-*cis*-violaxanthin, violaxanthin and β-cryptoxanthin.

Table 5. The effect of agricultural practice (conventional or organic) on the carotenoid concentrations of Clemenpons mandarin juices

Pigment ⁸	R.T. (min)	Experimental	Literature	Conventional	Organic
		λ_{max} (nm)		Concentration (mg l ⁻¹)	
Violaxanthin	13.9	418,444,471	414,442,472	0.21±0.06b	0.41±0.09a
Luteoxanthin-like ^a	16.4	400,424,450	400,424,450	0.13±0.04b	0.19±0.02a
9- <i>cis</i> -Violaxanthin ^a	18.1	328,414,438,466	328,414,438,466	0.22±0.04b	0.83±0.12a
Antheraxanthin	18.8	422,447,475	422,447,475	0.93±0.22b	1.18±0.13a
Luteoxanthin b ^a	20.1	396,419,445	397,419,445	0.14±0.03b	0.23±0.04a
Lutein	21.7	424,448,475	426,443,473	0.56±0.09b	0.88±0.14a
Mutatoxanthin-like ^a	22.2	408,430,454	409,430,451	1.08±0.25b	1.19±0.36a
<i>cis</i> -Antheraxanthin ^a	23.9	333,420,444,471	333,420,444,471	1.02±0.22b	1.35±0.29a
Zeaxanthin	24.7	428,454,480	428,452,481	0.46±0.13b	0.57±0.24a
Zeinoxanthin ^a	27.7	424,447,476	425,447,477	1.34±0.08b	1.64±0.13a
β-Cryptoxanthin	31.1	429,454,479	425,452,479	6.96±0.37b	8.31±0.25a
α-Carotene	35.1	424,448,479	424,449,476	0.31±0.05b	0.39±0.03a
β-Carotene	38.7	429,454,479	425,454,479	0.11±0.02a	0.15±0.03a
Total carotenoids				13.47±0.41	17.32±0.28

^aTentatively identified (carotenoids identified solely by comparison with the literature absorption maxima values, order of elution, and retention time (MELÉNDEZ-MARTÍNEZ et al., 2007b; LEE, 2001).

LEE and CASTLE (2001) concluded that total juice carotenoids increased as the fruit ripening progressed and ranged from about 1 mg l⁻¹ in unripe oranges up to 9 mg l⁻¹ in ripe fruits. Values found in the present experiment agreed with ripe mandarins (mean maturity index of 12.0), which have a very intense orange colour as previously described.

In Clemenpons juices, the most abundant carotenoids were β-cryptoxanthin, zeinoxanthin, *cis*-antheraxanthin, mutatoxanthin-like carotenoids, and antheraxanthin with mean contents of 7.64, 1.49, 1.19, 1.14, and 1.06 mg l⁻¹, respectively. MELÉNDEZ-MARTÍNEZ *and co-workers* (2007a) stated that the carotenoid profile of most foods depends on the variety of oranges, climatic factors, industrial processing and storage conditions, among others. But even considering these limitations, β-cryptoxanthin and antheraxanthin have been widely reported as being two of the major carotenoids found in orange juices (GOODNER et al., 2001; MELÉNDEZ-MARTÍNEZ et al., 2007a).

β-Cryptoxanthin has a dark orange, almost red, appearance that gives mandarin fruit and juices their distinctive colours (GOODNER et al., 2001). β-cryptoxanthin concentrations vary drastically among different citrus fruits, being large for all mandarin products and low for all orange products (YANO et al., 2005). In fact, GOODNER *and co-workers* (2001) concluded that β-cryptoxanthin can be used as a discriminating factor for orange, mandarin and hybrid classification. β-Cryptoxanthin represented a mean of 49.6% of all carotenoids found in Clemenpons juices (51.7 and 48.0% in traditional and organic juices, respectively). LEE and CASTLE (2001) reported that with the onset of peel colour development, the gradual synthesis of new pigments begin, in particular, β-cryptoxanthin, zeaxanthin, and antheraxanthin. These authors reported values of β-cryptoxanthin growing from 2.6% in unripe fruits up to 12.9% in ripe juices of Hamlin, Earlygold, and Blood sweet oranges.

The carotenoid previously identified as isolutein (lutein epoxide) in orange juice was isolated and identified as a 9 or 9'-*cis* isomer of antheraxanthin by MELÉNDEZ-MARTÍNEZ *and co-workers* (2005). *cis*-Antheraxanthin was the third most abundant carotenoid in Clemenpons juices and represented a mean of 7.68% of total carotenoids.

Hydrocarbon carotenoids such as α- and β-carotene were present in small concentrations (2.3 and 0.8%, respectively) in Clemenpons juices, independently of the agricultural practice.

Although approximately 700 carotenoids have been reported only those with an unsubstituted β-ring with an 11-carbon polyene chain, have pro-vitamin A activity (MELÉNDEZ-MARTÍNEZ et al., 2007b). This structural requirement is satisfied by around 60 carotenoids (RODRÍGUEZ-AMAYA, 2001). Vitamin A (retinol) can also be provided in the diet as other preformed forms as pro-vitamin A carotenoids, which are subsequently transformed into vitamin A (MELÉNDEZ-MARTÍNEZ et al., 2007c).

The retinol activity equivalents (RAE) of the samples analysed and discussed here referred to 1 l of mandarin orange juice. The bioavailability of carotenoids is influenced by many factors, such as amount, food matrix, age, existence of certain diseases or parasite infestation, intake of fat, vitamin E and fibre, protein and zinc status (FNBIM, 2000). According to this, it is difficult to accurately determine the retinol activity equivalent (RAE) of any food. In this study, calculations were performed, considering new guidelines according to the following formula (FNBIM, 2000):

$$RAE = \left(\frac{\mu\text{g } \beta\text{-carotene}}{12} \right) + \left(\frac{\mu\text{g } \alpha\text{-carotene} + \mu\text{g } \beta\text{-cryptoxanthin}}{24} \right)$$

The RAE values found in this study for traditional and organic mandarin orange juices were 312.1 and 375.0 RAE l⁻¹, respectively; these high values of RAE l⁻¹ support the general fact that mandarin products are very good sources of vitamin A and this statement is especially true for organic mandarin juices. These experimental values fit perfectly within the range previously described by MELÉNDEZ-MARTÍNEZ *and co-workers* (2007c) for the RAE l⁻¹ contents of different types of orange and mandarin juices marketed in Spain ranging from 9.7 to 359. Besides, these values are also higher than those reported by BELTRÁN-GONZÁLEZ *and co-workers* (2008) for Hernandina juices (217.5 and 253.8 RAE l⁻¹ for traditional and organic juices, respectively). Without any doubt the high levels of RAE in mandarin juices compared to other citrus juices are related to their higher concentrations of β -cryptoxanthin.

2.4. Sensory quality

The trained panel established that organic Clemenpons juice had slightly higher colour intensity, fresh mandarin juice aroma and sweetness than conventional juice; however, results were not statistically significant for sweetness. The exact values for colour, sweetness, acidity, fresh mandarin aroma, and floral aroma for the organic and conventional juices were as follows: 8.8±0.1, 6.5±0.1, 5.7±0.1, 9.0±0.1, 4.9±0.1 and 8.3±0.1, 6.3±0.1, 5.7±0.1, 8.4±0.1, 4.8±0.1, respectively (Fig. 1). No off-flavours were detected for any of the two juices under study.

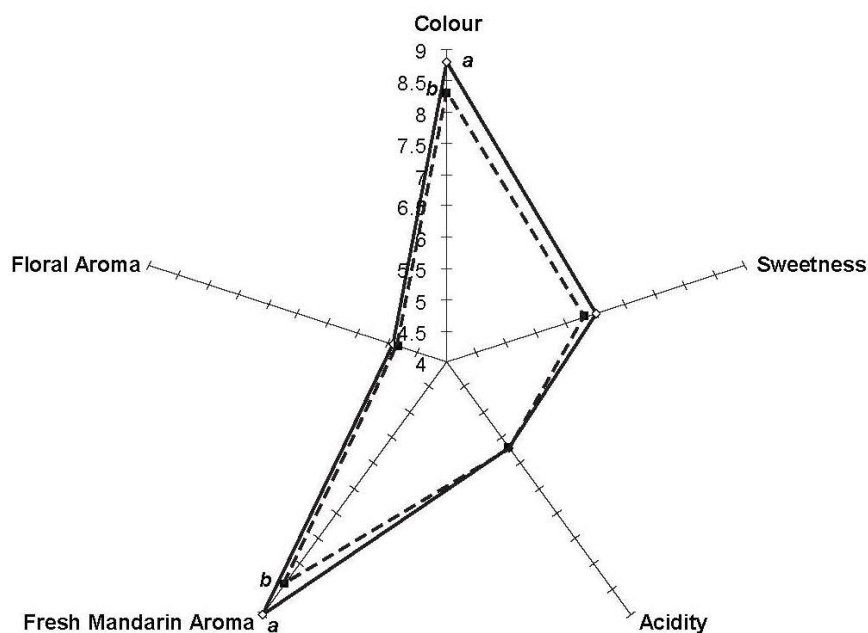


Fig. 1. The effect of agricultural practice (conventional or organic) on the sensory descriptive profiles of Clemenpons mandarin juices. Values of a sensory parameter with different letters were significantly different ($p < 0.05$), Tukey's multiple range test. —◇—: organic; -■-: tradicional

3. Conclusions

Freshly squeezed juices of Clemenpons mandarins had a very intense orange colour. If the most popular mandarin orange cultivar, Clemenules, is not available for juice making, Clemenpons is a good option. In general, mandarin orange juices are very good sources of potassium and β -cryptoxanthin. The organic juice had higher mineral and carotenoid contents and presented higher intensities of red, yellow and orange colours than the conventional juice. Besides, the sensory intensities of colour, sweetness, and fresh mandarin aroma were higher in the organic juice than in the conventional juice. Therefore, organic farming had positive effects on the quality of mandarin orange juices, cultivar Clemenpons.

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Antioxidant Activity, Color, Carotenoids Composition, Minerals, Vitamin C and Sensory Quality of Organic and Conventional Mandarin Juice, cv. Orogrande

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The effects of organic farming on antioxidant activity, CIE $L^*a^*b^*$ color, carotenoids composition, minerals contents, vitamin C and sensory quality of Orogrande mandarin juices were studied. Independent of the farming type, mandarin juices can be considered as good source of some important nutrients, such as potassium and antioxidant chemicals, for example, β -cryptoxanthin. Organic farming of mandarins resulted in juices with higher antioxidant activity, total carotenoids concentrations, minerals (Ca, K and Fe) contents, vitamin C content, more appealing and intense orange color and better sensory quality. For instance, organic Orogrande juice contained significantly ($p < 0.001$) higher total carotenoids content (22.7 ± 0.3 mg/L) than conventional juice (15.7 ± 0.4 mg/L); a similar pattern was observed for the antioxidant activity, with values being 0.076 ± 0.004 and 0.053 ± 0.003 mM Trolox m/L in organic and conventional juices, respectively. A trained panel stated that organic Orogrande juices had higher intensities of orange color, fresh mandarin and floral aromas than conventional juices.

Key Words: β -cryptoxanthin, mandarins, organic farming, Orogrande, juices, sensory evaluation

INTRODUCTION

During the last years, organic farming has experienced a strong growth due to several factors, including growing consumers' interest on: (i) food safety, (ii) healthy diets and (iii) respect for the environment (Burch et al., 2001).

Organic farming could be defined as the agricultural techniques, which exclude the use of synthetic chemicals, such as fertilizers, pesticides, antibiotics, etc., with the objective of preserving the environment, keeping or improving the fertility of soils and providing foods with all their natural properties (Canavari et al., 2002).

In the European Union (EU), the land area dedicated to organic foods is 3.5 million ha, which represents only 3% of the total EU useful agricultural area. Italy is the country within the EU with more land dedicated to

organic farming, followed by Germany, UK, Spain and France. Organic farming has become one of the most dynamic agricultural sectors, with organic agricultural products yielding an income of approximately 13–14 billion euros in 2005. In Spain, organic farming started about 25 years ago and nowadays is a quite promising sector, with about 350 000 ha being cultivated in 1999 (Foster and Lampkin, 2005).

Oranges and mandarin oranges are two of the agricultural products with the highest demand among fruits consumers. Spain is the second world producer of mandarin oranges and the main supplier to the international market (MAPA, 2005).

Because prevention of chronic illnesses is always a better strategy than their treatment, reducing the risk of heart diseases and cancer, through a healthier diet, has become a topic of the greatest interest for the health professionals, researchers, food technologists, food chemists and nutritionists. For this reason nowadays, the production of organic fruits and vegetables is being supported by most of the European governments and official institutions and organisms (Liu, 2003).

In general, citric products have high contents of natural antioxidant compounds, which are often related to reduction of neurodegenerative illness, certain types of cancer, heart diseases, etc. Therefore, consumption of

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citric products seems to help in maintaining and reinforcing the health of consumers (Thomasset et al., 2007) because of their high antioxidant activity (Vinson et al., 2001; Meléndez-Martínez et al., 2007a, c).

The synthesis and composition of these compounds depend on the species type and even on the fruit cultivar or variety. The farming type, the application of chemical products, such as fertilizers, pesticides and the type and abundance of irrigation will also affect both the biochemical composition (both qualitatively and quantitatively) and the activity of fruits (Meléndez-Martínez et al., 2007b, c).

Carotenoids provide fruits with their characteristic colors and have important biological functions. Although approximately more than 600 carotenoids have been reported, only 60 carotenoids have pro-vitamin A activity. Citrus fruits can have more than 115 different carotenoids. The main carotenoids found in mandarin oranges are: β -cryptoxanthin, violaxanthin, neoxanthin, lutein, antheraxanthin, α -carotene and β -carotene. From these compounds, β -carotene, α -carotene and β -cryptoxanthin are the ones with the highest pro-vitamin A activities (Meléndez-Martínez et al., 2007a). Carotenoids (C_{40} tetraterpenoids) are synthesized by plants from eight C_5 isoprenoid units, producing phytoene as the first C_{40} carotenoid (Rodríguez-Amaya, 2001), as a defensive mechanism against external aggressions. This is the main reason why organic oranges and mandarin oranges, which have not been treated with synthetic pesticides and/or chemicals to protect them against illnesses, usually have higher carotenoid contents than conventionally grown fruits (Heaton, 2001).

The main objective of this study was to assess the effect of different agricultural practices on total antioxidant activity, carotenoid composition, vitamin C, minerals and instrumental color of mandarin oranges (cultivar Orogrande).

MATERIALS AND METHODS

Fruit Material

Both conventional and organic mandarin oranges (*Citrus reticulata* L.), var. Orogrande, were grown in the same farm and under identical conditions of soil, irrigation and illumination in eastern Spain (Librilla, Murcia). The citrus rootstock was the same for both mandarin trees, Carrizo citrange (*Citrus sinensis* L.), and all selected trees were about 10-year-old and free of diseases. Fruits were collected in autumn (second week of October, 2007) when proper values of diameter, pH, total soluble solids content (SSC, °Brix) and maturity index (total soluble content/titratable acidity, SSC/TA) were reached (Table 1).

Table 1. Main properties of Orogrande mandarin oranges (at collection time) and fresh juices as affected by agricultural practice (conventional or organic).

Property	Farming type	
	Conventional	Organic
Weight (g)	94.3±6.9 a	88.5±4.7 a
Diameter (mm)	57.6±2.6 a	52.4±4.1 a
Soluble solid content (°Brix)	12.3±0.5 a	12.6±0.4 a
Titratable acidity (% citric acid)	0.97±0.05 a	0.94±0.04 a
Maturity Index (SSC/TA)	12.7±0.4 a	13.4±0.3 a
Vitamin C (mg/L)	366±18 b	419±25 a
Antioxidant Activity (mM Trolox/mL)	0.053±0.003 b	0.076±0.004 a
L^*	59.42±0.96 a	52.83±0.69 b
a^*	19.94±0.51 b	28.03±0.88 a
b^*	51.36±0.39 b	66.51±0.48 a
C_{ab}^*	55.09±0.42 b	72.17±0.71 a
h_{ab}	68.78±0.64 a	67.14±0.92 a

Mandarin orange fruits with the same letter, within the same row, were not significantly different at $p < 0.005$ for the evaluated property (Tukey's multiple range test).

Maturity index is the ratio between the solid soluble content and titratable acidity.

Orogrande Mandarin oranges were studied on conventional and organic farming. Organic production means that no synthetic chemicals were used in the production of these fruit trees and that only natural substances were used to control pest, weeds, etc. Farming of organic mandarin trees followed all rules established by the Board of Organic Agriculture of the Murcia Region (BOAM, 2009). A complete list of the materials used in both conventional and organic farming is included in Table 2.

Methods

Sample Preparation

Mandarins were collected and processed the same day. The exact weights of fruits processed were 8550 and 9240 kg of conventional and organic Orogrande mandarin oranges, respectively. Mandarin juices were processed in a commercial plant (Murcia, Spain) and were obtained using a Premium Juice Extractor (FMC Corporation, Florida, USA; FMC, 2009). This machinery leads to a juice with a low content of essential oils (Kimball, 2002) because after a proper calibration of fruits the contact between the juice and the skin of mandarins is minimized.

Freshly squeezed juices were treated in an Alfa Laval plate heat exchanger (Alfa Laval Iberia S.A., Madrid, Spain), for 20 s at a temperature of 98 °C. After this heat treatment, the juice was first transferred to a pre-cooler, which cooled the juice down to 30 °C using forced air and then to a cooler, which finally took the temperature

Table 2. Materials used in both conventional and organic farming (Source: Beltrán et al. 2008b).

Compound	Agricultural practice	
	Conventional	Organic
Soil fertilizers	Ammonium nitrate, calcium nitrate, ammonium sulphate, phosphoric acid, diammonium phosphate, potassium sulphate, potassium nitrate, potassium chloride	Manure, compost, and fulvic and humic acids
Foliar fertilizers	potassium phosphate, magnesium nitrate, urea, mixture of oligoelements	algae extracts, aminoacids
Herbicides	Bromacil, diuron, diquat, fluroxypyr, glyphosate, norflurazon, paraquat, sulfosate, simazine.	None; weeds are removed by mechanical methods
Pesticides	Malathion, dicofol, methidathion, clopidol	Neem oil, pheromone traps

down to 2°C. Heat-treated juices were stored in aseptic metallic deposits at a temperature of 4°C.

Physico-Chemical Analyses

The soluble solids content, SSC (°Brix), was determined using a portable refractometre Comecta, S.A., model C3 (Barcelona, Spain). Titratable acidity, TA (% citric acid), was determined in 10 mL of juice by titration to pH 8.2 ± 0.1 with a 0.1 N NaOH solution. The maturity index, MI, was calculated for each mix and expressed as the percentage of the ratio between the SSC and TA.

Vitamin C (reduced ascorbic acid) was measured following the AOAC Official Method 985.33 (Horwitz, 2000). Ascorbic acid was estimated by titration with colored oxidation-reduction indicator, 2,6-dichloroindophenol. EDTA was added as chelating agent to remove Fe and Cu interferences.

All physico-chemical analyses were analyzed in 20 fruits of each agricultural practice.

Instrumental Measurement of Color

Color determinations were made, at 25 ± 1°C, using a Hunterlab Colorflex® (Hunterlab, Reston, Virginia, U.S.A.). This spectrophotometer uses an illuminant D65 and a 10° observer as references. A sample cup for reflectance measurements was used (5.9 cm internal diameter × 3.8 cm height) with a path length of light of 10 mm. Blank measurements were made with the cup filled with distilled water against a reference white background (Pérez-López et al., 2006).

Color data are provided as CIE $L^*a^*b^*$ coordinates, which define the color in a three-dimensional space (Minolta, 1994). Color analyses were run in 6 replicates.

Antioxidant Activity

The oxygen radical absorbance capacity (ORAC) analyses were conducted using a Synergy HT multidetector microplate reader (Bio-Tek Instruments Inc.,

Winooski, VT), using 96-well polystyrene microplates with black sides and clear bottom. Fluorescence was read through the clear bottom, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The ORAC was determined as described by Dávalos (2004) with slight modifications (Lucas-Abellán et al., 2008). All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample. To avoid temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 200 µL of distilled water.

The results were expressed as relative fluorescence with respect to the initial reading. The area under the fluorescence decay curve (AUC) was calculated by the equation:

$$AUC = 1 + \sum_{i=1}^{120} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i . The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank to the sample AUC. The results of antioxidant capacity were expressed as mM of Trolox C per µL of juice.

Minerals Contents

A multi-place digestion block, Selecta Block Digest 20 (Barcelona, Spain), was used for sample mineralization. 15 mL of juice were treated with 5 mL of concentrated HNO₃ (65%, w/v) in Pyrex tubes, placed in the digestion block, and heated at 60°C for 60 min and at 130°C for 120 min (Carbonell-Barrachina et al., 2002). Solutions were left to cool to room temperature, transferred to a volumetric flask and diluted to a final volume of 25 mL with ultra-high-purity deionized water.

Determination of Ca, Mg, K, Cu, Fe, Mn and Zn in previously-mineralized samples was performed with a Unicam Solar 969 atomic absorption spectrometer

(Unicam Limited, Cambridge, U.K.). Minerals were analyzed in five replicates.

Instruments were calibrated using certified standards. In each analytical batch, at least one reagents blanks, one international reference material (CRM) and one spike were included to assess precision and accuracy for chemical analysis. The certified material selected for the current experiment was GBW07603 (bush, branches and leaves); this material was provided by LGC Deselaers S.L. (Barcelona, Spain) and produced by the Institute of Geophysical and Geochemical Exploration of China (GBW07603).

Carotenoid Compounds Extraction and Quantification

The quantification of total carotenoid content and carotenoids profiling were carried out following the methods previously described by Darnoko et al. (2000) and Andreu-Sevilla et al. (2008). Mandarin orange juices were extracted with acetone; samples were later saponified with 20% KOH-methanol. Finally, pigments were extracted with diethyl ether, evaporated in a rotary evaporator and taken up in a maximum of 10 mL of acetone.

The high-performance liquid chromatography system consisted of an HP-1100 series unit with a photodiode array detector equipped with HP ChemStation software (Hewlett Packard, Palo Alto, CA). The column used was a 250 mm \times 4.6 mm i.d., YMC C30, S-5 μ m (YMC, Schermbek, Germany). The mobile phase for this column was 81:15:4 methanol:methyl tertiary butyl ether (MTBE): H₂O (solvent A) and 91:9 MTBE: methanol (solvent B). The gradient elution was 100% A to 50% A and 50% B in 45 min followed by 100% B in the next 10 min and 100% A in the next 5 min at a flow rate of 0.8 mL/min (Darnoko et al., 2000; Andreu-Sevilla et al., 2008). Carotenoids were monitored at 450 nm and analyses were carried out in triplicate.

Identification was based on the order of elution, retention time and spectra of absorbance maxima of a particular peak. Standards of violaxanthin, antheraxanthin, β -cryptoxanthin and α - and β -carotene for quantification purposes were obtained from CaroteNature (Lupsingen, Switzerland); besides, standards of lutein and zeaxanthin were obtained from Extrasynthase (Genay, France).

The absolute concentrations of the main mandarin juices carotenoids (violaxanthin, antheraxanthin, lutein, zeaxanthin, β -cryptoxanthin, α -carotene and β -carotene) were worked out by external calibration in the range of 0–15 mg/L for β -cryptoxanthin and 0–6 mg/L for the rest of carotenoids. On the other hand, the levels of luteoxanthin-like, 9-*cis*-violaxanthin, luteoxanthin b, mutatoxanthin-like, *cis*-antheraxanthin and zeionoxanthin were calculated out using β -apo-8'-carotenol as internal standard (Hans-Dieter et al., 1999), due to lack of proper commercial standards.

Total carotenoid content was assessed as the sum of the content of the 13 individual carotenoids studied in this experiment.

Sensory Evaluation

Sensory analysis was performed by a trained panel to evaluate the quality of mandarin orange juices. A panel of 10 panellists, ages from 20 to 50 years (8 female and 2 male, all members of the Catholic University San Antonio of Murcia), with sensory evaluation experience, was trained in descriptive evaluation of citrus juice (Serrano-Megías et al., 2005).

The panel was selected and trained following the ISO standard 8586-1 (AENOR, 1997; Meilgaard, 1999). Further details on selection, training and validation of the panel can be found in Pérez-López et al. (2006).

Measurements were performed in individual booths with controlled illumination (750–1000 lux) and temperature ($23 \pm 2^\circ\text{C}$) (AENOR, 1997; Meilgaard, 1999). The individual products were scored for the intensities of color, sweetness, acidity, fresh mandarin juice aroma, floral aroma and off-flavor using a scale of 0–10, where: 0 = extremely slight intensity, and 10 = extremely high intensity.

Samples were presented in 50 mL plastic cups with lids. The entire experiment was repeated three times (all judges scored two juice samples on each session for a total of three sessions) and the sensory scores were presented as the overall means.

Statistical Analysis

All data were subjected to analysis of variance (ANOVA) and the Tukey's least significant difference multi-comparison test to determine significant differences among mandarin orange juices. Significance of differences was represented as $p < 0.001$. The statistical analyses were done using SPSS 14.0 (SPSS Science, Chicago, USA).

RESULTS AND DISCUSSION

Mandarins from the cultivar Orogrande were selected for this study because they present adequate technological (quite homogeneous in size and form, making peeling and squeezing easy) and sensory (intense sweetness) properties for juice making (García-Lidón et al., 1996).

No significant differences ($p < 0.05$) in quality parameters of the conventional and organic mandarins were found at picking time (Table 1). The main properties of the Orogrande mandarins selected for this experiment were: 91.4 g, 55.0 mm of diameter and a maturity index of 13.1 (mean values of conventional and organic mandarins). However, organic mandarins contained a significantly ($p < 0.001$) higher content of vitamin C than

conventional fruits, 419 ± 25 and 366 ± 18 mg/L, respectively (Table 1).

Antioxidant Activity

Organic farming resulted in significantly higher antioxidant activity compared to conventional farming, with values being 0.076 ± 0.004 mM Trolox/mL of juice and 0.053 ± 0.003 mM Trolox/mL, respectively (Table 1). One possible explanation for this experimental finding is that organic trees are not treated with synthetic pesticides and therefore these trees must induce the synthesis of natural protecting compounds, such as carotenoids, leading to a final increase in their natural antioxidant activity.

These results agreed with those previously reported for other agricultural products such as red grapes (Negro et al., 2003; Rivero-Pérez et al., 2008), tomatoes (Toor and Savage, 2006) and oranges (Arena et al., 2001; Riso et al., 2005).

Instrumental Measurement of Color

Beltran et al. (2008a) studied the effect of the mandarin cultivar on the color coordinates of mandarin juices and concluded that juice from Clemenules mandarins presented the higher values of coordinate a^* , and coordinate b^* and Chroma, C_{ab}^* , of eleven mandarin cultivars under study. In that study, the values of L^* , a^* , b^* and C_{ab}^* for the conventional Orogrande juice and the mean of the 11 mandarin orange cultivars studied were as follows: a) 50.75, 7.79, 28.42 and 29.47; and b) 51.85, 8.21, 28.91 and 29.52, respectively.

Juices from organic Orogrande mandarins presented better color characteristics than juices from conventional fruits (Table 1); juices are better considered by consumers when being less clear but having more intense color coordinates (a^* , b^* and C_{ab}^*). A change from traditional agricultural practices to more environmentally friendly organic farming practices resulted, in this particular study, in a significant ($p < 0.001$) increase in the intensity of the orange color of mandarin juices. This improvement in the color of Orogrande mandarin juices was related to significant ($p < 0.001$) increases in

some color coordinates a^* (8.09 units), b^* (15.15 units) and C_{ab}^* (17.08 units) and a decrease in the coordinate L^* (6.59 units).

Similar positive effects of organic farming on the color of Hernandina mandarin juices were reported previously by Beltran et al. (2008b), although the increases in the color coordinates caused by organic farming were around three units. On the other hand, no significant effects were found for Clemenules mandarins (Pérez-López et al., 2007). Considering the color data included in Table 1 and literature information, Orogrande cultivar can be considered as very sensitive cultivar to organic farming.

Mineral Contents

Certified values for Ca (%), Mg (%), K (%), Cu (mg/kg), Fe (mg/kg), Mn (mg/kg) and Zn (mg/kg) were: 1.81 ± 0.07 , 0.65 ± 0.03 , 1.38 ± 0.04 , 274 ± 10 , 9.3 ± 0.5 , 45 ± 2 and 37 ± 1 , respectively, while measured values for the same elements were: 1.79 ± 0.04 , 0.65 ± 0.02 , 1.42 ± 0.02 , 270 ± 12 , 9.4 ± 0.3 , 48 ± 3 and 35 ± 2 , respectively. These results show the goodness of the digestion and spectroscopy analyses of minerals.

Organic farming had a significant effect on the content of some of the analyzed elements, macro-nutrients (Ca and K) and micro-nutrients (Fe). In general, nutrients contents were higher in the juice from the organic mandarins (Table 3); however, differences were not always statistically significant. Mandarin orange juice is a very good source of potassium (mean of 3.2 g/L).

The concentrations of almost all nutrients analyzed in this study fall within the ranges reported in the literature for mandarin juices from different geographical areas, such as Florida, California, México and Brazil, and within the ranges suggested by the guidelines of AIJN (Ting and Rouseff, 1986; AIJN, 2009). Besides and as expected because we worked with natural freshly squeezed juices, the concentrations of Cu, Zn and Fe were below the maximum levels recommended by the Codex Stan 45-1981 of 5, 5 and 15 mg/L.

Table 3. Minerals contents of Orogrande mandarin orange juices as affected by type of farming (conventional or organic).

Agricultural practice	Minerals (mg/L)						
	Ca	Mg	K	Fe	Cu	Mn	Zn
Conventional	74.8 ± 3.8 b	109 ± 16 a	2986 ± 79 b	0.69 ± 0.07 b	0.19 ± 0.06 a	0.18 ± 0.04 a	0.31 ± 0.05 a
Organic	98.7 ± 5.1 a	138 ± 33 a	3325 ± 94 a	0.86 ± 0.09 a	0.17 ± 0.04 a	0.21 ± 0.02 a	0.37 ± 0.02 a

Mandarin orange fruits with the same letter, within the same column, were not significantly different at $p < 0.005$ for the mineral evaluated (Tukey's multiple range test).

Table 4. Carotenoids concentrations of Orogrande mandarin orange juices as affected by type of farming (conventional or organic).

Carotenoid	Farming type	
	Conventional	Organic
Vio/axanthin	0.23±0.06 b	0.58±0.13 a
Luteoxanthin-like	0.15±0.03 b	0.41±0.06 a
9-cis-Vio/axanthin	0.78±0.15 b	1.59±0.32 a
Antheraxanthin	1.35±0.29 b	2.87±0.24 a
Luteoxanthin b	0.15±0.03 b	0.28±0.05 a
Lutein	0.94±0.20 a	1.21±0.34 a
Mutatoxanthin-like	0.75±0.11 b	1.09±0.18 a
cis-Antheraxanthin	0.88±0.15 b	1.97±0.31 a
Zeaxanthin	0.41±0.11 a	0.62±0.23 a
Zeinoxanthin	2.08±0.14 b	2.92±0.39 a
β-Cryptoxanthin	7.55±0.11 b	8.34±0.09 a
α-Carotene	0.35±0.12 a	0.61±0.20 a
β-Carotene	0.09±0.03 a	0.18±0.06 a
TOTAL	15.7±0.4 b	22.7±0.3 a

Mandarin orange fruits with the same letter, within the same row, were not significantly different at $p < 0.005$ for the specific carotenoid (Tukey's multiple range test).

Total Carotenoid Content and Carotenoids Composition

Organic farming caused a positive effect on the total content of carotenoids in Orogrande juices, increasing the values of this quality parameter from 15.7 ± 0.4 in conventional juices to 22.7 ± 0.3 mg/L in organic juices (Table 4). A change from traditional agricultural practices to organic farming practices resulted in an increase of about 45% of total carotenoids content, which is a very significant improvement in the nutritional value of this juice. Lee and Castle (2001) concluded that total juice carotenoids increased as the fruit ripening progressed and ranged from about 1 mg/L in unripe oranges up to 9 mg/L in ripe fruits. Values found in the present experiment agreed with ripe mandarins (mean maturity index of 13.1), which have an intense orange color as previously described in the instrumental color section.

Organic farming significantly increased concentrations of 9 out of 13 identified carotenoids in Orogrande juice (Table 4); however, no specific effects of organic farming on the different carotenoid biosynthesis pathways can be proposed from results obtained in this experiment and more research is needed to reach such conclusions. The predominant carotenoids found in Orogrande juices were β-cryptoxanthin, zeinoxanthin, antheraxanthin, and cis-antheraxanthin with mean concentrations of 7.95, 2.50, 2.11 and 1.43 mg/L, respectively (Table 4). Meléndez-Martínez et al. (2007a) stated that the carotenoid profile of most foods depends on the fruit variety, climatic factors, industrial processing and storage conditions, among others. Even so,

β-cryptoxanthin has always been reported as the major carotenoid found in mandarin juices.

Although approximately 700 carotenoids have been reported only those with an unsubstituted β-ring with an 11-carbon polyene chain, have pro-vitamin A activity (Meléndez-Martínez et al., 2007a, c). This structural requirement is satisfied by around 60 carotenoids (Rodríguez-Amaya, 2001). Vitamin A (retinol) can also be provided in the diet as other preformed forms as pro-vitamin A carotenoids, which are subsequently transformed into vitamin A (Meléndez-Martínez et al., 2007a).

The retinol activity equivalents (RAE) of the samples analyzed and discussed here referred to 1 L of mandarin orange juice. The bioavailability of carotenoids is influenced by many factors, such as amount, food matrix, age, existence of certain diseases or parasite infestation, intake of fat, vitamin E and fiber, protein and zinc status (Thomasset et al., 2007). According to this, it is difficult to accurately determine the RAE of any food. In this study, calculations were performed, considering new guidelines according to the following formula (FNBIM, 2002):

$$RAE = \left(\frac{\mu\text{g } \beta - \text{carotene}}{12} \right) + \left(\frac{\mu\text{g } \alpha - \text{carotene} + \mu\text{g } \beta - \text{cryptoxanthin}}{24} \right)$$

The RAE values found in this study for Orogrande traditional and organic mandarin orange juices were 337 and 388 RAE/L, respectively; these high values of RAE/L support the general fact that mandarin products are a very good source of vitamin A and this statement is especially true for organic mandarin juices. These experimental values fit perfectly within the range previously described by Meléndez-Martínez et al. (2007a) for the RAE/L contents of different types of orange and mandarin juices marketed in Spain ranging from 9.7 to 359. This RAE/L range was significantly reduced when only orange juices were considered, 9.7 to 94.8. Without any doubt the high levels of RAE in mandarin juices compared to other citrus juices are related to their higher concentrations of β-cryptoxanthin.

Sensory Quality

The trained panel established that the quality of both juices was high but that organic juice had slightly higher intensities of color, fresh mandarin juice aroma and floral aroma than conventional juice. In fact, the exact values for color, sweetness, acidity, fresh mandarin aroma and floral aroma for the organic and conventional juices were as follows: 8.2 ± 0.1 , 6.5 ± 0.1 , 5.7 ± 0.1 , 8.4 ± 0.1 , 5.4 ± 0.1 and 7.7 ± 0.1 , 6.4 ± 0.1 ,

5.7 ± 0.1, 7.4 ± 0.1, 5.0 ± 0.1, respectively. No off-flavors were detected for any of the two juices under study.

CONCLUSIONS

If the most popular mandarin orange cultivar, Clemenules, is not available for juice making, Orogrande is a good option and it has been demonstrated that this cultivar is very sensitive to organic farming (high increases in values of color coordinates, total carotenoids content, etc.). In general, mandarin juices are very good sources of potassium and β-cryptoxanthin. Organic Orogrande mandarin juices were characterized by high antioxidant activity (0.076 mM Trolox m/L), intense orange color (C_{ab}^* 72.2), high content of total carotenoids (22.7 mg/L), high contents of nutrients such as calcium, potassium, iron and vitamin C content (419 mg/L). Besides, the sensory quality of the organic juice was better than that of the conventional juice based on intensities of color, fresh mandarin and floral aromas. Therefore, organic farming had positive effects on the quality of mandarin juices, cultivar Orogrande.

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Original article

Effects of β -cyclodextrin addition and farming type on vitamin C, antioxidant activity, carotenoids profile, and sensory analysis in pasteurised orange juices

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Summary The effects of organic farming, pasteurisation and addition of β -cyclodextrin on the content of vitamin C, colour, carotenoids and antioxidant capacity of orange juices were studied. After pasteurisation at 98 °C (20 s) and subsequently storage along 145 days at room temperature (20–25 °C), the loss of vitamin C content was around 30%. The effects of the thermal process on carotenoid were clearly observed in lutein (loss of 16% for organic and traditional 8%) and especially β -cryptoxanthin (loss of 30%). The colour changes were noticeable after the pasteurisation of orange juice and subsequent storage, with significant decreases being observed in lightness and the coordinate a^* , while increases were found for coordinates b^* , Hue^* and chroma. The antioxidant capacity was 0.075 ± 0.01 and 0.053 ± 0.01 mM mL^{-1} for organic and conventional, respectively, with losses around 40% being found at the end of the storage period. The addition of β -cyclodextrin caused no significant effects on the parameters under analysis. These data showed that strong thermal treatments, such as pasteurisation, adversely affect the nutritional and sensory quality of orange juices.

Keywords Antioxidant, β -cryptoxanthin, carotenoids, citrus, colour, orange juice.

Introduction

There is actually a change in consumers' habits towards healthier diets, such as the Mediterranean one, with increased consumption of horticultural products. While some fruits and vegetables are eaten as fresh products, many others are processed or preserved for economic, logistical reasons, to improve their digestibility, for culinary needs or to facilitate their use to certain groups of consumers (Cullum, 2003).

These new habits are reflected in a significant increased consumption of fruits. Fruit juices have experienced a high growth in recent years. Among other reasons, the increased consumption and export of juices can be attributed to significant progress in the cultivation and improvement in their processing, transport and distribution (Schieber *et al.*, 2001).

Orange juice is one of the most requested types of juices by consumers all over the world. According to Food and Agriculture Organization (FAO), Spain is the main Mediterranean producer of oranges and mandarins with a total production of 2800 t of oranges and 2100 t of mandarins in 2008 [Ministerio de medio ambiente y medio rural y marino (MARM). Retrieved 24 May, 2010 from: <http://www.marm.es/>].

Currently, consumers are increasingly worried about the quality of their food and require rigorous food safety. For this reason, organic products are increasingly under demand. Organic farming preserves the nutritional quality of foods by keeping high in micronutrients and bioactive substances through techniques that avoid the use of toxic compounds by enhancing crop rotation, use of natural fertilisers, etc. (Rembiakowska, 2007).

Citrus fruits have been recognised for their beneficial health effects according to different epidemiological and clinical intervention studies (Buiatti & Muñoz, 1996; Zullo *et al.*, 2000). These beneficial properties are because

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of bioactive substances present in these foods; among the bioactive compounds present in citrus fruits are carotenoids and vitamin C (Willer & Minou, 2005). Carotenoids prevent degenerative diseases because of their nutritional value because of vitamin A activity (Byers & Perry, 1992). These biological effects have been attributed to antioxidant properties of carotenoids through the deactivation of free radicals and the capture of singlet oxygen (Palozza & Krinsky, 1992). Citrus can have more than 115 different carotenoid compounds, including epoxy-carotenoids (violaxanthin and antheraxanthin), carotenols (beta-cryptoxanthin, lutein, zeaxanthin) and carotenes (α - and β -carotene) (Meléndez-Martínez *et al.*, 2007a).

Commercial juices, both traditional and organic, are preserved by pasteurisation, which consists in heating juices at approximately 95 °C for 20 s. During heat treatment, in addition to the inactivation of microorganisms, desirable constituents as nutrients, colour, aroma and texture are destroyed in different percentages (Lee & Coates, 2003), resulting in significant loss of product quality. To reduce food quality loss during food processing, natural molecules called cyclodextrins (CDs) are being used in the food industry (Szejtli, 1998). The applications of CDs in the food industry are many and varied, including prevention of unwanted smells, reduction in colour degradation and the development of hydrophilic functional foods, such as juices and nectars with hydrophobic nutrients (vitamins, minerals, carotenoids, flavonoids, etc.) (Szente & Szejtli, 2004). Cyclodextrins are considered since 1998 as GRAS (food ingredients that expert recognised as safe based on its long history of use in food) to a maximum of 2% in many food products (Buschmann & Schollmayer, 2002).

The main aim of this study was to study the effect of the pasteurisation step and the addition of β -cyclodextrin on the quality of orange juices from two different types of farming, conventional and organic, stored for 145 days at room temperature. Different quality parameters were studied during this period and included vitamin C content, colour, antioxidant capacity, carotenoids composition and sensory quality.

Materials and methods

Fruit material

Both traditional and organic oranges (*Citrus sinensis*, var. Navelina), were grown in the same farm and under identical conditions of soil, irrigation and illumination in southern of Spain (Huelva). The citrus rootstock was the same for both orange trees, Cleopatra mandarin trees, and all selected trees were about 12 years old and free of diseases. They were collected in winter (first week of December, 2009). Fruits were selected on the basis of their diameter, pH, total soluble solids content (SSC,

°Brix) and maturity index (total soluble content/titratable acidity, SSC/TA).

Navelina oranges were grown under both traditional and organic farming. Organic farming followed all rules established by the Board of Organic Agriculture of the Murcia Region (BOAM, 2011).

Sample preparation

Navelina oranges were collected and processed the same day, 6 December 2009. Orange juices were processed in a commercial plant (Cooperativa Agraria Naranjera de Los Alcores, S. C. A) (Sevilla, España) and were obtained using a Premium Juice Extractor (FMC Corporation, Houston, TX, USA) (FMC, Retrieved June, 2010 from: <http://www.fmctechnologies.com>). This machinery leads to a juice with a low content of essential oils.

Freshly squeezed juices, traditional and organic, were processed in a citrus processing plant. Once squeezed and before juices were treated in a Alfa Laval plate heat exchanger (Alfa Laval Iberia S.A., Madrid, Spain), for 20 s at a temperature of 98 °C, β -CD from TCI Europe NV (Tokyo Chemical Industry Co Ltd., Zwijndrecht, Belgium) were added to the traditional oranges juices samples (1.5% of 13 mM β -CD). In this way, three juices were under study: (i) traditional, (ii) traditional + β -CD and (iii) organic.

Samples were stored at room temperature (20 ± 2 °C) and under for 145 days. Samplings were conducted at 7, 30, 60, 100 and 145 days. At each sampling, the following parameters were analysed: total soluble solids, acidity, pH, vitamin C, colour, carotenoids, antioxidant capacity and sensory quality. All analyses were run in triplicate.

Physico-chemical analyses

The SSC (°Brix), was determined using a portable refractometer Comecta, S.A., model C3 (Barcelona, Spain). Titratable acidity (% citric acid), was determined in 10 mL of juice by titration to pH 8.2 ± 0.1 with a 0.1 N NaOH solution. The maturity index, MI, was calculated for each mix and expressed as the percentage of the ratio between the SSC and TA.

Vitamin C

Reduced ascorbic acid was measured following the AOAC Official Method 985.33 (Horwitz, 2000). Ascorbic acid was estimated by titration with coloured oxidation-reduction indicator, 2,6-dichloroindophenol. EDTA was added as chelating agent to remove Fe and Cu interferences. All physico-chemical analyses were analysed in twenty fruits of each agricultural practice.

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Instrumental measurement of colour

The colour of orange juice ranges from pale yellow at the beginning of the production season to red-orange at the end of this season. The colour depends on many factors, including the cultivar and climatic conditions, among many others (Heredia *et al.*, 2003). Colour determinations were made, at 25 ± 1 °C, using a Hunterlab Colorflex[®] (Hunterlab, Reston, VA, USA). This spectrophotometer uses an illuminant D₆₅ and a 10° observer as references. A sample cup for reflectance measurements was used (5.9 cm internal diameter \times 3.8 cm height) with a path length of light of 10 mm. Blank measurements were made with the cup filled with distilled water against a reference white background (Pérez-López *et al.*, 2006).

Colour data are provided as CIE L*a*b* coordinates, which define the colour in a three-dimensional space (Minolta, 1994). Finally, the colour differences (ΔE^*) between two points in the CIE L*a*b* space are worked out as the Euclidean distance between their localisations in the three-dimensional space defined by L*, a* and b*. Mathematically, it is, therefore, calculated by applying the formula $\Delta E^* = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$. Colour analyses were run in six replicates.

Antioxidant activity

All the reagents used for the determination of the antioxidant capacity, fluorescein (FL), 2,2 azobis [2 aminopropane] dihydrochloride (AAPH) and 6 hydroxyl 2,5,7,8-tetramethylchroman 2-carboxylic acid (Trolox C) were purchased from Sigma (Madrid, Spain).

The Oxygen Radical Antioxidant Capacity Assay (ORAC assay) was carried out on a Synergy HT multidetector microplate reader, from Bio-Tek Instruments Inc. (Winooski, VT, USA), using 96-well polystyrene microplates with black sides and clear bottom, purchased from Nalge Nunc International. Fluorescence was read through the clear bottom, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The oxygen radical absorbance capacity was determined as described by Dávalos *et al.* (2004) with slight modifications (Lucas-Abellán *et al.*, 2008). A blank with FL and AAPH using sodium phosphate buffer instead of the antioxidant sample and eight calibration solutions using Trolox C (6.25, 12.5, 15, 18.75, 21.25, 25, 27.5 and 31.25 μ M) as antioxidant was also used in each assay. All reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. To avoid a temperature effect, only the inner sixty wells were used for experimental purposes, while the outer wells were filled with 200 μ L of distilled water.

The results were expressed as relative fluorescence with respect to the initial reading. The area under the fluorescence decay curve (AUC) was calculated by the equation:

$$AUC = 1 + \sum_{i=1,14}^{i=120} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min, and f_i is the fluorescence reading at time i . The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. Results of antioxidant capacity were defined as millimolar of Trolox C per millilitre of juice.

Carotenoid compounds extraction and quantification

The analysis of the carotenoid composition requires an extraction with organic solvent followed by de-esterification of the carotenoid fraction. Juices (350 μ L) were extracted with 600 μ L of solvent (methanol/acetone/dichloromethane, 25:25:50 v:v:v), and centrifuged retaining the coloured phase. The final residue was finally extracted with 300 μ L of dichloromethane, centrifuged and again keeping the coloured phase, which was mixed with the previous one. This mixture was washed several times with deionised water to remove any trace of acetone. This phase containing the pigments, in various stages of esterification with fatty acids, was saponified with 15% KOH-methanol (MeOH) for 1 h at room temperature and washed with water to remove any trace of the base. The pigments were subsequently evaporated in a rotary evaporator at 30 °C, keeping the samples under nitrogen atmosphere at -18 °C until analysed.

The high-performance liquid chromatography system consisted of an HP-1100 series unit with a photodiode array detector equipped with HP ChemStation software (Hewlett Packard, Palo Alto, CA, USA). The column used was a 250 \times 4.6 mm i.d., YMC C30, S-5 μ m (YMC, Wilmington, NC, USA), and column temperature was kept at 17 °C. The mobile phase for this column was MeOH, methyl tertiary butyl ether (MTBE) and water; MeOH and MTBE contained butylated hydroxytoluene (BHT) (0.1%) and TEA (0.05%) to protect carotenoids during analysis (Mouly *et al.*, 1999). The gradient elution was: 0 min, 90% MeOH + 5% MTBE + 5% water; 12 min, 95% MeOH + 5% MTBE; 25 min, 89% MeOH + 11% MTBE; 40 min, 75% MeOH + 25% MTBE; 50 min, 40% MeOH + 60% MTBE; 56 min, 15% MeOH + 85% MTBE; 62 min, 90% MeOH + 5% MTBE + 5% water, at a flow rate of 1.0 mL min^{-1} (Hart & Scott, 1995). Carotenoids were monitored at 450 nm, and analyses were carried out in triplicate.

Identification of carotenoids was conducted by comparison with standards isolated from natural sources. Lutein, β -cryptoxanthin and β -carotene were obtained from spinach leaves (*Spinacia oleracea* L.) (Britton *et al.*, 1995).

Quantification of lutein, β -cryptoxanthin and β -carotene was carried out using calibration curves

(Rodríguez-Amaya, 2001). Zeaxanthin concentration was quantified using the β -carotene calibration curve because they have the same spectral properties (Meléndez-Martínez *et al.*, 2007b).

Sensory evaluation with trained panel

Sensory evaluation by a trained panel was used to evaluate the quality of oranges juices. A panel of ten panelists, ages 20–50 years (eight women and two men, all members of the Catholic University San Antonio of Murcia), with sensory evaluation experience, was trained in descriptive evaluation of citrus juice (Pérez-López *et al.*, 2006).

The panel was selected and trained following the ISO standard 8586-1 [Asociación Española de Normalización y Certificación (AENOR), 1997; Meilgaard *et al.*, 1999]. Further details on selection, training and validation of the panel can be found in Pérez-López *et al.* (2006).

Measurements were performed in individual booths with controlled illumination (750–1000 lux) and temperature (23 ± 2 °C) (Asociación Española de Normalización y Certificación (AENOR), 1997).

The individual products were scored for the intensities of colour, sweetness, acidity, fresh orange juice aroma and off-flavour using a scale of 0–10, where: 0 = extremely slight intensity and 10 = extremely high intensity.

Samples were presented in 50 mL plastic cups with lids. The entire experiment was repeated three times (all judges scored two juice samples on each session for a total of three sessions), and the sensory scores were presented as the overall means.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and the Tukey's least significant difference multi-comparison test to determine significant differences among orange juices. Significance of differences was represented as $P \leq 0.001$. The statistical analyses were performed using SPSS 14.0 (SPSS Science, Chicago, IL, USA) and figures using Sigma Plot 9.0 (SPSS Science).

Results and discussion

The Navelina variety of oranges was selected because of its early (November to January) and high productivity. Navelina oranges are highly appreciated because of their high quality, intense flavour, juiciness and suitability for the juice industry (Maccarone *et al.*, 1998).

Physico-chemical analyses

Organic and conventional orange juices, with or without CD, maintained average pH values of 3.1 ± 0.2 and maturity index of 13.4 ± 0.1 .

Vitamin C

In general, the ascorbic acid content in oranges depends upon a wide variety of factors, such as the chemical characteristics of the soil, the climate of the area of production, the amount of light received, cultivar, etc. (Nagy, 1980). In relation to this, it is well-known that thermal processing of orange juice in the industry has a negative effect, not only on the levels of ascorbic acid but also on other physical and chemical parameters (Farnworth *et al.*, 2001).

Initially, the ascorbic acid levels of the samples ranged from 475 ± 13 mg L⁻¹ for conventional orange juice to 570 ± 14 mg L⁻¹ for organic samples. After thermal processing (98 °C for 20 s), there was a mean reduction of 15% in all samples analysed, with values being 508 ± 21 and 400 ± 12 mg L⁻¹ for organic and conventional orange juices, respectively. Pérez-López *et al.* (2011) found vitamin C losses of 19% in canned mandarin segments under different preservation liquids. Otherwise, losses in vitamin C during storage time (145 days) were always below 10% (Fig. 1). Vitamin C content in organic orange juice was 17% higher than in traditional samples, independently of CD treatment. These results are similar to those described by Rapisarda *et al.* (2005), who found that organic orange juice contained 12% more vitamin C than conventional juices.

The loss of ascorbic acid during storage of the juice has been studied by several authors (Pérez-López *et al.*, 2011); experimental results proved that high temperature and storage time usually result in losses in the range of 17–20% losses for storage at room temperature and for a period of up to 4 months. This decrease in ascorbic

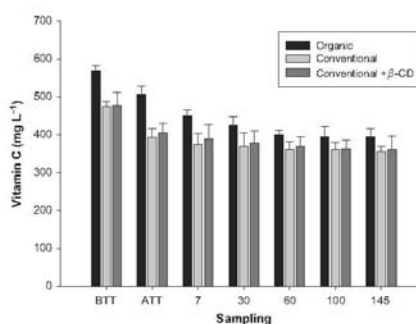


Figure 1 Vitamin C content on pasteurised orange juices along 145 days of storage. BTT: before thermal treatment (pasteurisation at 98 °C for 20 s) and ATT: after thermal treatment (pasteurisation at 98 °C for 20 s).

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acid occurs as a result of oxidation of acid leading to final products such as furfural and CO_2 (Graumlich *et al.*, 1987). Experimental results proved that ascorbic acid could be considered as an indicator of ageing of orange juice. The addition of β -CD to orange juice samples produced low or null effect on the vitamin C content of the samples.

CIEL*a*b* colour

The CIEL*a*b* coordinates measured were L^* (lightness), a^* (red-green), b^* (yellow-blue), C^* [colour purity; $C_{ab} = (a^{*2} + b^{*2})^{1/2}$], Hue angle* (colour; $h_{ab} = \arctan(b/a)$) and ΔE [colour differences; $\Delta E^* = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$].

In general, the main colour characteristics of fresh orange juice analysed (day 0) in this study were: $L^* = 87.3 \pm 0.4$, $a^* = 10.4 \pm 1.2$, $b^* = 60.0 \pm 0.8$ for organic orange juices and $L^* = 87.0 \pm 0.3$, $a^* = 6.8$

± 0.2 , $b^* = 59.2 \pm 0.4$ for conventional samples. These values are indicative of light yellowish-orange colours (Fig. 2). In most of the cases, organic juices presented a more intense and darker orange colour (lower values of L^* and higher of a^* , b^* and C^*) than the traditional juices, independently of the addition of β -CD.

After pasteurisation and storage of orange juice samples, there was a progressive and significant ($P < 0.001$) degradation of the parameter a^* (green-red), with values being 5.4 ± 0.4 for organic orange juices, 3.0 ± 0.6 for conventional samples and 4.1 ± 0.3 for conventional juices treated with β -CD. The parameter b^* (yellow-green) gradually changed to slightly higher ($P < 0.05$) values in all samples analysed (63.3 ± 0.3 , 64.8 ± 0.8 and 63.9 ± 0.6 for organic, conventional and conventional plus β -CD samples, respectively). The lightness, L^* , of juices decreased gradually ($P < 0.05$) after heat treatment and storage

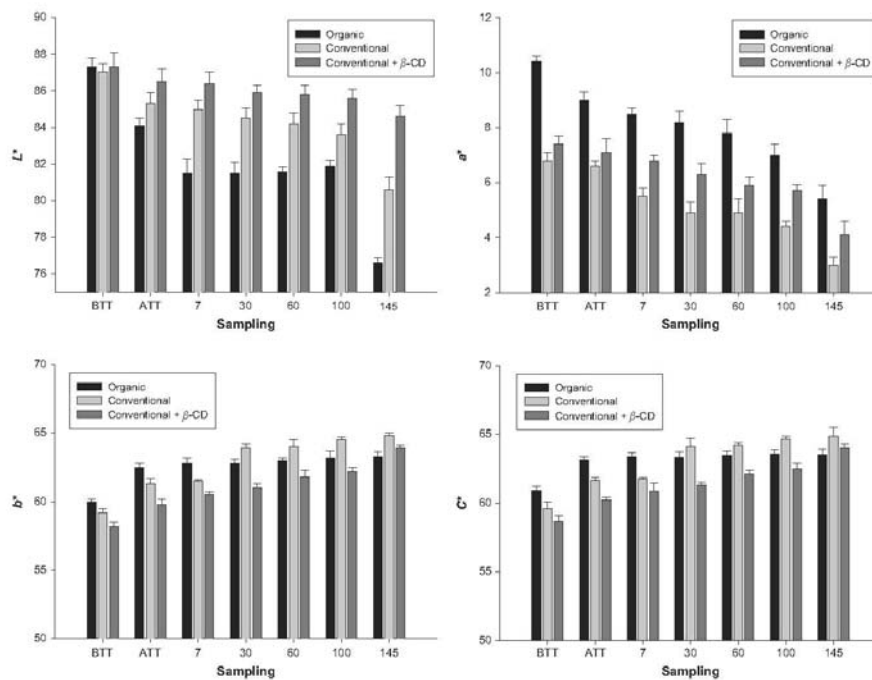


Figure 2 Changes in the CIEL*a*b* colour coordinates of three types of orange juices. BTT: before thermal treatment (pasteurisation at 98 °C for 20 s) and ATT: after thermal treatment (pasteurisation at 98 °C for 20 s).

of the orange juice (Fig. 2). This instrumental colour changes could be related to degradation of carotenoids (Odrizola Serrano *et al.*, 2008).

The chroma (C^*), which represents the colour purity, increased significantly ($P < 0.001$) from 60.9 ± 0.1 and 59.6 ± 0.2 for organic and conventional to 63.5 ± 0.3 and 64.9 ± 0.4 , respectively, after thermal treatment. Odrizola Serrano *et al.* (2008) observed similar increases on tomato juice because of the heat treatment during processing. Some researchers found similar results on fruits and vegetables after thermal treatments (Vikram *et al.*, 2005). The C^* values followed a similar trend to that described for the yellow-blue coordinate, b^* , during the storage of samples at room temperature.

The average value of Hue angle in fresh organic, conventional and conventional β -CD treated juices was 80.1 ± 0.8 , 83.4 ± 0.3 and 82.7 ± 0.8 , respectively. After pasteurisation and storage, orange juices Hue angle* increased between 4 and 5 units probably because of the change in the pigment profile of juices (Lemmens *et al.*, 2010). The values of the Hue angle were almost constant during the storage of samples at room temperature, with mean values for the storage period being 83.3 ± 0.5 , 85.8 ± 0.4 and 84.6 ± 0.5 for organic, traditional and traditional β -CD treated juices, respectively.

Carotenoids

The major carotenoid pigments, which are responsible for the colour of fresh and pasteurised orange juices, were measured by HPLC and are presented in Table 1. The total carotenoid contents in fresh organic and conventional samples were 5.17 ± 0.21 and 3.54 ± 0.36 mg L^{-1} , respectively. The main carotenoid found in orange juices was β -cryptoxanthin.

After pasteurisation, the total content of carotenoids decreased to 3.73 ± 0.32 and 2.50 ± 0.19 mg L^{-1} , respectively, with losses being around 30%. The carotenoids with the highest losses were lutein (16% and 8% for organic and conventional juices, respectively) and β -cryptoxanthin (mean losses of 30%). After storage of juices, the loss of carotenoids reaches values within the range 41–44%.

Sánchez-Moreno *et al.* (2003) assessed the influence of thermal treatments on degradation and isomerisation of lutein from processed vegetables. Torres Gama & De Sylos (2007) studied changes in carotenoid pigment content of Brazilian Valencia orange juices because of thermal processing and concentration and found that thermal effects on carotenoid pigment contents, especially β -cryptoxanthin and lutein, were clearly observed. Pasteurisation reduced the content of violaxanthin by 38% and lutein by 20%. Besides, the concentration process resulted in losses of lutein around 17%.

The vitamin A activity was estimated using the contents of β -cryptoxanthin and β -carotene because no α -carotene was found in samples under analysis (FNBIM, 2002):

$$\text{RAE} = \frac{C_{\beta\text{-car}}}{12} + \frac{C_{\beta\text{-crip}}}{24}$$

where $C_{\beta\text{-car}}$ and $C_{\beta\text{-crip}}$ are the concentrations ($\mu\text{g L}^{-1}$) of β -carotene and β -cryptoxanthin, respectively.

Retinol equivalents (RE) were 187, 126 and 130 $\mu\text{g L}^{-1}$ at day 0 for organic, conventional and conventional β -CD treated juices, respectively. Meléndez-Martínez *et al.* (2007c) obtained similar values for different types of orange juices. There were significant losses as a result of the pasteurisation step, around 30% in all types of juices. After the storage period, the losses reached 40%.

Table 1 Carotenoids profiles of orange juices under study

Storage time (days)	Carotenoids (mg L^{-1})											
	Lutein			Zeaxanthin			β -cryptoxanthin			β -carotene		
	Organic	Traditional	Tr. + β -CD*	Organic	Traditional	Tr. + β -CD	Organic	Traditional	Tr. + β -CD	Organic	Traditional	Tr. + β -CD
BTT**	0.74a [†]	0.49b	0.50b	0.27a	0.24a	0.24a	3.85a	2.80b	2.71b	0.31a	0.21b	0.21b
ATT***	0.62a	0.45b	0.46b	0.25a	0.23a	0.23a	2.58a	1.72b	1.75b	0.28a	0.19a	0.19a
7	0.60a	0.41b	0.42b	0.24a	0.22a	0.22a	2.53a	1.60b	1.68b	0.26a	0.18b	0.19b
30	0.58a	0.38b	0.40b	0.23a	0.21a	0.21a	2.41a	1.56b	1.66b	0.25a	0.18b	0.18b
60	0.56a	0.35b	0.37b	0.22a	0.19a	0.20a	2.28a	1.48b	1.62b	0.24a	0.17a	0.17a
100	0.54a	0.27b	0.29b	0.21a	0.18a	0.19a	2.21a	1.45b	1.62b	0.23a	0.16a	0.16a
145	0.50a	0.24b	0.25b	0.20a	0.16a	0.17a	2.11a	1.43b	1.51b	0.22a	0.15ab	0.15b

*Tr. + β -CD: traditional orange juice + β -CD; **BTT: before thermal treatment (pasteurisation at 98 °C for 20 s); ***ATT: after thermal treatment (pasteurisation at 98 °C for 20 s).

[†]Samples followed by the same letter, within the same row, were not significantly different at $P < 0.001$ for the attribute evaluated (Tukey's multiple range test).

CD, cyclodextrins.

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Table 2 Sensory analysis of orange juices throughout 145 days of storage

Storage time (days)	Sensory properties											
	Colour			Sweetness			Fresh mandarin aroma			Off-flavour		
	Organic	Tradit.	Tr. + β -CD *	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD
BTT**	8.8a [†]	7.3b	7.2b	6.3a	5.5b	5.4b	7.5a	6.3b	6.4b	0.9a	2.0b	2.4b
ATT***	8.2b	6.8c	7.0c	6.3a	5.3b	5.4b	7.4a	6.3b	6.3b	0.9a	2.2b	2.3b
7	8.2b	6.7c	6.8c	6.2a	5.2b	5.2b	7.3a	6.1b	5.9b	1.0a	2.3b	2.4b
30	8.1b	6.6c	6.7c	6.3a	5.3b	5.3b	7.1a	5.8b	5.8b	1.0a	2.3b	2.3b
60	8.0b	6.6c	6.7c	6.2a	5.3b	5.2b	7.1a	5.8b	5.7b	1.0a	2.3b	2.3b
100	8.0b	6.6c	6.7c	6.2a	5.2b	5.2b	7.0a	5.9b	5.7b	1.1a	2.2b	2.4b
145	7.9b	6.5c	6.7c	6.1a	5.2b	5.1b	6.9a	5.8b	5.7b	1.2a	2.3b	2.4b

*Tr. + β -CD: traditional orange juice + β -CD; **BTT: before thermal treatment (pasteurisation at 98 °C for 20 s); ***ATT: after thermal treatment (pasteurisation at 98 °C for 20 s).

[†]Samples followed by the same letter, within the same row, were not significantly different at $P < 0.001$ for the attribute evaluated (Tukey's multiple range test).

CD, cyclodextrins.

It can be concluded that pasteurisation and storage at room temperature negatively affected total carotenoids and vitamin A activity. Organic farming led to the juices with the highest carotenoid content at all stages studied. On the other hand, conventional juices treated with β -CD had carotenoid content and RE slightly higher than traditional juices, but these differences were not statistically significant.

Antioxidant activity

Figure 3 shows the changes in antioxidant activity (expressed as Trolox equivalents) in juice samples stored up to 145 days at room temperature using the ORAC-FL method. This method has been used to evaluate the

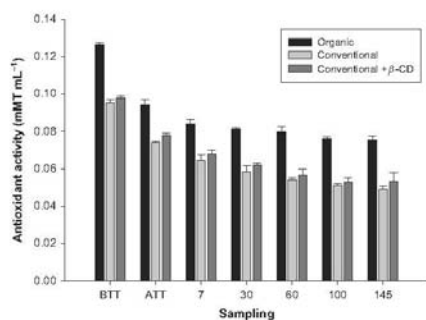


Figure 3 Changes in the antioxidant capacity of orange juices during 145 of storage. BTT: before thermal treatment (pasteurisation at 98 °C for 20 s) and ATT: after thermal treatment (pasteurisation at 98 °C for 20 s).

antioxidant capacity of many types of fruits and vegetables (Zapata *et al.*, 2007). The antioxidant capacities of fresh juices were significantly ($P < 0.001$) different and reached values of 0.126 ± 0.002 and 0.098 ± 0.009 mMT mL⁻¹ in organic and conventional juices, respectively. The analysis revealed losses in the juices after the heat treatment in the range 20–25%. The results presented agreed with previous data obtained by Piga *et al.* (2002). After the storage period (145 days), the antioxidant capacity ranged from 0.075 ± 0.001 mMT mL⁻¹ for organic juices to 0.053 ± 0.007 mMT mL⁻¹ in conventional samples. Global losses (pasteurisation and storage) of antioxidant activity can be established at 40–45%. There is a clear difference between the oranges from organic agricultural practices compared to those from conventional farming (Fig. 3), with antioxidant activity being always higher for organic samples. This could be explained by considering that organic farming uses no synthetic pesticides, and therefore, plants are exposed to different forms of stress, which will induce the accumulation of protective compounds such as carotenoids, thus presenting greater antioxidant capacity (Vian *et al.*, 2006). These results are consistent with those described for other fruits (Rapisarda *et al.*, 2005).

Encapsulation of bioactive compounds in orange juice had no effect on the antioxidant capacity of samples. Some authors have also obtained negative results on β -CD encapsulation of bioactive compounds in foods (López-Nicolás *et al.*, 2007).

Sensory evaluation

In general, the trained panel demonstrated that the juice with significantly ($P < 0.001$) higher intensities of the positive sensory attributes colour (8.2 in a scale from 0

to 10), sweetness (6.2) and fresh orange flavour (7.2) was the organic one (Table 2). No statistically significant ($P > 0.05$) differences were found between the traditional (colour: 6.7, sweetness: 5.3 and fresh orange flavour: 6.0, respectively) and the traditional treated (6.8, 5.3 and 5.9, respectively) juices. On the other hand, even though low intensities of off-flavours were detected, the values were always higher in the traditional juices (mean of 2.3) compared to the organic juices (1.0).

The only significant effect ($P < 0.01$) of the pasteurisation process was the reduction in the colour intensity of the juices. Finally, the storage of the juices at room temperature did not affect the sensory quality of the orange juices.

Conclusions

Experimental data proved that strong thermal treatments, such as pasteurisation, are not the best preservation methods. This statement is based on the significant reductions or losses in antioxidant compounds, such as carotenoids, together with severe reductions of the sensory quality of orange juice during storage at room temperature. However, and according to the literature, they are useful in reducing microbiological pollution and enzymatic activity. The addition of β -cyclodextrin did not imply a significant improvement in the storage behaviour of conventional orange juice. Therefore, it is recommended that alternative preservation methods must be considered for a proper preservation of the orange juice quality.

Acknowledgments

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EFFECTS OF FARMING PRACTICES ON THE QUALITY OF ULTRA-FROZEN MANDARIN JUICE

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ABSTRACT

The effects of the farming type (organic or conventional) and cyclodextrin addition on the quality (loss of ascorbic acid, carotenoids composition, color and antioxidant activity) of freshly squeezed, nonpasteurized, ultra-frozen mandarin juices were studied during a shelf life of 145 days. The vitamin C content was reduced from 540 and 470 mg/L to 515 and 447 mg/L in organic and conventional juices, respectively. Carotenoids were stable during the storage time (decreases being in the range 5–10%), with β -cryptoxanthin (7.21 ± 0.31 mg/L) being the most abundant compound, followed by lutein and zeaxanthin. The juices color was not affected by thermal treatment and these data were provided by both instrumental *Commission internationale de l'éclairage L**, *a**, *b** data and the sensory studies conducted. The antioxidant activity was 0.04 and 0.06 mMT/mL, at day 0, for the organic and conventional juices; the losses during the storage were below 18%. The addition of cyclodextrins did not have any significant effect on the parameters under study. This study showed that the nutritional and organoleptic quality of ultra-frozen juices is quite stable during the storage period.

PRACTICAL APPLICATIONS

The application of cyclodextrins in the food industry is very important as solubilizing agents, stabilizers and emulsifiers. The molecular encapsulation of lipophilic food ingredients with cyclodextrin improves the stability of flavors, vitamins, colorants and unsaturated fats, etc., both in physical and chemical sense leading to extended product shelf life, accelerated and long-term storage stability.

INTRODUCTION

Nowadays, both the consumption and therefore the manufacturing of fruit juices have experienced high growth because, among other things, of progress in the farming and improvement on fruit processing, transport and distribution (Schieber *et al.* 2001). Mandarin juice production in Spain is low compared with the production of orange juice, but is expected to be higher because of market saturation in fresh and to the pleasant taste, fresh aroma and the great nutritional value of mandarin. Spain currently produces 2.5 million tons of mandarins per year and production is increasing. The most widespread cultivar is Clementine (1.6 million tons), from which about 60% of production is exported to other coun-

tries, particularly to Northern European countries and the United States (Agroinformacion 2010).

Mandarin is the fruit that has more carotenoids in its composition, providing β -cryptoxanthin and β -carotene in outstanding amounts (Meléndez-Martínez *et al.* 2010). The antioxidant activity of these phytochemicals and vitamin C provide the food physiological properties that go beyond the proper nutrition. Antioxidants fight the harmful action of free radicals, substances responsible for the development of cardiovascular diseases, degenerative diseases and cancer (Sánchez-Moreno *et al.* 2003a). The synthesis and composition of these compounds can oscillate depending on the type of species, fruit variety, climatic factors, industrial processing and storage conditions (Meléndez-Martínez *et al.* 2010).

Farming practices, such as the presence or absence of certain pesticides, fertilizer or type of irrigation, can also affect the phytochemical composition of the fruit, both qualitatively and quantitatively (Meléndez-Martínez *et al.* 2010). Therefore, the high demand by health-concerned consumers is encouraging the development of products with improved nutritional quality, such as fruits and vegetables grown under organic systems.

Organic farming can be defined as a set of agricultural techniques that exclude the use of synthetic chemicals, including fertilizers, pesticides, antibiotics, etc., with the objective of preserving the environment, maintaining or increasing soil fertility and provide food with all its natural properties (Willer and Yussefi 2004).

Organic farming has become one of the most dynamic agricultural sectors of the European Union, with sales reaching 13–14 billion euros in 2005. In the European Union, the area of organic crops is 3.5 million hectares, 3% of the total agricultural area. Spain is one of the European countries with the largest organic area. Oranges and mandarins are one of the most demanded fruits by the consumer; Spain is the second producer of organic citrus worldwide, with the regions of Valencia and Murcia being the leaders in this industry (Mapa 2005).

In the recent years, a new type of industrial juice has been developed; after processing, the juice is ultra-frozen without being subjected to any concentration or thermal treatments. Deep freezing is one of the most effective and used preservation techniques. Quick freezing is a method of preservation that ensures food safety and the maintenance of the food sensory and nutritional properties (Meléndez-Martínez *et al.* 2009).

Besides to organic food, the market is requiring the use of new substances with high added value, which are able to lead to high nutritional value and taste, which is the case of the so-called cyclodextrins (CDs). These compounds can be used to improve the quality of current foodstuffs and also to promote the development of new products. There are three types of CDs: γ , β and α . β -CDs are considered as GRAS (safe food ingredients) up to a maximum content of 2% in food products since 1998.

CDs have a hydrophilic outer surface and are therefore soluble in water but at the same time have a hydrophobic internal cavity (Szejtli 1998), that may complex molecules with different degree of hydrophobicity, resulting in inclusion complexes.

In general, CDs are used in the food industry to: (1) stabilize light- or oxygen-sensitive compounds, volatile compounds; (2) increase the water solubility of poorly soluble compounds; (3) protect against microbial degradation; and (4) mitigate undesirable odours or flavors (Loftsson and Stefansson 1997; Szejtli 1998; Buschmann and Schollmayer 2002).

In the recent years, the effects of CDs on color of fruit juices has been widely studied, however, only few studies have evaluated the effects of CDs on juice aroma (López-Nicolás *et al.* 2009). In this particular study, β -CD (the most reported type of CD to complex different guest molecules) was used to encapsulate hydrophobic nutrients (carotenoids and flavonols) and aroma compounds of hydrophilic juices, perhaps increasing its stability in the stages of processing and storage, as well as their bioavailability (Mercader-Ros *et al.* 2009).

The main objective of this work is to study the effects of: (1) the complexation of phytochemicals, such as carotenoids, by β -CD; and (2) the farming type (organic or conventional) on quality properties, such as antioxidant activity, carotenoids profile and vitamin C, of ultra-frozen mandarin juices during their shelf life.

MATERIALS AND METHODS

Fruit Material

Both conventional and organic mandarin oranges (*Citrus reticulata* L.), var. Clementules, were grown in the same farm and under identical conditions of soil, irrigation, and illumination in Eastern Spain (Librilla, Murcia). The citrus rootstock was the same for both mandarin trees, Cleopatra mandarin trees, and all selected trees were about 10-year-old and free of diseases. Fruits were collected in winter (second week of February 2008). Fruits were selected on the basis of their diameter, pH, total soluble solids content ([SSC] °Brix) and maturity index (MI) (total soluble content/titratable acidity [SSC/TA]).

Clementules mandarin oranges were studied under conventional and organic farming. Organic production means that no synthetic chemicals were used in the cultivation of these fruit trees and that only natural substances were used to control pest, weeds, etc. Farming of organic mandarin trees followed all rules established by the Board of Organic Agriculture of the Murcia Region (Boam 2009).

Sample Preparation

Mandarin juices were processed in a commercial plant (Murcia, Spain) and were obtained using a Premium Juice Extractor (FMC Corporation, Jacksonville, FL) (Kimball 2002). This machinery leads to a juice with a low content of essential oils (FMC 2010). After the mechanical extraction, the juice was counter-currently treated with nitrogen to remove air and prevent oxidative processes and microbial contamination. Next, the content of pulp was adjusted and the juice was cooled to around 0°C. Finally, the product was bottled in high-density propylene plastic containers (1.15 L total capacity, 50 mL headspace) and passed through a freezing tunnel using liquid nitrogen as cryo-protective

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fluid, with which a rapid freezing is achieved. The mandarin juice manufactured did not contain additives, was not enriched with vitamins or any other kind of permitted substance, and was kept at a temperature equal to or below -18C over storage, delivery and retailing (Meléndez-Martínez *et al.* 2009).

β -CD from TCI (Europe) was added to the conventional mandarin juices samples. β -Cyclodextrin was added at a concentration of 1.5% to 2 L of freshly squeezed juice. Three types of mandarin juices were studied: conventional, conventional plus β -CD and organic.

Samples were stored at -18C in darkness and analyzed after 7, 30, 60, 100 and 145 days. Samples were thawed at 5C for about 8 h and in each sample the following parameters were analyzed: total soluble solids, titratable acidity, pH, vitamin C, color, carotenoids content and composition and antioxidant activity. All juices were prepared in triplicate.

Physicochemical Analyses

SSC ($^{\circ}$ Brix) was determined using a portable refractometer Comecta, S.A., model C3 (Barcelona, Spain). TA (% citric acid), was determined in 10 mL of juice by titration to pH 8.2 ± 0.1 with a 0.1 N NaOH solution. MI was calculated for each mix and expressed as the percentage of the ratio between the SSC and TA.

Vitamin C (reduced ascorbic acid) was measured following the AOAC Official Method 985.33 (Horwitz 2000). Ascorbic acid was estimated by titration with colored oxidation-reduction indicator, 2,6-dichloroindophenol. Ethylenediaminetetraacetic acid was added as chelating agent to remove Fe and Cu interferences.

All physicochemical analyses were analyzed in 20 fruits of each agricultural practice.

Instrumental Color

Color measurement was made, at $25 \pm 1\text{C}$, using a Hunterlab Colorflex[®] (Hunterlab, Reston, VA). This spectrophotometer uses an illuminant D65 and a 10^{P} observer as references. A sample cup for reflectance measurements was used (5.9 cm internal diameter \times 3.8 cm height) with a path length of light of 10 mm. Blank measurements were made with the cup filled with distilled water against a reference white background (Pérez-López *et al.* 2006).

Color data are provided as CIE L^* , a^* , b^* coordinates, which define the color in a three-dimensional space (Minolta 1994). Finally, the color differences (ΔE^*) between two points in the CIE L^* , a^* , b^* space are worked out as the Euclidean distance between their localizations in the three dimensional space defined by L^* , a^* and b^* . Mathematically,

it is therefore calculated by applying the formula $\Delta E^* = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$. Color analyses were run in six replicates.

Antioxidant Activity

All the reagents used for the determination of the antioxidant capacity, fluorescein (FL), 2,2-azobis-[2-aminopropane] dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C) were purchased from Sigma (Madrid, Spain).

The ORAC assay was carried out on a Synergy HT multidetector microplate reader, from Bio-Tek Instruments, Inc. (Winooski, VT), using 96-well polystyrene microplates with black sides and clear bottom, purchased from Nalge Nunc International (Rochester, NY). Fluorescence was read through the clear bottom, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The oxygen radical absorbance capacity was determined as described by Dávalos *et al.* (2004) with slight modifications (Lucas-Abellán *et al.* 2008). A blank with FL and AAPH using sodium phosphate buffer instead of the antioxidant sample and eight calibration solutions using Trolox C (6.25, 12.5, 15, 18.75, 21.25, 25, 27.5 and 31.25 μM) as antioxidant were also used in each. All reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. In order to avoid a temperature effect, only the inner 60 wells were used for experimental purposes, whereas the outer wells were filled with 200 μL of distilled water.

The results were expressed as relative fluorescence with respect to the initial reading. The area under the fluorescence decay curve (AUC) was calculated by the equation:

$$AUC = 1 + \sum_{i=1}^{n-1} \frac{f_i}{f_0}$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i . The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. The results of antioxidant capacity were defined as millimolar of Trolox C per milliliter of juice. Data processing was performed using SigmaPlot software package (Jandel Scientific, Erkrath, Germany).

Carotenoid Compounds Extraction and Quantification

The analysis of the carotenoid composition requires an extraction with organic solvent followed by de-esterification of the carotenoid fraction. Mandarin orange juices (350 μL) were extracted with 600 μL of solvent (methanol [MeOH]/acetone/dichloromethane, 25:25:50 v:v:v), centrifuged retaining the colored phase. The final residue was finally

extracted with 300 μ L of dichloromethane, centrifuged and again keeping the colored phase, which was mixed with the previous one. This mixture was washed several times with deionised water to remove any trace of acetone. This phase containing the pigments, in various stages of esterification with fatty acids, was saponified with 15% KOH-MeOH for 1 h at room temperature and washed with water to remove any trace of the base. The pigments were subsequently evaporated in a rotary evaporator at 30°C, keeping the samples under nitrogen atmosphere at -18°C until analyzed.

The high-performance liquid chromatography system consisted of an HP-1100 series unit with a photodiode array detector equipped with HP ChemStation software (Hewlett Packard, Palo Alto, CA). The column used was 250 \times 4.6 mm in internal diameter, YMC C30, S-5 μ m (YMC, Wilmington, NC), and column temperature was kept at 17°C. The mobile phase for this column was MeOH, methyl tertiary butyl ether (MTBE) and water; MeOH and MTBE contained BHT (0.1%) and TEA (0.05%) to protect carotenoids during analysis (Hart and Scott 1995). The gradient elution was: 0 min, 90% MeOH + 5% MTBE + 5% water; 12 min, 95% MeOH + 5% MTBE; 25 min, 89% MeOH + 11% MTBE; 40 min, 75% MeOH + 25% MTBE; 50 min, 40% MeOH + 60% MTBE; 56 min, 15% MeOH + 85% MTBE; 62 min, 90% MeOH + 5% MTBE + 5% water, at a flow rate of 1.0 mL/min (Mouly *et al.* 1999). Carotenoids were monitored at 450 nm and analyses were carried out in triplicate.

Identification of carotenoids was conducted by comparison with standards isolated from natural sources. Lutein, β -cryptoxanthin and β -carotene were obtained from spinach leaves (*Spinacia oleracea* L.) (Britton *et al.* 1995).

Quantification of lutein, β -cryptoxanthin and β -carotene was carried out using calibration curves (Rodríguez-Amaya 2001). Zeaxanthin concentration was quantified using the β -carotene calibration curve because they have the same spectral properties (Meléndez-Martínez *et al.* 2010).

Sensory Evaluation with Trained Panel

Sensory evaluation by a trained panel was used to evaluate the quality of mandarin orange juices. A panel of 10 panellists, ages 20 to 50 years (eight females and two males, all members of the Catholic University San Antonio of Murcia), with sensory evaluation experience, was trained in descriptive evaluation of citrus juice (Serrano-Megías *et al.* 2005).

The panel was selected and trained following the ISO standard 8586-1 (Aenor 1997; Meilgaard *et al.* 1999). Further details on selection, training and validation of the panel can be found in Pérez-López *et al.* (2006).

Measurements were performed in individual booths with controlled illumination (750–1,000 lux) and temperature (23 \pm 2°C) (Aenor 1997; Meilgaard *et al.* 1999).

The individual products were scored for the intensities of color, sweetness, acidity, fresh mandarin juice aroma and off-flavor using a scale of 0–10, where: 0 = extremely slight intensity, and 10 = extremely high intensity.

Samples were presented in 50 mL plastic cups with lids. The entire experiment was repeated three times (all judges scored two juice samples on each session for a total of three sessions) and the sensory scores were presented as the overall means.

Statistical Analysis

All data were subjected to analysis of variance and the Tukey's least significant difference multicomparison test to determine significant differences among mandarin orange juices. Significance of differences was represented as $P \leq 0.001$. The statistical analyses were done using SPSS 14.0 (SPSS Science, Chicago, IL) and figures using Sigma Plot 9.0 (SPSS Science).

RESULTS AND DISCUSSION

Clemenules mandarin oranges were chosen for this experiment because several studies have proved they are the best ones for juice manufacturing because of their high vitamin C content, intense orange color and aroma (Beltrán *et al.* 2008).

Physicochemical Analyses

All samples under assay presented the same pH (3.2 \pm 0.2) and MI (13.3 \pm 0.1°Brix/% citric acid) and these values remained stable throughout the experiment.

Vitamin C

The vitamin C content of fruits depends on several factors, including cultivar, farming type and MI (Howard *et al.* 2000; Lee and Kader 2000; Tudela *et al.* 2002).

The initial contents of vitamin C in organic and conventional juices were 540 and 470 mg/L, respectively. After 7 days of storage of the ultra-frozen mandarin juices vitamin C contents decreased about 5% and were 515 and 447 mg/L for juices nontreated and treated with β -CD, respectively. After the first week and until the end of the storage period (145 days), all samples experienced losses of vitamin C lower than 3%. Always organic juices had higher vitamin C than conventional samples and at the end of the experiment the difference was about 13%; these results agree with data previously published for strawberry and corn (Wang *et al.* 1996).

The fact that only a 3% loss of vitamin C was found proved that the ultra-freezing treatment was effective in maintaining the quality of mandarin juices. These results also agree with previous studies conducted with orange juices (Lee and

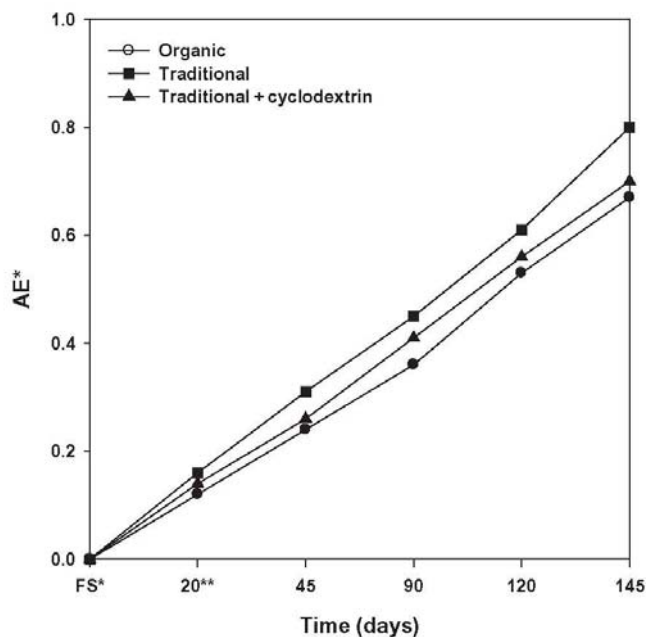


FIG. 1. CHANGES OF ΔE^* (TOTAL COLOR) OF ULTRA-FROZEN MANDARIN JUICES THROUGHOUT 145 DAYS OF STORAGE
*Freshly squeezed. **Storage time (-18°C)

Coates 1999; Farnworth *et al.* 2001; Sánchez-Moreno *et al.* 2003b; Meléndez-Martínez *et al.* 2009).

The addition of β -CD had no significant effect on vitamin C changes with time.

CIE L^* , a^* , b^* Color Coordinates

The pulp content has a direct effect on the measurement of reflectance and to avoid this effect all samples must have similar pulp content at the beginning of any experiment (Arena *et al.* 2001). The fiber contents of all mandarin juice samples were equilibrated by using a FMC FoodTech Quick Fiber device (FMC Corporation). In this way, juice samples presented a mean value for the centrifugable pulp content of 15.0 ± 1.1 g/L (no significant differences were found among juice samples).

The main color characteristics of the freshly squeezed mandarin juice (day 0) analyzed in this study were: $L^* = 75.88 \pm 0.31$; $a^* = 22.64 \pm 0.15$; $b^* = 62.37 \pm 0.19$ and were indicative of a deep orange color. The color coordinates at the end of the study (day 145) in organic, conventional and β -CD-treated conventional juices were: $L^* = 73.64 \pm 0.25$, $a^* = 23.12 \pm 0.42$, $b^* = 66.76 \pm 0.37$; $L^* = 72.34 \pm 0.29$, $a^* = 20.27 \pm 0.51$, $b^* = 67.35 \pm 0.44$; and $L^* = 72.15 \pm 0.62$,

$a^* = 19.92 \pm 0.23$, $b^* = 67.78 \pm 0.52$, respectively. The instrumental color data showed significant differences among mandarin juices as affected by farming type. The color of organic mandarin juice was more reddish ($+a$) than that of conventional samples. However, the addition of β -CD to the mandarin juices had not significant effects on the values of ΔE^* . As shown in Fig. 1, the values of ΔE^* increased from day 0 to day 145.

Color data showed differences among mandarin juices of 0.70 units in ΔE at day 145 of storage. Studies on the effects of thermal treatment on juices reported differences of about 3 units between fresh and pasteurized juices (Lee and Coates 2003).

On the other hand, studies by Meléndez *et al.* (2009) in ultra-frozen juices indicated that these products had an intense orange color ($L^* = 63.23 \pm 1.27$; $a^* = 16.8 \pm 0.56$; $b^* = 64.64 \pm 3.78$), because of the fact that juices were not subjected to neither high temperature nor concentration steps.

Carotenoids

Identification of carotenoids was based on their spectral parameters using the current rules. The precision of the caro-

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tenoids quantification is based on the absorption coefficients used to calculate the concentration of the standards (Davies 1976; Britton 1995).

Lutein, zeaxanthin, β -cryptoxanthin and β -carotene were measured because of their predominance in mandarin juice (Beltrán-González *et al.* 2008). All the studied carotenoids showed higher concentrations in organic mandarin juices than in conventional ones (Table 1). Initially, day 0, results of this study showed higher concentration of total carotenoids in organic mandarin juice, 8.54 ± 0.35 mg/L, than in conventional samples, 6.97 ± 0.26 mg/L. No differences were found between conventional and conventional + β -CD, 6.99 ± 0.22 mg/L. Finally, at day 145, there was a loss of approximately 10% of total carotenoids on all samples under analysis. The addition of β -CD did not represented any beneficial effect on the total concentration of carotenoids (Table 1); it is possible that the effects of the low temperatures used during the manufacturing of juices (ultra-freezing) were much higher than those from the addition of CDs and therefore, the effects of CDs was masked. On the other hand, some studies have previously reported beneficial effects of β -CD on the quality of juices subjected to pasteurization and sterilization protocols (Meier *et al.* 2001; Rodríguez-Amaya 2003; Jun 2009).

The most abundant carotenoid in all the juices was β -cryptoxanthin, representing more than 80% of the total concentration of carotenoids. The initial concentrations for the organic and conventional juices were 7.21 ± 0.31 and 6.09 ± 0.25 mg/L, respectively. The losses for both types of juices after 145 days of storage were about 10% (Table 1). β -cryptoxanthin is one of the main carotenoids found in orange and mandarin juices (Meléndez-Martínez *et al.* 2009). This carotenoid has provitamin A activity and provides juices with a dark orange, almost red color, which is a distinctive attribute of mandarin juices compared with orange juices.

Lutein and zeaxanthin presented lower concentration than β -cryptoxanthin, but always their concentrations were higher in organic than in conventional juices; again no significant effects were found after addition of β -CD. The losses caused by the storage period (145 days) were about 5% of the initial concentrations, and therefore their stability was high because of the ultra-freezing treatment. Some researchers showed that canning does not decrease the content of these carotenoids, in corn and some vegetables, and that freezing may increase carotenoid content in corn, which can further influence bio-availability and health benefits (Updike and Schwartz 2003; Scott and Eldredge 2005).

Finally, β -carotene was the studied carotenoid with the lowest concentration, 0.12 ± 0.03 and 0.09 ± 0.01 mg/L in organic and conventional juices, respectively; these concentrations only represented about 1.4% of the total concentration of carotenoids. These concentrations decreased about 15% after 145 days of storage, because of the high sensitivity

TABLE 1. CHANGES IN CAROTENOIDS COMPOSITION OF ULTRA-FROZEN MANDARIN JUICES THROUGHOUT 145 DAYS OF STORAGE

Storage time (days)	Carotenoids (mg/L)											
	Lutein			Zeaxanthin			β -Cryptoxanthin			β -Carotene		
	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD
0	0.61 \pm 0.08	0.38 \pm 0.06	0.38 \pm 0.11	0.60 \pm 0.08	0.41 \pm 0.03	0.43 \pm 0.09	7.21 \pm 0.31	6.09 \pm 0.25	6.09 \pm 0.32	0.12 \pm 0.03	0.09 \pm 0.01	0.09 \pm 0.01
20	0.60 \pm 0.12	0.37 \pm 0.08	0.37 \pm 0.06	0.59 \pm 0.03	0.39 \pm 0.08	0.41 \pm 0.04	7.14 \pm 0.22	6.08 \pm 0.32	6.08 \pm 0.17	0.12 \pm 0.01	0.09 \pm 0.03	0.09 \pm 0.02
45	0.58 \pm 0.16	0.37 \pm 0.11	0.36 \pm 0.07	0.59 \pm 0.04	0.37 \pm 0.05	0.37 \pm 0.08	7.12 \pm 0.17	5.98 \pm 0.41	5.98 \pm 0.12	0.11 \pm 0.04	0.08 \pm 0.02	0.08 \pm 0.01
90	0.58 \pm 0.05	0.37 \pm 0.14	0.36 \pm 0.02	0.59 \pm 0.09	0.36 \pm 0.09	0.36 \pm 0.03	6.95 \pm 0.27	5.87 \pm 0.26	5.87 \pm 0.29	0.11 \pm 0.02	0.08 \pm 0.01	0.08 \pm 0.02
120	0.58 \pm 0.15	0.37 \pm 0.03	0.36 \pm 0.08	0.59 \pm 0.06	0.35 \pm 0.07	0.35 \pm 0.09	6.75 \pm 0.19	5.79 \pm 0.34	5.79 \pm 0.24	0.10 \pm 0.03	0.08 \pm 0.02	0.08 \pm 0.03
145	0.58 \pm 0.22	0.37 \pm 0.10	0.35 \pm 0.05	0.58 \pm 0.09	0.34 \pm 0.05	0.35 \pm 0.04	6.45 \pm 0.24	5.47 \pm 0.39	5.47 \pm 0.15	0.10 \pm 0.02	0.07 \pm 0.01	0.07 \pm 0.02

Tr. + β -CD, conventional mandarin juice + β -cyclodextrin.

of β -carotene compared with other carotenoids, such as lutein (Siems *et al.* 1999).

According to literature there are several factors that can justify the fact that the concentrations of carotenoids were higher in organic than in traditional mandarin juices. For instance, Mäder *et al.* (2006) suggested that organic soils are richer in some nutrients and that can result in higher contents of some elements in plants and therefore in the products manufactured from them, such as juices. Asami *et al.* (2003) also indicated that plants not treated with pesticides produce higher concentrations of protective-compounds, such as carotenoids.

Retinol Activity Equivalents (RAEs)

RAEs of the samples analyzed and discussed here are referred to 1 L of mandarin juice. The bioavailability of carotenoids is influenced by many factors, such as amount, food matrix, age, existence of certain diseases, intake of fat, vitamin E and fiber, protein and zinc status (FNBIM 2002). In this study, α -carotene was not found in mandarin juices, calculations were performed, considering new guidelines according to the following formula (FNBIM 2002):

$$RAE = \frac{C_{\beta\text{-car}}}{12} + \frac{C_{\beta\text{-crip}}}{24}$$

where $C_{\beta\text{-car}}$ and $C_{\beta\text{-crip}}$ are the concentrations ($\mu\text{g/L}$) of β -carotene and β -criptoxanthin, respectively. Initially the RAE values found for organic and conventional, with and without β -CD, mandarin juices were 310 and 261 $\mu\text{g/L}$, respectively; these high values of RAE/L support the general fact that mandarin products are a very good source of vitamin A and this statement is especially true for organic mandarin juices (Beltrán *et al.* 2008). Finally, the RAE values lost for organic and conventional was about 11% after 145 days of storage, independently of the addition of β -CD (which had no effect on this quality parameter). These experimental values agreed well with the range previously described by Meléndez-Martínez *et al.* (2007) for the RAE/L contents of different cultivars of mandarin and orange juices marketed in Spain, ranging from 9.7 to 359. This RAE/L range was significantly reduced when only orange juices were considered, 9.7–94.8. Without any doubt the high levels of RAE in mandarin juices compared with others citrus juice are related to their high concentrations of β -criptoxanthin.

Antioxidant Activity

The antioxidant activity of all assayed samples was quite high during the 145 days of storage (Fig. 2). Organic farming led to significantly higher values of antioxidant activity than conventional farming, 0.06 ± 0.01 and 0.04 ± 0.01 mM Trolox/

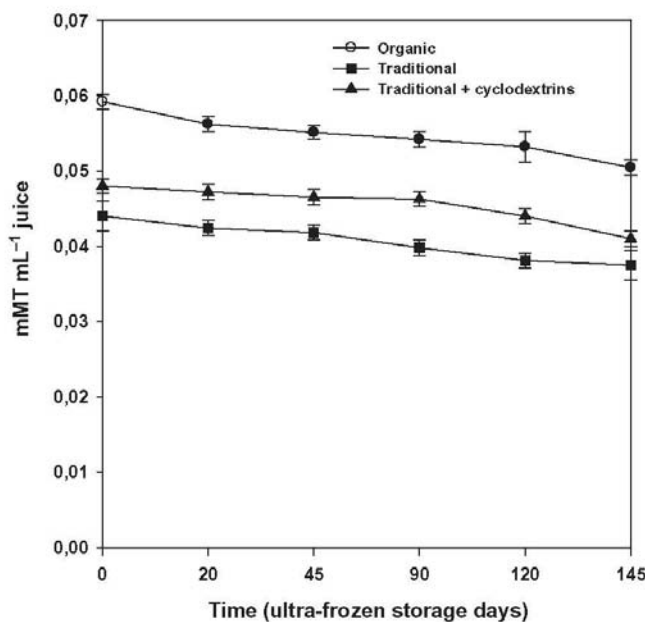


FIG. 2. CHANGES OF ANTIOXIDANT ACTIVITY VALUES OF ULTRA-FROZEN MANDARIN JUICES THROUGHOUT 145 DAYS OF STORAGE

TABLE 2. SENSORY ANALYSIS OF ULTRA-FROZEN MANDARIN JUICES THROUGHOUT 145 DAYS OF STORAGE

Storage time (days)	Sensory properties											
	Color			Sweetness			Fresh mandarin aroma			Off-Flavor		
	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD	Organic	Tradit.	Tr. + β -CD
0	8.2a	7.1b	7.0b	6.5a	5.5b	5.4b	7.2a	6.0b	6.0b	1.0a	2.3b	2.1b
20	8.0a	6.8b	7.0b	6.5a	5.6b	5.5b	7.1a	6.0b	6.0b	1.0a	2.2b	2.3b
45	8.0a	6.7b	6.8b	6.4a	5.4b	5.6b	7.1a	5.9b	5.9b	1.0a	2.3b	2.4b
90	8.0a	6.7b	6.8b	6.5a	5.5b	5.5b	6.9a	5.8b	5.8b	1.0a	2.1b	2.2b
120	8.0a	6.6b	6.8b	6.3a	5.6b	5.5b	6.9a	5.8b	5.7b	1.0a	2.0b	2.2b
145	8.0a	6.6b	6.7b	6.4a	5.5b	5.6b	7.0a	5.9b	5.7b	1.1a	2.0b	1.9b

Samples with the same letters were not significantly different at $P < 0.001$ for the attribute evaluated (Tukey multiple range test). Tr. + β -CD, conventional mandarin juice + β -cyclodextrin.

mL, respectively. Because of the fact that organic farming does not use synthetic chemicals as fertilizers, pesticides, etc., the plant synthesized natural protecting compounds (e.g., carotenoids), which in general have antioxidant activity. Results agreeing with this last statement have been reported by several authors (Negro *et al.* 2003; Riso *et al.* 2005; Toor and Savage 2006; Rivero-Pérez *et al.* 2008).

Again the addition of β -CD did not have any significant effect on the antioxidant activity of mandarin juices (Fig. 2).

Ultra-freezing of mandarin juices was very effective in protecting the antioxidant activity of juices and only 18% of the initial activity was lost after 145 days of storage (Fig. 2).

Sensory Evaluation

The trained panel established that the quality of the mandarin juices analyzed was high but organic juice had higher intensities than conventional samples for the following sensory attributes: color, sweetness, fresh mandarin juice and aroma. However, the addition of β -CD had no significant effect on the quality of conventional juices (Table 2).

CONCLUSIONS

This study proved that the conservation of mandarin juice by ultra-freezing is an interesting alternative to the heat treatments. Ultra-frozen juices were characterised by having high sensory (intense orange color and fresh mandarin aroma and low presence of off-flavors) and nutritional quality (high content of total carotenoids and antioxidant capacity) and being stable during storage (reduction of vitamin C and total carotenoids contents ranged from 5 to 10%). However, the addition of β -CD, at a concentration of 1.5%, did not result in any improvement on the quality of ultra-frozen mandarin juices. However, other concentrations and types of CDs must be used in future studies to determine the role of these protective compounds on ultra-frozen citrus juices.

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Effects of Cyclodextrin Type on Vitamin C, Antioxidant Activity, and Sensory Attributes of a Mandarin Juice Enriched with Pomegranate and Goji Berries

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Abstract: The effects of the addition of cyclodextrins (CDs), β -CD, or HP- β -CD (1%), on the protection of antioxidant compounds of mandarin juices enriched with pomegranate extract and goji berries juice, was studied. Juices were prepared and after their thermal treatment (98 °C, 30 s) they were stored at 4 °C during 75 d. Vitamin C content, CIE $L^*a^*b^*$ color, antioxidant capacity, retinol equivalents, and sensory properties were studied. Losses on vitamin C were higher (6%) for juices with β -CD than juices with HP- β -CD. Retinol equivalents degradation was lower (3.4%) in juices with HP- β -cyclodextrins than in those treated with β -CD. Lower losses were observed for the instrumental and sensory color intensity in juices with HP- β -CD addition. Finally, the antioxidant capacity was also higher in juices treated with HP- β -CD. Finally, the overall sensory quality of juices with HP- β -CD was the best one after 30 d of cold storage. Even though β -CD addition did not cause any improvement compared with control juice (without CD addition), the benefits of adding HP- β -CD to this particular juice were shown in almost all parameters under study.

Keywords: antioxidant activity, *Citrus reticulata*, color, mandarin juice

Practical Application: The present study deals with the practical aspects of the utilization of CDs and CD-complexes in the food industry. The molecular encapsulation of lipophilic food ingredients with CD are supposed to improve the stability of flavors, vitamins, colorants, and antioxidant activity, leading to extended product shelf life. The HP- β -CD treated mandarin juice enriched with pomegranate extract and juice of goji berries will have a more intense color, higher vitamin C content, retinol equivalents, and antioxidant activity during storage and shelf life than control juices, with no CD addition.

Introduction

Oxidative stress has been involved in the development of numerous chronic diseases and in the aging process (Halliwell and Whiteman 2004). Human diet provides different natural antioxidants, which can be considered as a supplement for the natural organism defenses. In this way, the beneficial effects of the consumption of a diet rich in vegetables have been mainly attributed to vitamins, carotenoids, and phenolic compounds (Prior 2003).

Functional products are designed with the objective of supplying a high amount of antioxidants and to reduce the risk of diseases associated with the oxidative stress (Bello 2006). The term "functional food" is related to an ingredient, which is able to improve the health and/or reduce the risk of diseases (Rafter 2002). The

best option is to consume functional foods in a normal diet to obtain the beneficial effects of both the traditional diet and the functional foods (Roberfroid 2002). The product under study here, mandarin juice enriched with pomegranate extract and goji berries juice, will easily fit in this option.

Bioactive compounds, such as vitamin C and polyphenols, have been proved to possess anti-inflammatory, antiatherogenic, antimicrobial, and antioxidant activities (Kris-Etherton and others 2002). These bioactive compounds may or may not remain active in heat-treated products, such as juices, marmalades, jellies, and so on (Zafrilla and others 2001).

Commercial juices are preserved by high temperature pasteurization. The main aim of this heat treatment is to inactivate toxic microorganisms; however, changes in desirable components such as nutrients, color, aroma, and texture, also take place (Lee and Coates 2003), and are often related to quality losses. To solve this problem and to improve the bioavailability of different compounds, natural molecules called cyclodextrins (CDs) are being used (Szejtli 1998). The applications of CDs are numerous in the food industry and the main benefits are: (1) prevention of undesirable aroma production, (2) prevention of color degradation, (3) masking of undesirable flavor compounds.

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β -Cyclodextrin and its derivatives are widely used in pharmacology and food industry, particularly 2-hydroxypropyl- β -CD (HP- β -CD). This compound presents higher water solubility, lower toxicity, and higher hydrophobic cavity than the original product, β -CD (Boudad and others 2001).

The main objective of this study was to evaluate the efficiency of 2 types of CDs (β -CD and HP- β -CD) in encapsulating antioxidant compounds from a functional food, mandarin juice enriched with pomegranate extract and goji berries juice. To reach this objective, vitamin C, retinol equivalents (RE), antioxidant capacity, CIE $L^*a^*b^*$ coordinates and sensory quality were analyzed in samples with and without CD, before and after pasteurization (at 98 °C during 20 s) and during cold storage (up to 75 d at 4 °C).

Materials and Methods

Product description

A product rich in bioactive compounds and with high antioxidant activity was prepared using fresh mandarin juice (96%), goji berries juice (2%), pomegranate extract (1%), and CD (1%). The ingredients selection was based on the high consumers' acceptance of mandarins and goji berries and the high availability of mandarin juice in Spain. Both pomegranate extract and goji berries were added due to their high antioxidant activity, according to the scientific literature (Gil and others 2000; Seeram 2008). The final enriched product must satisfy consumers' requirements and do not decrease the high acceptability of the original product. Our hypothesis was that the addition of pomegranate extract and goji berries juice, at the concentrations used, would not affect consumers' liking but will increase the antioxidant activity and shelf life of the mandarin juice.

Materials

Mandarins used for this study, *Citrus reticulata* cultivar Ortanique, were selected basing on their diameter, pH, total soluble solids content (SSC), titratable acidity (TA), and maturity index (SSC/TA). Goji berries were added as juice, supplied by "Ransom" company, made from concentrated juice, ascorbic acid and potassium sorbate (Ransom & Son Pic, Bradford, U.K.). Pomegranate, in powder extract, was supplied by Probelte Pharma (Murcia, Spain). HP- β -Cyclodextrin and β -CD were from TCI Europe (Zwijndrecht, Belgium). All other chemicals used were of analytical grade and from Panreac S.A. (Barcelona, Spain).

Sample preparation

Total of 3 types of juice were prepared: (1) juice without CD (control juice), (2) juice with β -CD (1%), and (3) juice with HP- β -CD (1%). Freshly squeezed mandarin juice, with the pulp content optimized, was mixed with the rest of ingredients, adding 1% of every type of CD to the corresponding container and shaking the juices for 1 h for a correct and full solubilization of the solid ingredients. All samples were subjected to a high pasteurization step (98 °C for 30 s) and later samples were kept under refrigeration temperature (4 °C) during the storage period (75 d). Samples were analyzed before the thermal treatment (BTI), after the thermal treatment (ATT), and after 7, 30, 46, 60, and 75 d of storage. In each sampling, total soluble solids, titratable acidity, pH, vitamin C, retinol equivalents, antioxidant capacity, and sensory attributes were analyzed. Total of 5 batches of 2 L were prepared for each juice, making a total of 10 L per juice.

Physico-chemical analyses

Total soluble solids content, SSC (°Brix), was determined using a portable refractometer Comecta, S.A., model C3 (Barcelona, Spain). Titratable acidity, TA (% citric acid), was determined in 10 mL of juice by titration to pH 8.2 ± 0.1 with a 0.1 N NaOH solution. The maturity index, MI, was calculated for each juice and expressed as the ratio between the SSC and TA. Five replications were carried out for all physico-chemical analyses.

Vitamin C

Reduced ascorbic acid was measured following the AOAC Official Method 985.33 (Horwitz 2000). Ascorbic acid was estimated by titration with colored oxidation-reduction indicator, 2,6-dichloroindophenol. EDTA was added as chelating agent to remove Fe and Cu interferences. Total of 5 replications were carried out for the vitamin C analysis.

Instrumental measurement of color

Color determinations were made, at 25 ± 1 °C, using a Hunterlab Colorflex[®] (Hunterlab, Reston, Va., U.S.A.). This spectrophotometer uses an illuminant D65 and a 10° observer as references. A sample cup for reflectance measurements was used (5.9 cm internal diameter \times 3.3 cm height) with a path length of light of 10 mm. Blank measurements were made with the cup filled with distilled water against a reference white background (Pérez-López and others 2006). Color data are provided as CIE $L^*a^*b^*$ coordinates. The chroma or color purity (C_{ab}^*) and the color differences (ΔE^*) were calculated using the following formulas: $C_{ab}^* = (a^{*2} + b^{*2})^{1/2}$ and $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$. The color analyses were run in 5 replicates.

Provitamin A content, expressed as retinol equivalents (RE), was mathematically estimated using CIE $L^*a^*b^*$ color coordinates, as described by Meléndez-Martínez (2005):

$$RE = 0.308382 \times L^* + 0.108054 \\ \times a^* + 0.337946 \times b^* + 0.004563$$

Antioxidant activity

The Oxygen Radical Absorbance Capacity (ORAC) assay was carried out on a Synergy HT multidetection microplate reader, from Bio-Tek Instruments Inc. (Winooski, Vt., U.S.A.), using 96-well polystyrene microplates with black sides and clear bottom, purchased from Nalge Nunc Intl. (Hereford, U.K.). Fluorescence was read through the clear bottom, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The ORAC was determined as described by Dávalos and others (2004) with slight modifications (Lucas-Abellán and others 2008). All reaction mixtures were prepared in triplicate, and at least 3 independent assays were performed for each sample.

The results were expressed as relative fluorescence with respect to the initial reading. The area under the fluorescence decay curve (AUC) was calculated by the equation:

$$AUC = 1 + \sum_{i=114}^{i=120} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i . The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to

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the blank. The results of antioxidant capacity were defined as mM of Trolox C per 100 mL of juice.

Sensory evaluation with trained panel

Sensory evaluation by a trained panel was used to study the quality of the juices under study. A panel of 10 panelists, ages 20 to 50 y (8 female and 2 male, all members of the Catholic Univ. San Antonio of Murcia), with sensory evaluation experience, was trained in descriptive evaluation of citrus juices (Sarano-Megias and others 2005). The panel was selected and trained following the ISO standard 8586-1 (AENOR, 1997; Pérez-López and others 2006).

Measurements were performed in individual booths with controlled illumination (750 to 1000 lux) and temperature ($23 \pm 2^\circ\text{C}$).

The individual products were scored for the intensities of color, sweetness, fresh mandarin flavor, off-flavor and overall quality using a scale of 0 to 10, where: 0 = extremely slight or no intensity and 10 = extremely high intensity.

Samples were presented in 50 mL plastic cups with lids. The entire experiment was repeated 3 times (all judges scored 3 juice samples on each) and the sensory scores were presented as the overall means.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and the Tukey's least significant difference multi-comparison test to determine significant differences among treatments. Significance of differences was represented at $P \leq 0.05$. The statistical analyses were conducted using SPSS 14.0 (SPSS Science, Chicago, Ill., U.S.A.) and figures were prepared using Sigma Plot 9.0 (SPSS Science).

Results and Discussion

Physico-chemical analyses

The pH, SSC, TA, and maturity index values of all juices under study were 3.4 ± 0.2 , $12.3 \pm 0.4^\circ\text{Brix}$, $0.98\% \pm 0.03\%$ citric acid, and 12.6 ± 0.2 , respectively. These values remained constant throughout the experiment and there were not significant differences ($P > 0.05$) among juice samples due to CD addition or storage time.

Vitamin C

Changes in vitamin C have been considered as an indicator of the enzymatic and non-enzymatic degradative reactions, which take place during processing and/or storage (Skrede 1996). For

instance, vitamin C maintenance has been used as indicator of shelf life in chilled orange juices. It has been considered that juices with 50% of the initial vitamin C are at the end of their shelf life (Shaw 1992).

Figure 1 shows the degradation of ascorbic acid after the heat treatment and during the cold storage at 4°C . Initially, the ascorbic acid levels were of $255 \pm 7 \text{ mg L}^{-1}$ in all samples. After the pasteurization heat treatment (98°C for 30 s), the levels were of $250 \pm 5 \text{ mg L}^{-1}$ for the control juice and 245 ± 6 and $255 \pm 6 \text{ mg L}^{-1}$ for juices treated with β -CD or HP- β -CD, respectively. Vitamin C losses were basically affected by storage time and only slightly by the pasteurization treatment; the losses due to the storage time were about 33% to 35%. The highest final content of vitamin C was found in juices treated with HP- β -CD, $185 \pm 5 \text{ mg L}^{-1}$. Nuñez-Delgado and others (1997) added HP- β -CD to a high antioxidant activity matrix and concluded that HP- β -CD acted as secondary antioxidant, preserving the antioxidant capacity of the matrix due to the extra-protector effect of HP- β -CD against the ascorbic acid oxidation.

CIE $L^*a^*b^*$ color

The initial color coordinates before the thermal treatment were $L^* = 39.91 \pm 1.31$, $a^* = 28.28 \pm 2.01$, $b^* = 44.16 \pm 1.56$ for all the samples, corresponding to an intensive orange color (Table 1).

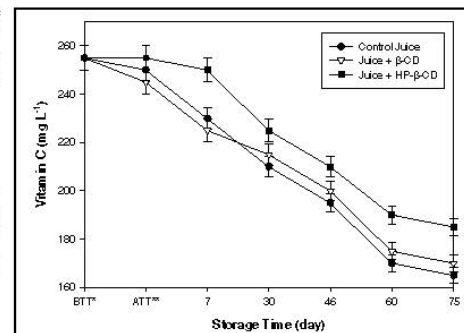


Figure 1—Changes with storage time (75 d) of the vitamin C content (mg L^{-1}) of juices under study. BTT* = before thermal treatment (pasteurization 98°C for 30 s); ATT** = after thermal treatment.

Table 1—Changes with time of color coordinates (L^* , a^* , b^* , and C_{ab}^*) in mandarin juices without or with cyclodextrin addition, before and after thermal treatment, and after 7, 30, 46, 60, and 75 d of cold storage at 4°C .

Storage time (d)	Color coordinates											
	L^*			a^*			b^*			C_{ab}^*		
	Control juice	Juice + β -CD	Juice + HP- β -CD	Control juice	Juice + β -CD	Juice + HP- β -CD	Control juice	Juice + β -CD	Juice + HP- β -CD	Control juice	Juice + β -CD	Juice + HP- β -CD
BTT*	39.9 a A ¹	39.9 a A	39.9 a A	28.3 a A	28.3 a A	28.3 a A	44.2 a A	44.2 a A	44.3 a A	52.4 a A	52.4 a A	52.4 a A
ATT**	39.5 a A	39.7 a A	39.0 a A	26.3 ab AB	25.8 bc B	27.9 ab A	43.2 a A	43.4 a A	44.1 a A	50.6 bc B	50.5 b B	52.2 a A
7	39.5 a A	39.8 a A	38.8 a B	24.9 b B	24.8 c B	26.4 b A	43.0 a AB	40.9 b B	44.1 a A	49.7 bc AB	47.9 cd B	51.4 ab A
30	39.6 a A	40.0 a A	38.7 a B	23.9 b B	24.4 c B	26.4 b A	41.7 b B	39.3 c B	44.2 a A	48.1 c B	46.3 d B	51.4 ab A
46	39.6 a A	40.0 a A	38.4 a B	24.0 b B	24.5 c B	26.3 b A	41.1 b B	39.3 c B	43.6 a A	47.6 c B	46.3 d B	50.9 b A
60	39.6 a A	39.9 a A	38.4 a B	24.0 b B	24.3 c B	26.4 b A	41.0 b B	39.3 c B	43.2 a A	47.5 c B	46.2 d B	50.6 b A
75	39.3 a A	39.9 a A	38.4 a B	24.0 b B	24.1 c B	26.4 b A	41.2 b B	39.3 c B	43.1 a A	47.7 c B	46.2 d B	50.5 b A

Values followed by the same "small" letter, in the same column (effect of thermal treatment and storage time), were not significant different ($P < 0.05$), Tukey's multiple-range test. Values followed by the same "capital" letter, in the same row (effect of CD addition) and within the same color coordinate, were not significant different ($P < 0.05$).

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Lightness (L^*) was constant in all the juices, with only a degradation of 2% to 3% at the end of the storage time (Table 1). Progressive degradations, with thermal treatment and storage time, of a^* (green-red) and b^* (blue-yellow) were found in all the samples. The losses during the thermal treatment were of approximately 2 units for control juices and those treated with β -CD, and of only 1 unit for juices treated with HP- β -CD. However, at the end of the storage period, losses of a^* and b^* reached up to 4 units.

Changes occurred in coordinates a^* and b^* in all the analyzed samples causing also changes in the chroma coordinate (C_{ab}^*), which represents the color purity. The chroma values decreased from 52.4 ± 0.65 (before thermal treatment) to 47.7 ± 0.15 , 46.2 ± 0.58 , and 50.5 ± 0.86 for control samples and juices treated with β -CD and HP- β -CD, respectively (Table 1). The experimental results presented here showed that the color of the juices was mainly affected by the storage time. Some researchers found similar results on fruits and vegetables after thermal treatments and storage (Vikram and others 2005).

After pasteurization, the ΔE^* values were 2.3, 3.3, and 0.97 for control juices and samples treated with β -CD and HP- β -CD, respectively (Figure 2). The biggest changes on ΔE^* were due to the storage time, with values increasing up to 5.36 ± 0.58 , 6.08 ± 0.58 , and 2.66 ± 0.58 , for control juices and samples treated with β -CD and HP- β -CD, respectively (increments of ΔE^* higher than 2 units will cause noticeable visual differences). It can be easily observed in Figure 2 that ΔE^* were lower ($P < 0.001$) in samples treated with HP- β -CD than those from samples treated with β -CD. Others researchers (López-Nicolás and others 2007) obtained similar results in peach juices; these researchers found nonsignificant effects on juice quality after the addition of β -CD but reported positive effects in juices treated with other types of CD, such as maltosyl- β -CD or α -CD. On the other hand, some studies (Lee and Coates 2003) showed significant effects on ΔE^* due to thermal treatment of juices.

Provitamin A content

Initially, the provitamin A content reached a value of 30.29 ± 0.15 ER L^{-1} (Figure 3). After the thermal treatment, ER losses were minimum (about 1% to 3%) in all the samples. The effects of the storage time accounted for decreases of about

5% to 6% in control juices and those treated with β -CD and only of 3.4% in juices treated with HP- β -CD. It can be concluded that both the heat treatment and the storage time negatively affected the total carotenoids and therefore the provitamin A, although reductions of ER were lower than 5%. Meléndez-Martínez and others (2007) obtained similar values for different types of oranges. Finally, Mercader-Ros and others (2010b) reported better protection of bioactive compounds in HP- β -CD than in β -CD, similarly to what is reported in the current study.

Antioxidant activity

Figure 4 shows the total antioxidant activity of the fruit juices and its changes during the storage at 4 °C. The ORAC method has been used to evaluate the antioxidant capacity of many types of fruits and vegetables, such as red fruits (Wada and Ou 2002), tomato (Zapata and others 2007), and orange and apple (Wang and others 1996). The antioxidant capacity of the freshly squeezed juices was 25.9 ± 0.4 mMT per 100 mL (Figure 4). The analysis revealed that losses in the juices after the heat treatment ranged

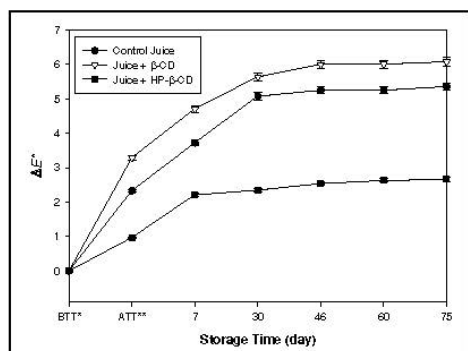


Figure 2—Changes with storage time (75 d) of the total difference color (ΔE^*) of juices under study. BTT* = before thermal treatment (pasteurization 98 °C for 30 s); ATT** = after thermal treatment.

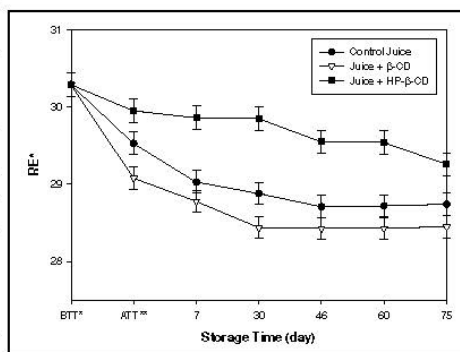


Figure 3—Changes with storage time (75 d) of the retinol equivalents (RE*) of juices under study. BTT* = before thermal treatment (pasteurization 98 °C for 30 s); ATT** = after thermal treatment.

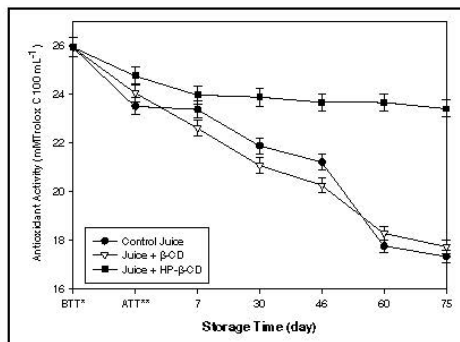


Figure 4—Changes with storage time (75 d) of the antioxidant capacity (mM of Trolox C 100 mL⁻¹ juice) of juices under study. BTT* = before thermal treatment (pasteurization 98 °C for 30 s); ATT** = after thermal treatment.

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Table 2—Sensory analysis of mandarin juices, without or with cyclodextrin addition, before and after thermal treatment, and after 7, 30, 46, 60, and 75 d of cold storage at 4 °C.

Storage time (d)	Sensory properties											
	Color			Fresh mandarin flavor			Off-flavor			Overall quality		
	Control juice	Juice + β -CD	Juice + HP- β -CD	Control juice	Juice + β -CD	Juice + HP- β -CD	Control juice	Juice + β -CD	Juice + HP- β -CD	Control juice	Juice + β -CD	Juice + HP- β -CD
BTT	8.6 a A ¹	8.4 a A	8.8 a A	9.0 a A	8.0 a A	7.9 a A	0.5 c A	0.5 b A	0.7 b A	8.8 a A	8.6 a A	8.8 a A
ATT	8.0 a A	7.8 b A	7.7 b A	8.5 b A	8.1 a A	7.3 a B	0.7 b A	0.5 b A	0.6 b A	8.3 a A	8.1 ab A	8.5 a A
7	8.1 a A	7.7 b A	7.8 b A	8.5 b A	7.7 b AB	7.3 a B	1.0 a A	1.0 a A	1.0 a A	8.3 ab A	7.9 bc A	8.5 a A
30	6.5 b B	6.5 c B	7.8 b A	8.4 b A	7.8 b AB	7.2 a B	1.0 a A	1.1 a A	1.2 a A	7.5 b AB	7.1 c B	7.8 b A
46	6.5 b B	6.4 c B	7.8 b A	8.4 b A	7.5 c B	7.2 a B	1.0 a A	1.0 a A	1.2 a A	7.5 b AB	6.9 c B	7.9 b A
60	6.4 b B	6.3 c B	7.6 b A	8.3 b A	7.5 c B	7.1 b B	1.1 a A	1.1 a A	1.1 a A	7.4 b AB	6.9 c B	7.7 b A
75	6.4 b B	6.3 c B	7.6 b A	8.2 b A	7.4 c B	7.0 b B	1.1 a A	1.1 a A	1.1 a A	7.4 b AB	6.7 c B	7.6 b A

Values followed by the same "small" letter, in the same column (effect of thermal treatment and storage time), were not significant different ($P < 0.05$), Tukey's multiple-range test. Values followed by the same "capital" letter, in the same row (effect of CD addition) and within the same color coordinate, were not significant different ($P < 0.05$).

from 8.6% in control juices to 4.5% in juices treated with HP- β -CD. After the storage period (75 d) the antioxidant capacity ranged from 17.8 ± 0.4 mMT 100 mL^{-1} in control juices and juices treated with β -CD and 23.4 ± 0.5 mMT 100 mL^{-1} in juices treated with HP- β -CD, with total reductions (pasteurization and storage) being approximately 31.5% and 9.75%, respectively. There is a clear difference ($P < 0.001$) between the group of 2 samples (control juices and samples treated with β -CD) and juices treated with HP- β -CD. This could be explained considering that HP- β -CD presented a structure much more favorable for the complexation of antioxidant compounds than β -CD. Therefore a higher effectiveness, encapsulating higher contents of bioactive compounds and the juice being more stable, was observed for HP- β -CD than for β -CD (Mercader-Ros and others 2010a).

Sensory evaluation

There were no significant effects ($P > 0.05$) of the 3 factors under study: (1) the CD type, (2) the heat treatment, and (3) the storage time, on the intensity of sweetness (mean value of 7.5 ± 0.1). A similar situation ($P > 0.05$) was observed for the off-flavors intensity (Table 2); however, during the first 7 d of cold storage the intensity of the off-flavors slightly increased.

Before the pasteurization step, the addition of β -CD or HP- β -CD to the juice samples did not cause any significant variation ($P > 0.05$) on the sensory parameters under study. However, significant changes ($P < 0.01$) in the color intensity were detected after 30 d of cold storage; juices treated with HP- β -CD had the highest intensity of orange color (Table 2). This finding agreed with the instrumental data (a^* , b^* , and C_{ab}^*) previously reported (Table 1). López-Nicolás and others (2009) showed that the higher CD concentration, the better the color of pear juices due to the reduction of browning; however, the addition of β -CD had no significant effects on the sensory color intensity.

The juice with the highest intensity ($P < 0.01$) of fresh mandarin flavor was the control one (with no CD addition), followed by juice with β -CD and, finally juice with HP- β -CD (Table 2). The binding between CD and the aroma compounds depends on both hydrophobicity of the guest molecules and their geometric accommodation into the CD cavity. Some researchers showed that β -CD is the most versatile CD for the stabilization of aroma compounds (Reineccius and others 2006). Finally, the overall quality of juices was evaluated by the trained panel after integration of color, taste and flavor attributes. The quality of the 3 juices under study was statistically ($P > 0.05$) similar until day 30, at which the quality of the juice treated with HP- β -CD was quantified being slightly higher ($P < 0.05$) than that of the control and β -CD-

treated juices; this same trend was kept until the end of the storage period (75 d).

Conclusions

Cyclodextrin addition caused both positive and negative effects on the composition and quality of the juice under study (mandarin juice enriched with pomegranate extract and goji berries juice). Juices treated with β -CD presented higher intensities of typical aroma (fresh mandarin) than those treated with HP- β -CD and especially control juices. However, juices treated with HP- β -CD presented the highest values of color intensity, antioxidant activity, vitamin C content, retinol equivalents, and overall quality, especially at the end of the cold storage period (75 d). The final conclusion is that HP- β -CD was more efficient than β -CD in improving the quality of mandarin juice enriched with pomegranate extract and goji berries juice.

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Detection of chemical residues in tangerine juices by a duplex immunoassay

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ABSTRACT

A rapid duplex ELISA for the simultaneous determination of two of the most widely used organophosphorous insecticides in tangerine juices is described. To accomplish this aim, two individual enzyme-linked immunosorbent assays for chlorpyrifos and fenthion pesticides were integrated into one ELISA test. The strategy uses 96-well plates with specific wells coated with the corresponding haptized conjugate. The optimized duplex ELISA was accomplished within 40 min achieving a detection limit of $0.20 \pm 0.04 \mu\text{g/L}$ and $0.50 \pm 0.06 \mu\text{g/L}$, for chlorpyrifos and fenthion, respectively in tangerine juice samples. The determination of residues of both pesticides was carried out by simple sample dilution, without any extra sample clean-up procedure. Results of testing precision, stability, and selectivity demonstrated that the assay provided reliable analytical performances for the simultaneous determination of residues of chlorpyrifos and fenthion in fruit juice samples below the established European maximum residue limits (MRL). In addition, the accuracy and reliability of this duplex bioanalytical method is demonstrated by analyzing blind spiked juice samples and the results, correlated well with those achieved using a well-established GC/MS method (recoveries between 95% and 106%).

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1. Introduction

Fresh vegetables, fruits and juices are the important part of a healthy diet because of the presence of significant amount of nutrients and minerals in them. However, at the same time, they can also turn out to be a source of toxic chemical substances as pesticides [1]. Among various pesticide classes, organophosphorous pesticide (OPPs) group is the most widely used class of agricultural pesticides [2–4]. In recent years, many studies have proven OPPs to be mutagenic, carcinogenic, cytotoxic, genotoxic, teratogenic and immunotoxic [5–10]. In addition, OPPs have tendency to bind to the enzyme acetyl cholinesterase, and to disrupt nerve functioning which further result in paralysis and death [11–12]. In this sense, accidental exposure of humans and animals to such chemicals may result in a potentially lethal cholinergic poisoning [13].

The increasing public concern about the possible health risk of pesticide residues in the diet has profoundly modified crop production strategies with emphasis on food quality and safety.

Apart from this, the widespread concern for health of society has led to the strict regulation of MRL of pesticide residues in food [14].

Chlorpyrifos (CLP), *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate and fenthion (FTN), *O,O*-dimethyl-*O*-[3-methyl-4-(methylthio)phenyl]phosphorothioate are two of the most used OPPs insecticides to control a variety of soil insects and arthropods, on a wide range of crops including peaches, nectarines, vegetables, cereals, and citrus fruits. Since these are absorbed by the fruits and vegetables and turned out to be noxious when consumed by human beings, both gas and liquid chromatography techniques have been traditionally applied to the detection of organophosphorous residues in fruits and juices [15] coupled to selective detectors [16,17] or mass spectrometer detectors [18,19]. Although chromatographic techniques provide low limits of detection, preliminary sample treatment is the bottle neck, making the analytical procedure laborious, and the number of samples analyzed is few due to economic issues. In this context, there has been increased interest in the development and implementation of rapid and cheaper screening methods, including immunoassays, for detection of pesticide residues. Having in mind that children consume relatively large quantities of fruit juices and that they are more susceptible to chemicals, since they are in the early stage of development, determining pesticide residues in fruit juices has a

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particular importance. In this sense, a rapid, sensitive and selective assay would be of great interest to detect pesticide residues in routine analysis as a screening method. Immunochemical techniques began recently to gain acceptance as a fast and cost-effective tools for separating and/or detecting trace amounts of chemicals such as pesticides [20]. ELISA methods are the most widely used immunoassays due to high sample throughput, although they are limited sometimes by their high specificity. Hence, immunoassay techniques are often used to detect a single analyte. On the other hand, orthogonal hapten–antibody/receptor combinations seem to be ideal for creating array structures with different immunoreagents for different analytes [21,22]. This kind of parallelization reaches its physical and economical limits with the increasing number of analytes that could be measured at the same time. The combination of generic immunoassays for simultaneous detection of CLP and FTN below the MRLs on a single assay could be an excellent tool for pesticide residue screening. Here we report the development and evaluation of a duplex ELISA combining immunoreagents specifically developed to detect two OPPs. The duplex ELISA has been evaluated by testing natural and commercial juice samples contaminated (or not) with both pesticides and the results discussed in comparison with those achieved using a well-established GC/MS method.

2. Material and methods

2.1. Chemicals

Analytical standards of chlorpyrifos, fenthion, chlorpyrifos-methyl, chlorpyrifos-oxon, fenclorophos, bromophos, trichloronate, diazinon, dichlorvos, fenitrothion, fenthion-sulphoxide, malathion and the chlorpyrifos metabolite 3,5,6-trichloro-2-pyridinol (TCP) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of pesticides were prepared in N,N-dimethylformamide (DMF) and kept at $-20\text{ }^{\circ}\text{C}$ until use. Standards were daily prepared in water by serial dilutions from the stock (6.90 mg/L for chlorpyrifos and 3.30 mg/L for fenthion) using borosilicate glass tubes. Peroxidase-labeled goat anti-rabbit immunoglobulins (GAR-HRP), poly(oxyethylensorbitan)monolaurate (Tween 20), O-phenylenediamine (OPD) and the proteins bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma Chemical Co., (St. Louis, MO). Coating buffer (CB) was 50 mM sodium carbonate–bicarbonate buffer pH 9.6 and working buffer was 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, containing 0.05% Tween-20, pH 7.4 (PBS-T). The developing buffer was 25 mM sodium citrate and 62 mM sodium phosphate, pH 5.4. The enzymatic substrate solution was prepared in developing buffer dissolving OPD (2.0 mg/mL) and H_2O_2 (0.012%). All other reagents used were analytical or biochemical grade.

Polyclonal anti-chlorpyrifos (C2-II) and anti-fenthion (F7-I) antibodies and the coating conjugates OVA-C5 and OVA-F1 [23,24] were used as immunoreagents.

2.2. Apparatus

Polystyrene microtiter plates were purchased from Costar (Cambridge, MA, USA). Washing steps were carried out using a 12-channel microplate washer from Nunc (Roskilde, Denmark). Absorbance was read with a Multilabel Counter 1420 microplate reader from Wallac (Turku, Finland) at dual wavelength mode. The mean absorbance value of triplicate wells was processed. Using Sigmaplot software package (Jandel Scientific, Erkrath, Germany), ELISA absorbance values of standards were plotted vs. the logarithm of analyte concentration and fitted to a four-parameter logistic equation [25].

Magnetic stir bars (20 mm length \times 0.5 mm film thickness) coated with poly(dimethylsiloxane) (PDMS), a desorption unit TDS-2 connected to a programmed temperature vaporization (PTV) injector (CIS-4), and a TDSA autosampler were all supplied by Gerstel (Mulheim/Ruhr, Germany). The PTV injector was installed in an Agilent 6890 GC-5973MS system (Agilent Technologies, Palo Alto, CA).

2.3. Duplex ELISA protocol

Flat-bottomed polystyrene ELISA plates were segmented in two sections (CLP and FTN) and coated with 100 μL /well of OVA-C5 at 0.1 mg/L and OVA-F1 at 1.0 mg/L for chlorpyrifos and fenthion analysis, respectively. After 16 h at $4\text{ }^{\circ}\text{C}$, plates were washed six times with 10 mM PBS-T pH 7.4, and then 50 μL of a mixture of pesticide standards or samples in PBS-T and 50 μL of a cocktail serum solution, containing specific polyclonal antibodies against chlorpyrifos and fenthion were added. After 15 min, the plates were washed as before and then 100 μL of GAR-HRP (1/4000 dilution in PBS-T) was added to each well and the solution incubated for 15 min. Next, the solution was washed away as above described and finally, 100 μL /well of substrate solution was added and the enzymatic reaction was stopped after 10 min by addition of 2.5 M H_2SO_4 (100 μL /well). The absorbance was read at 490 nm as test wavelength and 650 nm as reference wavelength.

2.4. Immunoassay optimization

The influence of several critical parameters such as ionic strength, pH, surfactant concentration, BSA and time of competition on assay performances was studied. Criteria used to evaluate the assay performances were sensitivity (IC_{50}), maximum absorbance (A), dynamic range (DR, established between the pesticide concentrations producing 20% and 80% colour inhibition) and limit of detection (LD, estimated at 10% inhibitory concentration).

To evaluate the specificity of polyclonal antibodies, different chemically related and unrelated compounds were tested. Four calibration curves for each compound were obtained in optimized ELISA conditions. The cross-reactivity (CR) values were calculated according to the following equation:

$$\text{CR} (\%) = \frac{(\text{IC}_{50})_{\text{chlorpyrifos or fenthion}}}{(\text{IC}_{50})_{\text{related - compound}}} \times 100 \quad (1)$$

2.5. GC/MS determinations

GC–MS analyses were performed in a 6890 GC gas chromatography coupled to a 5973 MS system (Agilent Technologies, Palo Alto, CA), with electron impact ionization detection. A HP5-MS capillary column (Agilent 190915 433E) with a 0.25 mm i.d., 30 m and 0.25 μm film thickness was used for the determination of organophosphorous pesticides. The pollutants were extracted from 100 mL of mandarin juice samples using magnetic stir bars (20 mm length \times 0.5 mm film thickness) coated with poly(dimethylsiloxane) (PDMS). The bars were previously conditioned in an empty thermal desorption tube at $300\text{ }^{\circ}\text{C}$ for 4 h with a helium flow rate of 50 mL/min. After stirring the sample for 24 h at 900 rpm, the bars were thermally desorbed in the splitless mode at $280\text{ }^{\circ}\text{C}$ for 6 min, transferring the analyte to the PTV injector at a 75 mL/min helium flow rate. Sodium chloride (20% m/v) was used as the matrix modifier. The temperature program was 2 min at $70\text{ }^{\circ}\text{C}$ then, increased to $200\text{ }^{\circ}\text{C}$ at a rate of $30\text{ }^{\circ}\text{C}/\text{min}$, hold for 1 min, and increased to $280\text{ }^{\circ}\text{C}$ at $3\text{ }^{\circ}\text{C}/\text{min}$, with a final isothermal period of 2 min. The PTV injector and interface temperatures were held at $280\text{ }^{\circ}\text{C}$. The detection was made in the full scan mode m/z range 50–400, and for confirmation of the spectral data, the

characteristic ions for chlorpyrifos (m/z 314, 199, 197, 97) and fenthion (m/z 278, 169, 125, 109) were used.

2.6. Juice samples analysis

Fruit juices were purchased from local supermarkets and fresh juices obtained after squeezing raw tangerines. For matrix effects and recovery studies, any sample pre-treatment was used. Spiked juice samples containing chlorpyrifos or fenthion were analyzed by ELISA as described in Section 2.3. In order to avoid matrix effects, juice samples were simply diluted (1/10, v/v) in assay buffer and a set of standard curves were obtained and compared with those carried out in assay buffer. The pH value of diluted samples was checked and corrected if necessary.

The effect of thermal treatment of juices on pesticide concentration was evaluated using the optimized ELISAs. For that, 12 samples of 100 mL of natural or commercial juices were fortified with known amounts of pesticide (10, 40 and 60 $\mu\text{g/L}$) derived from stock solutions and heated (60, 90 and 120 $^{\circ}\text{C}$) for 30, 40 and 60 s, respectively.

The samples were then analyzed by ELISAs and GC-MS. In order to assess assay reproducibility, triplicates of each fortification level were performed.

3. Results and discussion

3.1. Duplex ELISA conditions

Several points should be considered before using a single-target system in multiplexed assays. First, the antisera selectivity and sensitivity, and second, the carrier protein used in the coating conjugates is another variable to be marked. For these reasons, the development of a multianalyte ELISA requires an accurate selection of the suitable hapten structures. In this work, two specific haptens conjugates were immobilized by physisorption in distinct areas of a microplate, and a mixture of different specific polyclonal antibodies for chlorpyrifos and fenthion were used in an indirect ELISA-format, to determine simultaneously both analytes. An illustration of the working strategy is shown in Fig. 1.

In regard to the selectivity, both single-target systems were specific, since each antiserum only recognized its particular coating conjugate. In our case, since immunogens used to raise the antibodies were prepared with BSA, a different carrier protein was employed for coating the conjugate preparation, avoiding non-specific interactions and false positives. This criterion was matched by preparing ovalbumin based coating conjugates. In a second set of experiments, cross-reactivity studies were performed by measuring signal intensity variation to the presence of mixed analytes. In these experiments, single-target assays were carried out and cross-reactivity calculated as percentage of inhibition. In all cases, signal variation was below 0.01%, indicating high selectivity to the mixed analytes. This fact is essential when considering single assay candidates to be applied in multiplexed analysis.

Furthermore, the effect of pH, ionic strength and surfactant concentration and incubation time on assay performances (signal and IC_{50}) was studied at room temperature. First, the effect of pH of the working buffer was studied and observed that IC_{50} values and maximum signal (A) for the duplex ELISA varied 0.49–1.35 $\mu\text{g/L}$ and 0.95–1.80 absorbance units (A.U.), respectively, within a pH range from 4.0 to 9.0, being pH 7.5 the optimum for keeping acceptable signal and good sensitivity for both analytes. Furthermore, the effect of the ionic strength (10–200 mM) on the assay performance was tested, showing that for the duplex assay, IC_{50} and absorbance values decreased gradually as buffer salt concentration increased, as it had been previously observed for other

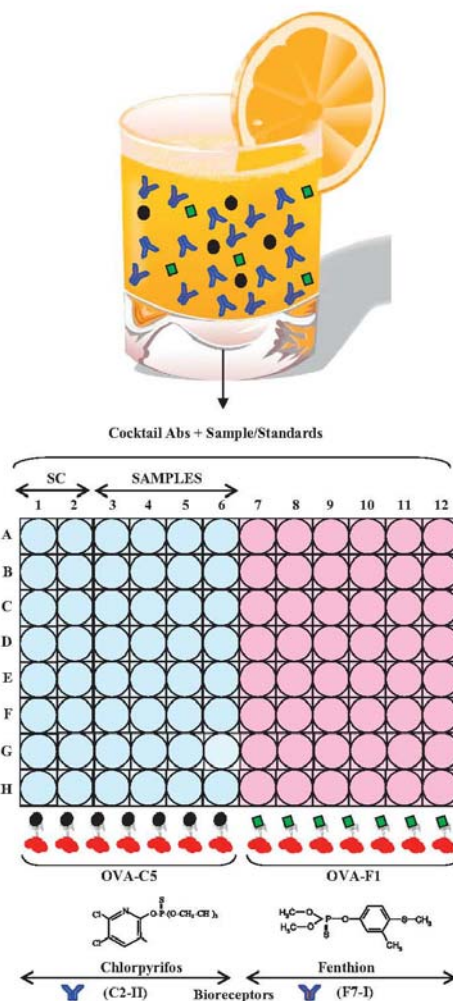


Fig. 1. Duplex enzyme-linked immunosorbent assay (dELISA). Different sections of the microtiter plate are coated with coating antigens of the two pesticides. The immunoreagents/bioreceptors and the analytes are then distributed through the whole plate as a mixture of reagents. Standard curves (SC) were carried out in columns 1,2 and 7,8 for chlorpyrifos and fenthion, respectively. Juice samples (32) were analyzed per duplicate.

organophosphorus immunoassays [23]. In this study, the concentration of PBS selected as a compromise between signal response and IC_{50} was 10 mM. The effect of the addition of detergents in the duplex ELISA sensitivity was also studied. In this study (data not shown), a negative influence (lower signal and lower sensitivity) was observed if the surfactant concentration increased over 0.05%. According to these results, 10 mM PBS, containing 0.05% Tween 20 was the optimum competition buffer for duplex ELISA systems.

Also, the influence of the incubation time on the competition step (15, 30, 45, and 60 min) was investigated. As can be seen in Figs 1–3, good analytical performances were obtained after 15 min competition in terms of sensitivity and signal response, taking 40 min the whole duplex assay.

Taking into account the selected conditions, a robust calibration curve ($n=50$) was constructed. Fig. 2 depicts the calibration graphs of the competitive optimized ELISAs. The errors bars—each point represents the mean \pm standard deviation—at each calibration point, show the assay to assay variability expected from small differences in timing, temperature, or reagent age over the 50 assays. The assay for chlorpyrifos shows an IC_{50} of $3.0 \pm 0.3 \mu\text{g/L}$, a working range between 0.3 and $25.1 \mu\text{g/L}$ and a LOD of $0.2 \mu\text{g/L}$. For fenthion, the analytical sensitivity was $6.6 \pm 0.2 \mu\text{g/L}$, a working range from 0.8 to $58 \mu\text{g/L}$ and a LOD of $0.7 \mu\text{g/L}$.

3.2. Determination of matrix interference

Duplex ELISA is not completely free from interferences caused by unidentified compounds of the food matrix notwithstanding antibody's high selectivity. Thus, it is advisable to determine the importance of these matrix interferences before the application of the assay to real samples. The easiest and most immediate way to minimize and to overcome matrix interferences is sample dilution.

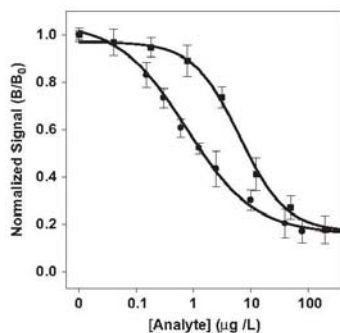


Fig. 2. Calibration curve ($n=50$) for the optimized duplex ELISA. (•) Chlorpyrifos, (◼) Fenthion.

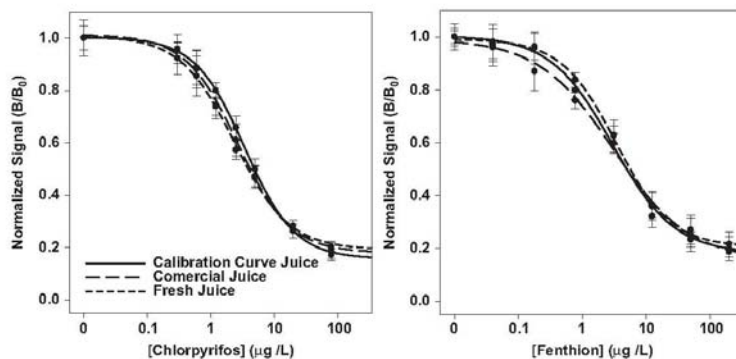


Fig. 3. Influence of matrix interference (Samples were analyzed without previous filtration) on the CLP and FTN assay in duplex ELISA.

As described in Section 2.3, juice samples were fortified and properly diluted in assay buffer (1/10, 1/20 and 1/50), and a set of standard curves were obtained and compared with those carried out in assay buffer. No significant matrix interference was observed only when filtered juice samples were 50-fold diluted and both calibration curves were very similar (data not shown). However, when diluting the sample 10-fold in PBS-T, significant matrix interference was found. Since our purpose was to optimize a rapid OPPs assay—omitting juice filtration step—a model matrix (MMx) with different commercial and natural tangerine juices from Murcia and Valencia was elaborated. As can be seen in Fig. 3, the curve parameter obtained for MMx correlated well with those obtained in both fresh and commercial juices, thus minimizing matrix interferences. As shown in Table 1, the standard curve in matrix show—for both pesticides—small differences for limit of detection and sensitivity (expressed as IC_{50}) values, with those obtained previously in assay buffer (PBS). Thus, to prevent erroneous results in the quantification of OPPs concentration, all juice samples were tested using a standard curve in matrix instead of that obtained with buffer (PBST), showing the potential to analyze tangerine juice samples by duplex ELISA, with a simple sample pre-treatment below the legislated levels.

The data correlated well with those obtained by Watanabe et al. [26], which evaluate matrix interference coming from juice samples in a commercial ELISA for imidacloprid. In apple and grape juice samples, no significant matrix interference was observed only when diluting at 10-fold (20-fold in the assay).

3.3. Cross-reactivity

The selectivity of the optimized ELISAs for CLP and FTN, was evaluated by assessing its response to several analyte-related compounds. As described in Section 2.4, cross-reactivity was defined as the percentage ratio between the IC_{50} value of the target analyte (CLP or FTN) and the IC_{50} value of the cross-reacting compound.

As shown in Table 2, none of the OPPs related compounds gave cross-reactivities for CLP higher than 0.2%, with the exception 2.5% fenclorophos and 12% for chlorpyrifos-methyl. As it had been previously reported in other works, some authors have described ELISAs where chlorpyrifos-methyl is recognized to the same extent (CR 102%) as chlorpyrifos [25,27,28], presumably because the methyl groups were better accommodated by the antibody binding sites. Only the immunoassay described by Lawruk et al. [29] showed a lower CR (26%) for this compound—but higher than that

optimized by us—probably because the hapten used for immunization maintains both ethoxyl groups in the phosphate ester. Also, a very specific assay was obtained for fenthion, since all CR values were lower than 4% -fenthion-, better than that described in the literature for Zhang et al. (406%) [30] or Kim et al. (14%) [31], and in the same order as that reported by Brun et al. [23].

3.4. Analysis of juice samples by dELISA

To overcome a particular disadvantage of immunoassays—often used to detect a single analyte—the developed duplex ELISA was evaluated by analyzing juice samples for simultaneous determination of chlorpyrifos and fenthion in a single way, thus increasing the number of analytes that could be measured at the same time. In this sense, the accuracy of the proposed methodology for the determination of chlorpyrifos and fenthion in juice

samples was studied using blind samples (see Section 2.6). Before addition step, samples were checked by GC–MS for incurred chlorpyrifos or fenthion residues. Each sample was evaluated in four separate assays, by triplicate, on the same day to verify reproducibility and minimize chlorpyrifos and fenthion degradation. Table 3 summarizes the accuracy of the chlorpyrifos and fenthion immunoassay in juice samples. In all cases, the amount of chlorpyrifos and fenthion added was completely recovered, with average percentage of recovery ranging from 85% to 97% for CLP and 96% to 117% for FTN, across the fortification range. For GC–MS the average recovery was in the range 89–104% (CLP) and 90–105% (FTN), very close to the results achieved by proposed duplex ELISA. Also, the accurate recovery of the fortified juice samples suggests that no matrix effects or interferences were present in the samples analyzed, and the proposed method was accurate across the OPPs concentrations tested. Such results are considered very well for residue methods [32], especially considering that the application of any immunoassay in complex matrices such as food and crops, is more difficult than to water samples [33].

Table 1
Values of more representative parameters of standard curves in assay buffer and juice for duplex ELISA for chlorpyrifos and fenthion.

dELISA	LOD	DR	IC ₅₀	Scope	A _{max}	r ²	
Chlorpyrifos	PBST	0.2	0.3–25.1	3.0 ± 0.3	0.98	1.30	0.981
	Juice ^a	0.2	0.3–9.0	2.2 ± 0.3	1.06	0.95	0.998
Fenthion	PBST	0.7	0.8–58	6.6 ± 0.2	1.08	1.86	0.992
	Juice	0.5	0.6–40	3.2 ± 0.2	0.90	1.42	0.997

Values are expressed in µg/L.

^a Juice diluted 1/10 (without previous filtration).

Table 2
Cross-reactivity (%) of duplex ELISA.

Compound	Duplex ELISA	
	Chlorpyrifos	Fenthion
Chlorpyrifos	100	1.0
Fenthion	< 0.2	100
Diazinon	0.2	2.0
Malathion	< 0.2	4.0
Chlorpyrifos-methyl	12	< 0.2
Chlorpyrifos-oxon	< 0.2	< 0.2
Dichlorvos	< 0.2	< 0.2
Fenchlorphos	2.5	< 0.2
Bromophos	< 0.2	< 0.2
Fenitrothion	< 0.2	3.0
Fenthion-sulphoxide	< 0.2	1.0
TCP	0.2	< 0.2
Trichloronate	< 0.2	< 0.2

Table 3
Determination of chlorpyrifos and fenthion residues in blind samples by duplex ELISA and GC–MS techniques.

Concentration (µg/L)	Chlorpyrifos				Fenthion			
	dELISA		GC–MS		dELISA		GC–MS	
	Value ± SD	R (%)	Value ± SD	R (%)	Value ± SD	R (%)	Value ± SD	R (%)
0	< LOD		< LOD		< LOD		< LOD	
10	8.5 ± 1.0	85	8.9 ± 0.5	89	9.7 ± 3.2	96	9.0 ± 1.3	90
40	38.2 ± 1.1	96	43.2 ± 0.9	108	46.8 ± 2.3	117	44.3 ± 0.8	110
60	58.1 ± 0.8	97	62.1 ± 1.1	104	64.5 ± 1.4	107	63.2 ± 1.0	105
Mean recovery		93		100		107		102

Values as mean ± S.D. (n = 12); n.d. non-detected; and R recovery.

3.5. Effect of thermal treatment of juices on pesticide concentration

The citrus juices, mainly orange has been an economically important food product in the world, around 50% people take one a day. However, the application of OPPs has resulted in pesticide residues in fruit and deterioration of juice quality. Several methodologies have been developed for dissipation of OPPs residues in citrus concentrates, however the effect of heat treatment (pasteurization) on the dissipation of pesticides in tangerine juice, to the best of our knowledge, has not been reported. In this sense, 12 samples of 100 mL of natural or commercial juices were fortified with known amounts of CLP and FTN (10, 40 and 60 µg/L) derived from stock solutions and heated (60, 90 and 120 °C) for 30, 40 and 60 s, respectively. The samples were then conditioned by diluting 1/10 (v/v) in assay buffer and checked for chlorpyrifos or fenthion with both duplex ELISA and GC–MS methods. As can be seen in Table 4, the chlorpyrifos and fenthion concentrations determined by duplex ELISA or GC–MS were well correlated. Regarding the effect of heat treatment, we observed that the behaviour of both pesticides was similar, since the concentration slightly declined in the same way under the same conditions. Although some evidences supported the positive effect of increased heat power and treatment time on the degradation of OPPs pesticides [34,35], for each thermal treatment there were no changes in pesticide concentration over time, maybe due to slight time variations (20 s) tested, according to a soft pasteurization process. In this sense, in Table 4 we set the average value—pesticide concentration—for each thermal treatment.

Table 4
Recovery values obtained for chlorpyrifos and fenthion in tangerine juices (with or without heat treatments), by ELISA and GC-MS techniques.

Pesticide	OPP added (µg/L)	Duplex ELISA								GC-MS							
		ST		TT (60 °C)		TT (90 °C)		TT (120 °C)		ST		TT (60 °C)		TT (90 °C)		TT (120 °C)	
		Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)	Mean value	R (%)
Chlorpyrifos	10	12.6 ± 0.5	126	12.2 ± 0.3	122	11.5 ± 0.2	115	11.2 ± 0.3	112	12.5 ± 0.1	115	11.4 ± 0.2	114	10.9 ± 0.1	109	9.6 ± 0.2	96
	40	44.2 ± 0.1	110	44.8 ± 0.3	112	42.3 ± 0.1	106	38.6 ± 0.2	96	45.1 ± 0.2	113	44.1 ± 0.2	110	43.9 ± 0.2	110	40.2 ± 0.1	100
	60	69.6 ± 0.1	116	67.3 ± 0.6	112	63.2 ± 0.2	105	61.5 ± 0.5	102	66.2 ± 0.4	110	66.0 ± 0.6	110	63.6 ± 0.3	106	58.9 ± 0.5	98
	Mean recovery		117		115		109		103		113		111		108		98
Fenthion	10	11.7 ± 0.4	117	11.2 ± 0.2	112	10.8 ± 0.5	108	9.2 ± 0.1	92	11.5 ± 0.2	115	11.6 ± 0.4	116	11.4 ± 0.5	114	9.8 ± 0.1	98
	40	47.5 ± 0.2	119	48.0 ± 0.2	122	47.0 ± 0.4	118	46.6 ± 0.2	117	44.2 ± 0.4	111	44.6 ± 0.2	112	44.0 ± 0.2	110	38.2 ± 0.3	95
	60	67.5 ± 0.3	112	62.2 ± 0.1	107	63.1 ± 0.4	105	58.3 ± 0.4	97	64.6 ± 0.3	108	63.3 ± 0.2	106	63.9 ± 0.3	107	58.4 ± 0.2	97
	Mean recovery		116		114		110		102		112		111		110		97

Values as mean ± S.D. (n = 12); n.d. non-detected; R recovery; ST without thermal treatment; and TT thermal treatment.

4. Conclusions

The performance of the developed duplex ELISA shows the analytical potential for the rapid and quantitative determination of chlorpyrifos and fenthion in tangerine juices. The methodology allows the analysis of a dozen of samples in about 40 min, reducing the total time required in traditional ELISA immunoassay, also providing selective and sensitive responses. The duplex ELISA performs very well in juice samples without any prior sample treatment other than dilution 1/10 (v/v). The analytical performances (accuracy and precision), were comparable to those obtained by the gas chromatographic method for the analysis of juice samples, with mean recoveries for both pesticides close to 100%. In addition, the soft heat treatment (low temperatures and short exposition times) does not suppose a significant influence in the reduction of pesticide concentration. Further work will address the employ of generic immunoreagents, to test in the same plate, different insecticide families, as well as, the miniaturization of the developed and proposed immunochemical multiplexed protocols on a microarray system. Moreover, these results will be the base for developing a powerful multianalyte immunosensor system.

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Arrays on disc for screening and quantification of pollutants



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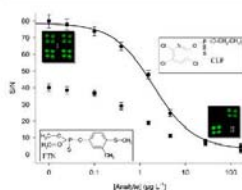
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HIGHLIGHTS

- Disc-arrays were developed for quantitative determination of pollutants.
- Dozens of samples were analyzed in 40 min after simple dilution.
- High-throughput multiplexed screening of organic contaminants at a very competitive value.
- User-friendly and robust methodology with high applicability for monitoring programs, in situ analysis, in low-level labs or outside the lab setting.

GRAPHICAL ABSTRACT



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ABSTRACT

A rapid compact disc based methodology for screening and quantification of organic pollutants in mandarin juices is presented. The assay is established on the coating conjugate indirect competitive principle and developed under disc-array configuration. The detection is based on the acquisition of attenuated reflective signals that were proportional to optical density of the immunoreaction product. The competitive assay is applied to quantify simultaneously, in a selective manner, non-systemic insecticides in mandarin juices. The detection limits were 0.2 and 0.1 $\mu\text{g L}^{-1}$ and the sensitivity 2.1 and 1.5 $\mu\text{g L}^{-1}$, for chlorpyrifos and fenthion, respectively. Pollutants were directly quantified after sample dilution in a total time of 40 min. Also, the implementation of positive and negative controls into the array configuration served as an automatic quality control test. The effect of thermal treatment on pesticide dissipation was studied and found that it was insignificant under the studied conditions. Recovery intervals ranged from 96–105% to 94–103%, for chlorpyrifos and fenthion, respectively and were similar to those obtained with gas chromatography coupled to mass spectrometry. In the current configuration, 64 samples can be simultaneously analyzed on a disc at a very competitive value, demonstrating its potential for high-throughput multiplexed screening applications for controlled monitoring programs in low-level labs or outside the lab setting.

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1. Introduction

According to the National Research Council (NRC) reports, dietary intake represents the major cause of organic pollutants exposure for infants and children [1], and this could produce a serious health risk for this population group. In order to ensure

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that this type of pollutants do not move into food chain, the European Food Safety Authority (EFSA) assesses the dietary intake of them at pre- and post-regulation level. In this context, the European Commission has proposed extensive food control programs that basically define maximum residue limits (MRLs) and highest residue measured (HRM) to establish the lowest consumption threshold [2]. Fruit juices that are manufactured as pasteurized and concentrated beverages are the most frequent source of diary fresh fruit intake [3]. Due to the rigorous legislations regulating the allowable amount of contaminants in fresh and manufactured beverages, the presence, in particular, of pesticide residues in commercial juice is one of the problems faced by the citrus sector since also it becomes an obstacle to free competition within the European Union and sea side trade. Therefore, there is an increasing demand on food manufacturers and farmers to ensure safe food supplies.

The reference methodologies for the determination of pesticide residues employ sophisticated and expensive instrumentation such as gas or liquid chromatography coupled with different detectors [4–6] and requires tedious sample pretreatments, making these methodologies unfeasible for rapid, cost-effective and in situ analysis. For this reason, food industries demand for more portable, fast and cheap technologies to simplify the operational procedures and increase the number of analysis. In this line, immunoanalytical tools are easily adaptable to different formats and analytical scenarios, allowing for simplification of sample preparation and representing cost-effective and simpler methodologies [7–9]. An example of this type of analytical methodology is represented by lateral flow immunochromatographic assays that remain the current preferred option in terms of rapidity for point-of-care screening and in situ analysis [10–14]. In most of the cases, however, this type of technology analyzes one analyte in one strip, giving qualitative visual readouts and resulting expensive for screening applications. The scaling-up of the methodology is also a tough topic and it is not commonly approached due to limited sensing surface. Also, the feasibility of adapting the manufacturing process to a resource-limited setting remains challenging.

In the search for more powerful analytical tools, planar microarray technology go beyond since it can be easily adapted to high throughput tests, show multiplex capabilities and allow quantitative analysis with high potential applicability in life sciences [15–17]. In this line, arrays on compact disks have shown great potential when they are applied in the areas of food safety and environmental analysis [18,19]. Arrays on disc show high working capacity and present the additional advantage of being scanned by disc drives. Besides, standard disc drives are commercially available at very low price, offering robustness and portability. These characteristics make this technology a suitable tool for quantitative analysis for further use in industries and in coordinated control programs. For these reasons, the aim of this study was to develop a rapid and sensitive analytical methodology based on multiplexed arrays on commercial discs (DVD-R) for the simultaneous determination of organic pollutants using a standard DVD drive. As a proof of concept, the methodology has been applied to the simultaneous quantification of chlorpyrifos and fenthion in mandarin juices. These non-systemic insecticides, commonly used to control different pests on foliage in fruit, were chosen as model systems to test the capabilities of the arrays on disc to determine pesticide residues in mandarin juices. The results were validated in terms of sensitivity, selectivity, precision and robustness by gas chromatography coupled to mass spectrometry (GC-MS). The performances shown by arrays on discs might be of great interest in routine testing for food safety and quality control in monitoring programs and for industries with low equipment settings.

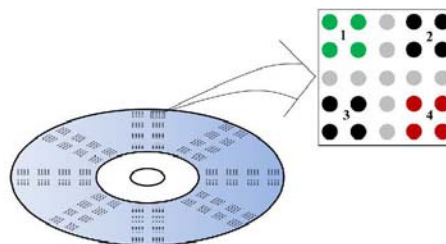


Fig. 1. Layout of arrays on DVD. The disk holds 64 arrays of 5×5 spots, organized in four blocks of 2×2 spots and 9 negative spots (in gray), simulating a virtual cross. Blocks correspond to fenthion (1; spots in green) and chlorpyrifos (4; spots in red) systems, BSA (2) and RlgG (3) as positive controls (spots in black), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Experimental

2.1. Chemicals

Standard chlorpyrifos and fenthion were purchased from Ciba-Geigy (Barcelona, Spain). Bovine serum albumin (BSA), ovalbumin (OVA), gold labeled goat anti-rabbit immunoglobulins (GAR-Au) and silver enhancer solutions (A and B) were from Sigma-Aldrich (Madrid, Spain). Printing buffer was 0.1 M sodium carbonate buffer, pH 9.6, assay buffer was 10 mM sodium phosphate buffer, 0.05% Tween 20, pH 7.5 (PBST) and both were filtered through a 0.22 μm pore size disk before use.

2.2. Microarraying

DVD-R disks were purchased from MPO Ibérica (Madrid, Spain). The discs were first conditioned by gentle ethanol washing, water rinsing, and dried by centrifugation. The coating conjugates OVA-C5 and OVA-F1 [20] used for the determination of chlorpyrifos and fenthion, respectively, were diluted in printing buffer and placed into a 384-well plate to dispense 25 nL of solution onto the disc with a noncontact printing device (AD 1500 BioDot, Inc., Irvine, CA) in microarray format. The disk layout (Fig. 1) consisted in a total of 64 microarrays arranged in blocks, each one of 5×5 spots. Within each microarray, the blocks correspond to chlorpyrifos and fenthion systems, two positive and several negative spot controls. In this configuration, spots are 500 μm in diameter with a track pitch (center to center distance) of 1.0 mm, achieving an array density of 4.0 spot/ mm^2 . Discs were printed on the polycarbonate surface under controlled temperature and relative humidity (25 °C and 90%, respectively).

2.3. Microimmunoassay protocol

The assays on DVD-arrays were based on an indirect competitive format. First, coating conjugate (OVA-C5 for chlorpyrifos and OVA-F1 for fenthion), BSA (25 mg L^{-1}) and nonimmunized rabbit sera (RlgG) (1/1000 dilution) solutions were arrayed onto the polycarbonate surface of the disc. BSA and RlgG were used as positive controls for the first and second immunoreaction steps, respectively, and ovalbumin solution (50 mg L^{-1}) was included as a negative control. After 16 h at 4 °C, the disk was thoroughly washed with PBST, rinsed with deionized water, and dried by centrifugation at 800 rpm. For single-target assay optimization, 12 μL of polyclonal sera [20] (1/1000 dilution for C2-II and 1/500 for F7-I) in PBST, with or without analyte, was dispensed over the array using a

micropipette. After 15 min incubation at room temperature the disk was washed with PBST buffer and then rinsed with deionized water. Next, 1.0 mL of gold-labeled secondary antibody solution (1/50 in PBST) was dispensed onto the disk, and it was evenly distributed along with the whole disc surface with a 12 cm diameter 0.6 mm thick dummy plastic disc. After 15 min at room temperature, the cover surface was removed, and the disk washed and dried as before. The immunoreaction was developed by homogeneously distributing 1.0 mL of silver enhancer solution onto the disk as before, and the reaction was stopped by washing the disk with water after 8 min. For multiplexed assays, 12 μ L of a solution prepared with 5 μ L of PBST or sample with and without analyte, conditioned with 40 μ L of PBST with 5 μ L of the mixed sera solution (cocktail) were dispensed on to disc, covering the whole array. The total volume of 50 μ L was used to analyze samples per triplicate. Next, the immunoassay was performed as described above. The total assay took 40 min. Juice samples were spiked with chlorpyrifos and fenthion within the working range and diluted 1/10 in PBST before analysis. Also, mandarin juice without spiking was used as blank sample.

To evaluate the selectivity of the duplex assay, different chemically related and unrelated compounds were tested. Three calibration curves for each compound were obtained in the optimized working conditions. The cross-reactivity was calculated by comparing the assays response at the midpoint (IC_{50}) and expressed as a percentage.

For quantitative determination, discs were read by the prototype as previously described [21]. Basically, the DVD drive (LG Electronics Inc., Englewood Cliffs, NJ), was controlled by custom software (Diskpick), running on a personal computer and connected to it through a USB2.0 universal serial bus interface. During the DVD scanning, the laser hits the immunoreaction products that modify the reflection properties of the DVD surface, attenuating the laser beam intensity that reaches the photodiode of the pickup. The analog signals were directly acquired from the photodiode of the DVD drive and related to optical density of the immunoreaction product. For the indirect competitive based immunoassay, the optical density is inversely proportional to analyte concentration. Inhibition curves were mathematically analyzed by fitting experimental results to a sigmoidal four-parameter logistic equation.

2.4. Instrumentation and chromatographic conditions

GC-MS analyses were performed in a 6890 GC gas chromatography coupled to a 5973 MS system (Agilent Technologies, Palo Alto, CA) with electron impact ionization detection. A HP5-MS capillary column (Agilent 19091S 433E) with a 0.25 mm i.d., 30 m and 0.25 μ m phase film diameter was used for the determination of organophosphorous pesticides. The pollutants were extracted from 100 mL of mandarin juice samples using magnetic stir bars (20 mm length \times 0.5 mm film thickness) coated with poly(dimethylsiloxane) (PDMS). The bars were previously conditioned in an empty thermal desorption tube at 300 $^{\circ}$ C for 4 h with a helium flow rate of 50 mL min^{-1} . After stirring the sample for 24 h at 900 rpm, the bars were thermally desorbed in the splitless mode at 280 $^{\circ}$ C for 6 min, transferring the analyte to the PTV injector at a 75 mL min^{-1} helium flow rate. Sodium chloride (20%, w/v) was used as the matrix modifier. The temperature program was 2 min at 70 $^{\circ}$ C then, increased to 200 $^{\circ}$ C at a rate of 30 $^{\circ}$ C min^{-1} , hold for 1 min, and increased to 280 $^{\circ}$ C at 3 $^{\circ}$ C min^{-1} , with a final isothermal period of 2 min. The PTV injector and interface temperatures were held at 280 $^{\circ}$ C. The detection was made in the full scan mode- m/z range 50–400 and for confirmation of the spectral data, the characteristic ions for chlorpyrifos (m/z

314, 199, 197, 97) and fenthion (m/z 278, 169, 125, 109) were used.

3. Results and discussion

3.1. Single-target assays

The majority of reported bioanalytical based screening methods for pollutants analyze one analyte at one time. In our study, we design arrays on discs with the goal of using it as high-throughput analysis system and for simultaneous determination of pollutants residues. For that, the disc included 64 arrays arranged in four blocks of 2×2 spots. Two blocks were specifically used for CLP and FTN quantification, whereas the rest of the blocks served as automatic quality controls (see Fig. 1).

In order to demonstrate the multianalyte capability, previously developed immunoreagents were used to set up the methodology on DVDs. For that, the selected [20] coating conjugate/sera pairs were used for developing the arrays on DVDs. As far as optimization is concerned, a wide interval of coating conjugate concentration (ranging from 0.10 to 50 mg L^{-1}) was tested against serial dilutions of polyclonal sera (1/500–1/50,000 dilution) C2-II and F7-I for CLP and FTN assays, respectively. Optimal coating conjugate concentrations and sera dilutions were selected on the basis of obtaining good signal intensity ($S/N > 35$) and reproducibility ($< 15\%$) by check-board titration in a competitive format. The optimum concentration was found to be 25 mg L^{-1} for OVA-C5 and 40 mg L^{-1} for OVA-F1 and 1/1000 and 1/500 sera dilution for C2-II and F7-I, respectively. Regarding detection antibody, the optimal concentration was obtained diluting the gold labeled secondary antibody (GAR-Au) 1/50 fold in PBST.

Selectivity studies were performed under the optimal immunoreagent concentrations and found that single-target systems were selective, since each serum only recognized its specific coating conjugate. Signal-to-noise ratio (S/N) values were 76.4 ± 4.8 and 37.2 ± 2.9 for single-target CLP and FTN assays, respectively. In contrast, nonspecific recognition was negligible showing S/N values below the limit of quantification ($S/N < 5$). These results are at the same level of selectivity as those reported by the respective ELISA plate methods. In a second set of experiments, cross-reactivity studies were performed by measuring signal intensity variation to the presence of high analyte concentrations ($> 10 \text{ mg L}^{-1}$). In these experiments, single-target assays were carried out and cross-reactivity calculated as percentage of inhibition. In all cases, signal variation was below 0.01%, indicating high selectivity to analyte solutions. This fact is essential when considering single assay candidates to be applied in multiplexed analysis. Furthermore, the selectivity of the assay on disc was evaluated using a set of organophosphorus insecticides and metabolites because of their similar structure to chlorpyrifos and fenthion and several non-chemically related pesticides due to their widespread agricultural and domestic use. As shown in Table 1, none of the tested pesticides and metabolites did interfere with the detection of chlorpyrifos and fenthion. Concretely, chlorpyrifos methyl (10%) and fenclorphos (3.5%) were the only related compounds that may interfere with CLP assay and malathion (5.3%), fenitrothion (3.8%), diazinon (2.2%) and fenthion-sulfoxide (1.6%) with FTN quantification. These results indicate that the methodology based on arrays on disc is highly selective for the simultaneous detection of chlorpyrifos and fenthion. It is also interesting to note that the use of specific antibodies against other pesticides or metabolites or employing generic antibodies could spread the spectrum of targets.

In the disc-array configuration, negative controls served for checking for non-specific signals, whereas positive controls

Table 1
Cross-reactivity (%) of the arrays on disc.

Compound	Array	
	Chlorpyrifos	Fenthion
Chlorpyrifos	100	1.0
Chlorpyrifos-methyl	10	<0.1
Fenclorophos	3.5	<0.1
Chlorpyrifos-oxon	<0.1	<0.1
Fenthion	<0.1	100
Malathion	<0.1	5.3
Fenitrothion	<0.1	3.8
Diazinon	0.2	2.2
Fenthion-sulfoxide	<0.1	1.6
Dichlorvos	<0.1	<0.1
Glyphosate	<0.1	<0.1
Atrazine	<0.1	<0.1
Bromophos	<0.1	<0.1
Trichlorpyr	0.2	<0.1
Trichloronate	<0.1	<0.1

were included to normalize assay intra and inter assay signals. For instance, disk area variations that might cause a slight lower/higher-than-normal reading at the controls would have the same effect on the pesticides array block. The signal intensity for the positive controls ($S/N = 85–88$) was constant to different analyte concentrations. Also, inter and intra disc reproducibility, measured as relative standard deviation (RSD) was below 9% and 7%, respectively, demonstrating the applicability of positive controls for signal normalization.

The sample volume was also another parameter studied. For these experiments, four volumes (6, 12, 18, and 20 μL) were compared in terms of signal intensity and reproducibility. For the blanks (absence of analyte), signals were significantly similar for all the tested volumes, varying from 10,870 to 11,950 (arbitrary units), for 6 and 20 μL , respectively. The test used to determine significant differences was a single factor ANOVA analysis, obtaining a level of significance (R) of 0.06. In terms of signal reproducibility, the best results were obtained with 12, 18, and 20 μL solutions (RSD below 12%). The larger volumes generate a drop that with time spread out occupying circular areas very close each other. For these reasons, 12 μL sample solution was selected for further analysis, covering the whole array surface and preventing cross-contamination between flanking samples.

3.2. Determination of matrix interference

Food matrices can modify the response of the arrays on disc, eventually impeding an adequate quantification of target analytes. This type of interference is basically dependent on the commodity under consideration. In the case of fruit juices, matrix interference on competitive immunoassays can be overcome by simple dilution prior to analysis. Due to the assay sensitivity achieved in PBST (IC_{50} 1.9 and 1.6 $\mu\text{g L}^{-1}$ for CLP and FTN, respectively), a dilution step did not compromise detectability level required for screening small organic pollutants such as pesticides in monitoring programs. To study the matrix effect on sensitivity, commercial mandarin juices were diluted 5, 10, 20 and 50 (v/v) fold in PBST. This study was performed using CLP as a model analyte. The diluted samples were used to prepare CLP standard curves, from 0.1 to 1000 $\mu\text{g L}^{-1}$, in order to determine the extent of matrix interference. As it can be seen in Fig. 2, the IC_{50} of the majority of the assays ranged from 1.9 to 2.1 $\mu\text{g L}^{-1}$ (curves A, B, C and D). In contrary, IC_{50} of the 5 fold diluted curve varied 50% with respect to that obtained in PBST. From this study, it was found that any significant negative effects in terms of sensitivity and reproducibility (t -student) could be observed after a simple 10-fold dilution (curve D) in comparison to that obtained in working buffer (curve A). This is very important

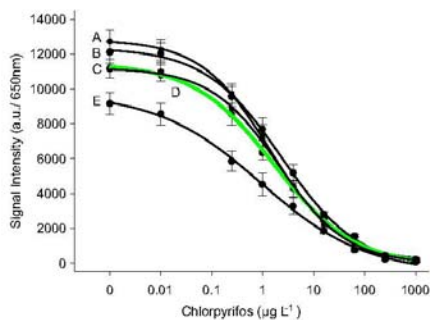


Fig. 2. Juice matrix interference on chlorpyrifos competitive assay. Standard curves are made in PBST (A), mandarin juice diluted 50 (B), 20 (C), 10 (D; curve in green) and 5 (E) fold in PBST. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

when transferring the method to industry that demands easy and practical sample treatment procedures.

3.3. Multiplexed competitive assays

Simultaneous determination of CLP and FTN was performed by an all-in-one reaction assay. For these experiments, specific single-target assays were integrated into one by preparing a unique mixed-sera solution. For that, the cocktail solution had the same sera concentration as that used in single-target assays. Signal-to-noise ratios were 78.6 ± 5.2 and 39.4 ± 3.3 for CLP and FTN, respectively, being these values similar to those for the corresponding single-target assays (see page 8). In particular, S/N variation ranged from 2.8% to 5.5%, indicating an acceptable difference between single and multiplexed assays.

The calibration curves for duplex assay in 10 fold diluted mandarin juice are shown in Fig. 3.

The plots displayed the mean of 24 curves (3 discs) performed on different days, averaging a total of 96 spots per concentration of analyte. The intradisk RSD for all the analyte concentrations

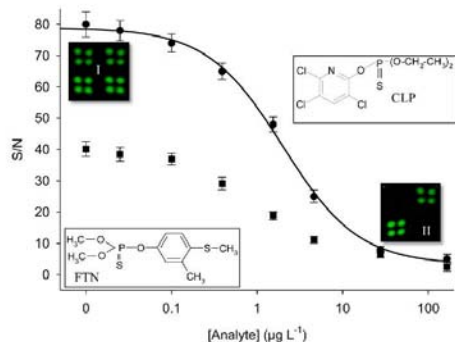


Fig. 3. Calibration curves of competitive multiplexed assays on discs for chlorpyrifos (CLP, ●) and fenthion (FTN, ■) in 10-fold diluted mandarin juice. Panels correspond to the images obtained in absence (panel I) and presence of 150 $\mu\text{g L}^{-1}$ of analytes (panel II). The insets show the chemical structures of chlorpyrifos (CLP) and fenthion (FTN).

Table 2
Determination of chemical residues in mandarin juice samples by duplex disc-arrays and GC-MS technique.

Disc-array								
Sample	Analyte found ($\mu\text{g L}^{-1}$)							
	NHT		HT60		HT90		HT120	
	CLP	FTN	CLP	FTN	CLP	FTN	CLP	FTN
S0	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
S1	22.0 ± 0.1	<LD	22.4 ± 0.3	<LD	21.2 ± 0.2	<LD	19.2 ± 0.2	<LD
S2	<LD	10.7 ± 0.4	<LD	11.2 ± 0.2	<LD	10.8 ± 0.1	<LD	9.2 ± 0.1
S3	21.5 ± 0.2	9.8 ± 0.2	22.0 ± 0.1	9.7 ± 0.1	20.1 ± 0.1	9.4 ± 0.3	18.9 ± 0.3	9.1 ± 0.2
S4	11.4 ± 0.1	20.8 ± 0.2	10.9 ± 0.1	20.2 ± 0.2	10.1 ± 0.1	19.2 ± 0.2	10.1 ± 0.1	19.1 ± 0.2
S5	<LD	40.6 ± 0.4	<LD	41.1 ± 0.3	<LD	40.2 ± 0.3	<LD	39.1 ± 0.2
S6	41.4 ± 0.3	10.5 ± 0.1	40.9 ± 0.3	10.2 ± 0.1	40.2 ± 0.3	9.2 ± 0.1	38.6 ± 0.3	9.0 ± 0.1
S7	10.3 ± 0.1	40.9 ± 0.3	10.5 ± 0.1	40.4 ± 0.3	9.1 ± 0.1	39.6 ± 0.4	9.3 ± 0.1	39.4 ± 0.4
S8	39.4 ± 0.4	<LD	39.9 ± 0.3	<LD	38.2 ± 0.4	<LD	38.9 ± 0.4	<LD
S9	20.2 ± 0.2	20.8 ± 0.2	20.4 ± 0.2	19.5 ± 0.2	19.8 ± 0.2	19.6 ± 0.1	18.7 ± 0.2	18.4 ± 0.2
MR (%)	105	103	106	102	99	98	96	94
GC-MS								
S0	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
S1	19.9 ± 0.1	<LD	20.2 ± 0.1	<LD	19.9 ± 0.2	<LD	18.1 ± 0.2	<LD
S2	<LD	10.3 ± 0.4	<LD	10.3 ± 0.2	<LD	10.1 ± 0.1	<LD	9.8 ± 0.1
S3	20.2 ± 0.2	9.9 ± 0.1	19.0 ± 0.1	9.8 ± 0.1	20.0 ± 0.1	9.8 ± 0.1	19.9 ± 0.2	9.7 ± 0.2
S4	10.2 ± 0.1	20.1 ± 0.2	10.1 ± 0.1	20.0 ± 0.1	9.8 ± 0.1	19.8 ± 0.2	9.9 ± 0.1	19.6 ± 0.2
S5	<LD	40.1 ± 0.2	<LD	39.8 ± 0.2	<LD	39.9 ± 0.3	<LD	39.8 ± 0.2
S6	40.4 ± 0.3	10.2 ± 0.1	40.7 ± 0.3	9.2 ± 0.1	40.8 ± 0.3	9.8 ± 0.1	39.6 ± 0.3	9.8 ± 0.1
S7	10.1 ± 0.1	40.1 ± 0.2	10.4 ± 0.1	40.2 ± 0.3	10.1 ± 0.1	39.9 ± 0.3	9.9 ± 0.1	40.4 ± 0.3
S8	39.8 ± 0.3	<LD	39.2 ± 0.3	<LD	39.4 ± 0.2	<LD	39.7 ± 0.3	<LD
S9	19.8 ± 0.2	20.1 ± 0.2	20.6 ± 0.2	19.8 ± 0.2	19.1 ± 0.2	19.9 ± 0.1	19.7 ± 0.2	18.8 ± 0.2
MR (%)	101	101	101	99	99	100	99	98

CLP, chlorpyrifos; FTN, fenthion. MR, mean recovery; LD, limit of detection; NHT, samples were not heat-treated; HT, heat treated, the figures appended correspond to the temperature; S0, non-spiked sample; S1, 20 $\mu\text{g L}^{-1}$ CLP; S2, 10.0 $\mu\text{g L}^{-1}$ FTN; S3, 20 $\mu\text{g L}^{-1}$ CLP and 10.0 $\mu\text{g L}^{-1}$ FTN; S4, 10 $\mu\text{g L}^{-1}$ CLP and 20.0 $\mu\text{g L}^{-1}$ FTN; S5, 40 $\mu\text{g L}^{-1}$ FTN; S6, 40 $\mu\text{g L}^{-1}$ CLP and 10.0 $\mu\text{g L}^{-1}$ FTN; S7, 10 $\mu\text{g L}^{-1}$ CLP and 40.0 $\mu\text{g L}^{-1}$ FTN; S8, 40 $\mu\text{g L}^{-1}$ CLP; S9, 20 $\mu\text{g L}^{-1}$ CLP and 20.0 $\mu\text{g L}^{-1}$ FTN.

ranged from 6.3% to 8.6%, while the mean interdisk RSD varied from 9.2% to 12.3%. As analyte concentration increased, the signal-acquired intensity varied from the highest (absence of analyte) to the background signal. The resulting silver deposit from the highest analyte concentration was minimal ($S/N < 3$), indicating a small degree of nonspecific response. The analytical performances measured as limit of detection, sensitivity (IC_{50}) and working interval were 0.2 and 0.1 $\mu\text{g L}^{-1}$, 2.1 and 1.5 $\mu\text{g L}^{-1}$, and 0.4–8.2 and 0.2–6.3 $\mu\text{g L}^{-1}$, for CLP and FTN, respectively. No significant differences were detected for any of them in terms of sensitivity between the assay performed individually or duplexed and with that obtained by ELISA plate, using the same immunoreagents. In both cases, the high sensitivity reached for chlorpyrifos and fenthion allows for the quantitative analysis below the maximum EU residue limit for mandarin juice (2 mg L^{-1} for chlorpyrifos and 3 mg L^{-1} for fenthion).

3.4. Mandarin juice sample analysis

Commercial bottled juice samples were purchased from local markets of Valencia. Also, raw mandarins were bought and squeezed in the lab to obtain fresh juice samples. All samples were stored at -80°C till the analysis.

Several methodologies have been tested for dissipation of pesticide residues in citrus concentrates [22]; however the effect of heat treatment on pesticide dissipation in mandarin juice, to the best of our knowledge, has not been reported. For this study, ten samples of 100 mL of natural and commercial juices were spiked at different levels within the working interval with known amounts of CLP and FTN (10, 20 and 40 $\mu\text{g L}^{-1}$). Before spiking, samples were analyzed by GC-MS for incurred chlorpyrifos or fenthion residues and confirmed that were free of pesticide contamination. To study the effect of thermal treatment on pesticide dissipation, samples

were heated at 60, 90 and 120 $^\circ\text{C}$ for 60 s. Then, the samples were left for 20 min to reach room temperature. Before analysis, samples were conditioned by adding the appropriate volume of sera cocktail and working buffer solution and then, the duplex assay was performed as described. Each sample was also analyzed by triplicate on the same day and on the same disc to verify reproducibility and minimize pesticide degradation. Finally, the disk was read by the DVD drive, obtaining quantitative results. As is shown in Table 2, recovery intervals were 96–105% and 94–103%, for chlorpyrifos and fenthion respectively. These results are in good agreement with those obtained by GC-MS. Accordingly, the developed duplex arrays on disc provided results that were statistically comparable to those obtained by GC-MS for the analysis of mandarin juices at trace levels by simple dilution without filtration.

Regarding the effect of heat treatment, it might be concluded that the behavior of both pesticides is similar. Dissipation of pesticide residues from juice matrix under the studied heat treatments is insignificant. These results are very important since the proposed methodology can be a suitable methodology to analyze agrochemical residues on both unprocessed and processed mandarin juice samples.

4. Conclusions

To the best of our knowledge, this is the first example of using arrays on disc in combination with a standard DVD drive for quantification, in a selective manner, pesticide residues in juice samples at $\mu\text{g L}^{-1}$. Results of optimization experiments demonstrated that a simple sample dilution eliminates matrix interferences, without compromising the detectability level required for the determination of agrochemical residues below the established maximum residue levels. Compact disc-based methodology is user-friendly (no chemical solvents are required), affordable and robust, with

high applicability to in situ analysis and monitoring programs. The capability for analyzing large number of samples; in this disc configuration 64 juices can be simultaneously tested; provides results in a quick way (40 min) at a competitive value. Also, the low volume of sample required in combination with the good assay sensitivity and selectivity make this methodology very promising for other applications where sample is limited such as in diagnostics and clinical chemistry. In summary, arrays on disc as platform and DVD drives as chemical detector is a powerful and cost-effective analytical tool with high potential for screening applications. Also, it might be of great interest in routine testing for food safety and quality control, ensuring similar results in terms of sensitivity, selectivity and reproducibility to reference methodologies.

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**CAPÍTULO IV. RESUMEN GLOBAL DE
LOS RESULTADOS OBTENIDOS**

En este trabajo se han abordado principalmente dos aspectos, por un lado, el estudio de los parámetros de calidad del zumo, con el fin de introducir en el mercado productos saludables y diferenciados, y por otro, la seguridad e inocuidad del zumo, para garantizar que los elaborados estén libres de sustancias contaminantes, en consonancia con las demandas del consumidor final.

1. ESTUDIO DE LA CALIDAD DEL ZUMO DE MANDARINA

Los cítricos han sido reconocidos por sus efectos beneficiosos para la salud, en numerosos estudios epidemiológicos y de intervención clínica. Estas propiedades beneficiosas se deben a diferentes nutrientes o no nutrientes -sustancias bioactivas-, como los carotenoides y el ácido ascórbico que presentan en su composición.

La mandarina es uno de los frutos con mayor contenido en carotenoides, fundamentalmente, β -criptoxantina y β -caroteno. El tratamiento térmico al que son sometidos los zumos comerciales puede provocar pérdidas importantes en este tipo de compuestos. Por ello, la industria alimentaria estudia diferentes alternativas que puedan solventar este problema, como la ultracongelación del zumo, que elude la agresividad del tratamiento térmico o bien, la protección de estos compuestos mediante el empleo de agentes encapsulantes como las ciclodextrinas (CDs).

En general, las frutas y hortalizas son muy ricas en compuestos saludables, entre ellas, la granada y las bayas de Goji están siendo objeto de estudio por el elevado contenido en sustancias anticarcinógenas, antioxidantes, antiinflamatorias, antiaterogénicas y antimicrobianas que contienen. Por esta razón, enriquecer los zumos mediante extractos de estas frutas podría ser una buena estrategia para la obtención de alimentos diferenciados, ricos en compuestos antioxidantes (vitamina C, carotenoides, compuestos fenólicos) y capaces de aportar beneficios a la salud del consumidor.

Artículos I y II

En primer lugar, se evaluó la calidad de los zumos de mandarina -en términos de nutrientes y no nutrientes-, que favorecen el mantenimiento de la salud, al igual que otras propiedades de aceptabilidad -atributos sensoriales-, obtenidos con dos variedades distintas (clemenpons y orogrande), procedentes de cultivo tradicional y ecológico. Este trabajo previo, permitió establecer una relación directa entre la calidad del zumo de mandarina y las técnicas de cultivo, recomendando el ecológico. Se determinó el contenido en minerales, carotenoides, color y diferentes parámetros sensoriales en zumos elaborados con mandarinas procedentes de cultivo tradicional y ecológico. Respecto a los minerales analizados, sólo se observaron diferencias significativas en dos macronutrientes, calcio y potasio y el hierro en cuanto a micronutrientes. En general, el zumo procedente de cultivo ecológico mostró un contenido de carotenoides totales del 45%, aproximadamente, el doble del encontrado en el zumo convencional.

Con respecto a los atributos sensoriales, la agricultura ecológica tuvo también efectos positivos sobre diferentes parámetros. Así, el color se mantiene estable en zumos de mandarina ecológico, ya que se observó un aumento significativo de la coordenada a^* (color rojo), b^* (color amarillo) y Cab^* y una disminución de la coordenada L^* (luminosidad). En especial las mayores diferencias (superiores a 10 unidades respecto al cultivo tradicional) se observaron en zumos obtenidos con la variedad orogrande. Se puede afirmar, en base a estos resultados, que en zumos obtenidos con mandarinas cultivadas bajo sistema ecológico se duplica el contenido en carotenoides, con respecto a las mismas variedades procedentes de cultivo tradicional, estando íntimamente relacionado este parámetro con el color final del zumo.

Estos resultados, corroboraron los obtenidos por jueces entrenados mediante ensayos hedónicos, al evaluar otros atributos sensoriales en el zumo de mandarina ecológico y tradicional, valorando mejor los ecológicos por su mayor intensidad de color, aroma a mandarina fresca y dulzor.

El siguiente paso de la investigación fue evaluar el contenido en vitamina C y la capacidad antioxidante de estos zumos. En este apartado, el estudio se centró en una sola variedad de clementina (orogrande), por ser muy productiva y originaria de la región de Murcia.

En consonancia con los resultados obtenidos en el estudio previo, se observó que las muestras de zumo ecológico contenían un 15% más de vitamina C que los zumos de cultivo tradicional. Además cuando se evaluó la capacidad antioxidante por el método ORAC-FL, se obtuvieron valores más altos en los zumos procedentes de agricultura ecológica. La capacidad antioxidante que presentaron las muestras de zumo ecológico fue aproximadamente el doble (43,4%), que la encontrada en el convencional, hecho que se podría relacionar directamente con el contenido en carotenoides.

En resumen, se puede afirmar que los tratamientos seguidos en cultivos ecológicos favorecen la protección de ciertos compuestos bioactivos –entre ellos carotenoides y ácido ascórbico-, sensibles a los plaguicidas, mejorando no sólo el color, sabor o aroma de los zumos, sino también su capacidad antioxidante.

Artículos III y IV

Avanzando un poco más en los objetivos propuestos en esta Tesis, se estudio el efecto de la técnica de conservación en diferentes parámetros (contenido en vitamina C, carotenoides, capacidad antioxidante, color y calidad sensorial). Para ello, se seleccionó la pasteurización (98 °C, 20 s) como tratamiento térmico y la ultracongelación (-80 °C) como tratamiento alternativo. Los zumos fueron conservados convenientemente (temperatura ambiente para los zumos pasteurizados y -80 °C para los zumos ultracongelados), analizando los parámetros seleccionados a lo largo de su vida útil (145 días). Además, como vía para proteger los compuestos bioactivos, se adicionaron –o no- a los zumos distintos tipos de CDs, evaluando su eficacia.

Al final del periodo de almacenamiento, las diferencias entre las muestras sometidas a los tratamientos de conservación indicados, fueron notables. Las pérdidas de vitamina C en los zumos tratados por ultracongelación fueron del 8%, mientras que en los sometidos a tratamiento térmico el descenso fue bastante acusado (30%).

En zumos ultracongelados, se observaron mermas no significativas respecto a su concentración inicial (entre el 5-10%) en el total de carotenoides. Por el contrario, la pasteurización y almacenamiento a temperatura ambiente provocó pérdidas cercanas al 16%, llegando incluso al 30% tras 145 días de almacenamiento en el caso de la β -criptoxantina.

En consonancia con estos datos, las muestras ultracongeladas no sufrieron pérdidas significativas de color, manteniéndose estables durante todo el periodo de almacenamiento. Las muestras analizadas al final del estudio, revelaron diferencias de color de tan sólo 0,70 unidades respecto a las muestras analizadas antes del tratamiento. En contraposición, las muestras sometidas a pasteurización y almacenadas a temperatura ambiente, sí sufrieron variaciones apreciables de color, la mayor disminución se produjo en el parámetro a^* (rojo) con pérdidas de hasta 5 unidades respecto a las muestras sin tratamiento térmico, justificando este hecho por la degradación de carotenoides.

La ultracongelación de los zumos, mantuvo la capacidad antioxidante, con mermas -al final del estudio-, que no superaron el 18%, inferiores a las encontradas en zumos pasteurizados (40%).

Respecto al enriquecimiento de los zumos con β -ciclodextrinas como vía para proteger los compuestos bioactivos, no se observaron diferencias significativas respecto a las muestras control. Este resultado podría justificarse por la inespecificidad de las mismas frente a diferentes compuestos presentes en una matriz compleja como el zumo y, por otra parte, a la baja solubilidad de las β -CDs en medios acuosos, lo que podría dificultar su efectividad en medios hidrosolubles como los zumos, observando precipitados en el fondo del envase.

Los ensayos realizados indicaron que las técnicas de ultracongelación no sólo evitan el desarrollo de microorganismos, la actividad enzimática o las pérdidas de nutrientes, sino que también mantienen las características sensoriales y organolépticas de los zumos estudiados. Por tanto, se recomienda la ultracongelación en la industria de zumos, ya que garantiza la inocuidad y no provoca mermas significativas en nutrientes y propiedades organolépticas de los mismos.

Artículo V

Para concluir con los objetivos de esta primera parte de la Tesis, se elaboró una bebida a base de zumo de mandarina, bayas de Goji y granada con la finalidad de incrementar el aporte de antioxidantes naturales en la dieta del consumidor. En este estudio, a diferencia de los anteriores, se evaluó la efectividad de las ciclodextrinas nativas (β -CDs), poco solubles y las modificadas (Hp- β -CDs) cuya solubilidad es mayor en disoluciones acuosas que la respectiva nativa, para proteger los compuestos bioactivos presentes en el zumo.

La bebida se elaboró con zumo de mandarina recién exprimido (98%), zumo de bayas de Goyi (2%), extracto de granada (60 mg) y ciclodextrinas tipo β y Hp- β -CDs (1%). Se hicieron suficientes réplicas, para evaluar diferentes parámetros de calidad antes y después del tratamiento térmico (98 °C, 30s), tras un periodo de almacenamiento de 75 días a 4 °C, observando diferencias significativas en el contenido en vitamina C, capacidad antioxidante y parámetros organolépticos.

Los resultados más favorables se obtuvieron en las muestras que contenían ciclodextrinas modificadas Hp- β -CDs, manteniendo concentraciones de ácido ascórbico del 98% tras el tratamiento térmico. Las determinaciones puntuales a lo largo del estudio revelaron que las modificadas preservaron un 8% más de ácido ascórbico que las nativas.

En cuanto al color, se evidenciaron mermas proporcionales al tiempo de almacenamiento. De nuevo, las muestras que contenían Hp- β -CDs en su formulación mostraron pérdidas de color menos acusadas que las que contenían β -CDs.

Al inicio del estudio, la bebida mostró una capacidad antioxidante cinco veces superior a la de un zumo de mandarina tradicional, con valores que alcanzaron los 25,94 mMT/100 mL de bebida. Tras el tratamiento térmico y a lo largo del periodo de almacenamiento, se observó una disminución generalizada de la capacidad antioxidante, siendo bastante menos acusada en las bebidas que contenían ciclodextrinas modificadas. A tiempo final (día 75), la pérdida de capacidad antioxidante de las muestras con ciclodextrinas nativas (β -CDs) respecto al control fueron del 31,5%. Sin embargo, las muestras que contenían ciclodextrinas modificadas (Hp- β -CDs) reflejaron pérdidas inferiores al 10 % (9.75%).

Para valorar la aceptación de estas bebidas en el mercado, se realizó un análisis sensorial con un panel de consumidores no entrenados. La cata se realizó antes, durante y al finalizar del periodo de almacenamiento. El panel de consumidores detectó variaciones significativas de color respecto a la muestra inicial, especialmente al final del estudio. Las bebidas con ciclodextrinas Hp- β -CDs fueron las mejor valoradas, coincidiendo con las medidas instrumentales de variación de color total realizadas a lo largo de este estudio.

Sin embargo, y a diferencia de lo que cabía esperar, cuando se analizaron los parámetros organolépticos “aroma a mandarina” y “flavor”, la bebida mejor valorada fue la que no presentaba ningún tipo de ciclodextrinas en su composición.

Esto podría explicarse, como se ha comentado anteriormente, al considerar que las ciclodextrinas no son selectivas para determinados compuestos y pueden complejar cualquier tipo de aroma ya sea agradable como indeseable (off-flavor). Por ello, las muestras que no presentaban ningún tipo de ciclodextrinas fueron, sensorialmente, las mejor valoradas, para los parámetros organolépticos estudiados, ya que al no complejar ningún tipo de aroma eran las que más se asemejaban al zumo de mandarina natural.

Sin embargo, las bebidas con ciclodextrinas tipo Hp- β -CDs fueron las mejor valoradas respecto al color, tanto a nivel instrumental como sensorial y a diferencia de las otras muestras, su capacidad antioxidante y contenido en vitamina C se mantuvo más estable durante todo el estudio.

2. ESTUDIO DE LA SEGURIDAD E INOCUIDAD DEL ZUMO DE MANDARINA

La demanda de zumos de fruta ha experimentado un acusado incremento en los últimos años, sin embargo, la presencia de residuos de productos fitosanitarios (fungicidas e insecticidas, mayoritariamente) en el zumo de naranja y mandarina comercial es uno de los principales problemas que se le plantea al sector citrícola.

Habitualmente, el control de plaguicidas y sus residuos se lleva a cabo por métodos convencionales. Los principales inconvenientes de esta metodología analítica derivan de la etapa de preparación de muestra, niveles de recuperación de analito y consumo de disolventes orgánicos, entre otros. Por ello, el control de este tipo de sustancias exige el empleo de métodos analíticos que mejoren los existentes, minimizando costes y aumentando la efectividad, ya que la detección y cuantificación de estos productos y sus metabolitos es, en muchos casos complicada, no permitiendo dar respuestas inmediatas.

Ante estas exigencias, la necesidad de respuestas analíticas rápidas y fiables es incuestionable. Destacan por sus excelentes prestaciones: sensibilidad, selectividad, sencillez, rapidez, bajo coste y tratamiento de muestra mínimo, los métodos inmunoquímicos. De hecho, su interés para la detección de plaguicidas en el campo agroalimentario ha aumentado extraordinariamente en los últimos años. Las principales razones de este auge se deben a la existencia de una extensa

variedad de formatos, mayor número de reactivos y sobre todo, a su mayor distribución y acceso comercial.

Así, el objetivo general de esta segunda parte de la Tesis fue el desarrollo de una metodología analítica sencilla, efectiva y rápida, aplicable al análisis multianalito de residuos de insecticidas organofosforados en zumos procedentes de mandarina. Para ello, se optimizó un inmunoensayo enzimático en formato indirecto competitivo sobre dos tipos de plataformas analíticas, i) placa ELISA y ii) disco compacto (DVD-R), para la determinación simultánea de dos de los principales residuos que suelen encontrarse en los zumos procedentes de cítricos, clorpirifos y fentión.

Artículo VI

La mayoría de métodos inmunoquímicos desarrollados para detectar contaminantes son unianalito, siendo una de las principales desventajas atribuidas a los inmunoensayos. Así, con el propósito de solventar esta limitación, planteamos el desarrollo de un ELISA competitivo en formato indirecto que permitiera la detección simultánea de más de un plaguicida organofosforado, utilizando dos sistemas (clorpirifos y fentión) para la demostración del concepto.

El ELISA optimizado mostró buena sensibilidad y una respuesta lineal en el intervalo 0,3-9,0 y 0,6-40,0 $\mu\text{g/L}$, para clorpirifos y fentión, respectivamente

Se consiguió eliminar el tratamiento previo al que son sometidas las muestras antes de este tipo de análisis, demostrando que una simple dilución en tampón fosfato (1/10 (v/v)) era suficiente para eliminar posibles interferencias de la matriz. Las recuperaciones de estos analitos en muestras de zumo fortificadas previamente, fueron muy interesantes (93-107%), similares a las recuperaciones obtenidas aplicando la metodología analítica de referencia.

Otro de los objetivos marcados en el planteamiento inicial, fue reducir el tiempo de ensayo (40 minutos) respecto a un ELISA convencional (130 minutos), manteniendo una buena especificidad.

Finalmente se estudió la influencia del tratamiento térmico aplicado habitualmente a los zumos, en la concentración de clorpirifos y fentión, no encontrando variaciones significativas al tratarse de tratamientos poco agresivos (temperaturas inferiores a 120 °C y tiempos cortos).

En este primer estudio se comprobó la validez del ELISA multianalito desarrollado para determinar cuantitativamente clorpirifos y fentión en zumos de

mandarina de forma rápida y efectiva. La metodología permite el análisis de 12 muestras simultáneamente en 40 minutos. Los resultados que se obtuvieron muestran el gran potencial de las técnicas inmunoquímicas, apoyando su empleo en la implementación de Planes de Vigilancia efectivos, mediante programas de control que aseguren la inocuidad de los alimentos y ofrezcan confianza al consumidor.

Artículo VII

En base a los resultados previos, se abordó la puesta a punto de sistemas de ensayo a escala micrométrica, utilizando como plataforma analítica discos compactos (DVD-R). En este caso, se trata de modificar la superficie de policarbonato de los discos compactos para imprimir los reactivos de captura, los conjugados correspondientes a clorpirifos y fentión, ya que utilizamos formato indirecto.

Estos reactivos actúan como elementos de captura de los anticuerpos específicos depositados en la plataforma - junto a las muestras- para el desarrollo de la etapa de competición. El revelado se lleva a cabo mediante un segundo anticuerpo marcado con oro, generando un precipitado oscuro en el disco tras amplificar con plata. Aquellas muestras de zumo de mandarina que contienen analito (clorpirifos y/o fentión), permiten correlacionar la intensidad de la señal con la extensión de la reacción de reconocimiento biológico. Los DVDs fueron escaneados con el lector de Cds, generando una imagen a escala de grises que puede ser cuantificado utilizando un programa informático.

Los resultados que se obtuvieron fueron similares a los obtenidos mediante ensayo unianalito, con límites de detección de 0,2 y 0,1 $\mu\text{g L}^{-1}$ y una sensibilidad de 2,1 y 1,5 $\mu\text{g L}^{-1}$ para clorpirifos y fentión, respectivamente.

Para asegurar el correcto desarrollo de los ensayos y descartar posibles errores durante las etapas de análisis, se incluyeron en cada micro-matriz controles positivos y negativos. Esta estrategia es útil para corregir la variabilidad del ensayo; por ejemplo, variaciones internas del disco pueden originar mayor o menor señal en la lectura de los controles, que tendrá el mismo efecto sobre la matriz del ensayo, proporcionando información cualitativa sobre el desarrollo del mismo y permitiendo descartar puntos o concentraciones erróneas.

La cuantificación de los plaguicidas se realizó directamente sin tratamiento previo de las muestras, reduciendo el tiempo de ensayo a 40 minutos. Las concentraciones de clorpirifos y fentión que se adicionaron a los zumos fueron determinadas mediante la metodología DVD, obteniendo unas recuperaciones entorno al 100%, equiparándose con la metodología de referencia.

Las ventajas de la metodología para la detección de productos fitosanitarios sobre DVD comercial, respecto a otros ensayos de diagnóstico, ofrece prestaciones competitivas en cuanto a sensibilidad, rendimiento, coste del proceso y tiempos de análisis, siendo compatible con otras técnicas bien establecidas.

Los límites de detección alcanzados son del orden de los kits comerciales, lo que da una idea del gran potencial de los Cds como herramienta de análisis masivo.

En resumen, la metodología basada en el empleo de tecnología Cd y dispositivos de disco compacto como plataforma analítica es sensible, portátil, de bajo coste y fácil de operar. Además, ha demostrado ser un sistema muy versátil en ensayos *in situ* simultáneos y multianalito, como ha quedado demostrado en su aplicación en la detección de clorpirifos y fentión en zumos de mandarina.

3. CONCLUSIONES

Los estudios llevados a cabo en la presente Tesis Doctoral han llevado a las siguientes conclusiones:

1. Las variables de calidad estudiadas no mostraron diferencias significativas, entre las variedades de mandarina obtenidas bajo el mismo sistema de cultivo.
2. Se observaron mayores concentraciones en los parámetros de calidad evaluados, cuando el zumo se elaboró con mandarinas procedentes de cultivo ecológico.
3. La conservación de los zumos de mandarina por ultracongelación, no provocó mermas significativas en la concentración de nutrientes o atributos sensoriales, mientras que el tratamiento térmico mostró efectos deletéreos sobre estos parámetros.

4. De las ciclodextrinas estudiadas, Hp- β -CDs son las más efectivas ya que estabilizaron la concentración de vitamina A (equivalentes de retinol), color y actividad antioxidante en los zumos de mandarina a lo largo de la vida útil del elaborado.
5. La adición de extracto de granada a la bebida a base de zumo de mandarina y bayas de Goji, mejoró el potencial antioxidante con respecto al zumo de mandarina, teniendo así una nueva bebida con alto contenido en compuestos fenólicos, adaptada a las tendencias actuales de consumo.
6. Se han optimizado inmunoensayos de alta sensibilidad y especificidad en diferentes soportes, para la determinación de los plaguicidas clorpirifos y fentión en zumos de mandarina.
7. Se han desarrollado protocolos de tratamiento de muestra que requieren una simple dilución del zumo, previa a la determinación de plaguicidas mediante inmunoensayo.
8. Se han optimizado protocolos de inmunoensayo en formato ELISA, que permiten determinar la concentración de plaguicidas en zumo de mandarina en 40 minutos, reduciendo en 90 minutos el tiempo de ensayo requerido en ELISAs convencionales.
9. Se ha desarrollado un inmunoensayo ELISA con las mismas prestaciones que los correspondientes ELISAs unianalito, para la determinación simultánea de clorpirifos y fentión en zumos de mandarina de forma sensible, rápida (40 minutos) y reproducible
10. Se ha puesto a punto una metodología muy novedosa basada en tecnología de disco compacto. Ello ha permitido disponer de una nueva herramienta analítica aplicable al desarrollo de microinmunoensayos.
11. Utilizando esta tecnología, se han puesto a punto con éxito ensayos específicos para la determinación de residuos de clorpirifos y fentión en zumos de mandarina.
12. Los micro-ELISAs desarrollados se han aplicado con éxito a la cuantificación de residuos de clorpirifos y fentión en decenas de muestras de zumo de mandarina, utilizando tratamientos de muestra rápidos (dilución) y sencillos, obteniendo resultados en 40 minutos.

13. La técnica desarrollada garantiza resultados similares en términos de sensibilidad, selectividad y reproducibilidad a las metodologías de referencia.
14. Los micro-ELISAs abren un amplio abanico de posibilidades para la implementación de estos dispositivos en la determinación de numerosas sustancias y su aplicación posterior a campos como el clínico, agroalimentario o medioambiental.
15. El tratamiento térmico suave aplicado a los zumos de mandarina, no provocó mermas significativas en la concentración de plaguicidas objeto de estudio.
16. Las concentraciones de residuos de plaguicidas determinadas por ambos tipos de inmunoensayo (ELISA dúplex y array en disco compacto) y cromatografía en zumos de mandarina, mostraron una buena correlación.

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CAPÍTULO VI. APÉNDICE

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