Universidad Miguel Hernández

Escuela Politécnica Superior de Orihuela

ESTUDIO Y CARACTERIZACIÓN DE ANTOCIANINAS EN DIFERENTES MATERIALES VEGETALES Y SU ESTABILIDAD AL PROCESADO

José Antonio Hernández Herrero

2014

ESTUDIO Y CARACTERIZACIÓN DE ANTOCIANINAS EN DIFERENTES MATERIALES VEGETALES Y SU ESTABILIDAD AL PROCESADO

Tesis doctoral realizada por D. José Antonio Hernández Herrero, Ingeniero Agrónomo, en el Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández de Elche, para la obtención del grado de Doctor.

D. José Antonio Hernández Herrero.

Orihuela, _____ de _____ de _____.

Mª JOSÉ FRUTOS FERNÁNDEZ, Profesora Titular del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández de Elche

HACE CONSTAR:

Que el presente trabajo ha sido realizado bajo mi dirección, y recoge la labor realizada por el Ingeniero Agrónomo D. José Antonio Hernández Herrero para optar al grado de Doctor.

Dra. Mª José Frutos Fernández.

Orihuela, _____ de _____ de _____.

D. JOSÉ RAMÓN DÍAZ SÁNCHEZ, Dr. Ingeniero Agrónomo, Catedrático de Escuela Universitaria y Director del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández de Elche

Certifica:

Que da su conformidad a la lectura de la Tesis Doctoral presentada por D. José Antonio Hernández Herrero, titulada "*Estudio y caracterización de antocianinas en diferentes materiales vegetales y su estabilidad al procesado*" que se ha desarrollado dentro del programa de doctorado de "*Ciencias y Tecnologías Agrarias y Alimentarias*" de este departamento, bajo la dirección de la Dra. Mª José Frutos Fernández, la cual considera conforme en cuanto a forma y contenido para que sea presentada para su correspondiente exposición pública.

Y para que así conste a los efectos oportunos firmo el presente certificado en Orihuela a_____ de _____ de _____.

Dr. José Ramón Díaz Sánchez.

Este trabajo ha sido financiado por la Conselleria de Cultura y Educación de la Generalitat Valenciana (proyecto GV00-087-13).

AGRADECIMIENTOS

En primer lugar quisiera agradecer a María José Frutos, mi directora de tesis y guía a lo largo de estos años en la universidad, su dedicación y enseñanzas han sido fundamentales para la elaboración de este trabajo.

A los técnicos de laboratorio del Departamento, Marisol Pina y Javier Vives, y a Carmen Ballester, del Servicio de Instrumentación Científica, por su siempre amable colaboración.

A nivel personal, me gustaría agradecer a mi hermano Alberto el haber estado siempre a mi lado, y a María José Martínez por el tiempo dedicado a la revisión de los textos.

Por último, agradecer a todas aquellas personas que a lo largo de estos años en la universidad me han alentado en la consecución del presente trabajo.

A mi familia

PRÓLOGO

La presente Tesis Doctoral se ha elaborado siguiendo la normativa de la Universidad Miguel Hernández de Elche para la "Presentación de Tesis Doctorales como un conjunto de publicaciones", y se ha dividido en los siguientes apartados:

- Resumen.
- Introducción.
- Objetivos.
- Publicaciones, este apartado consta de tres artículos publicados y un cuarto enviado:

    - Degradation kinetics of pigment, colour and stability of the antioxidant capacity in juice model systems from six anthocyanin sources. *International Journal of Food Science & Technology,* 2011, *46*, 2550–2557.
    - Colour and antioxidant capacity stability in grape, strawberry and plum peel model juices at different pHs and temperatures. *Food Chemistry,* 2014, *154*, 199-204.
    - Influence of rutin and ascorbic acid in colour, plum anthocyanins and antioxidant capacity stability in model juices. *Food Chemistry* **(enviado)**.
    - Effect of concentrated plum juice on physicochemical and sensory properties of yoghurt made at bench top scale. *International Journal of Dairy Technology,* 2014, *67(1)*, 123–128.

- Resultados y Discusión, donde se resumen los resultados y discusión más relevantes de las cuatro publicaciones.
- Conclusiones.
- Bibliografía, en la que se recogen únicamente las referencias que aparecen citadas en el apartado "Introducción", puesto que las referencias correspondientes a "Resultados y Discusión" se encuentran citadas en las publicaciones adjuntas.

Este documento no incluye el apartado "Materiales y Métodos", puesto que éstos se encuentran descritos en las diferentes publicaciones.

ÍNDICE

ÍNDICE DE TABLAS

ÍNDICE DE FIGURAS

Resumen

RESUMEN

Las antocianinas son un colorante natural en la escala del rojo, que posee como valor añadido una elevada capacidad antioxidante responsable de diferentes efectos beneficiosos para la salud. El mayor problema de las antocianinas es su inestabilidad frente a diversos factores, tales como el pH, luz, vitamina C, oxígeno y actividad de agua entre otros. En el presente trabajo se ha evaluado el potencial de diferentes materiales vegetales como fuente de un colorante alimentario natural basado en antocianinas. Para ello, el estudio fue dividido en cuatro fases de investigación, las cuales se corresponden con los cuatro artículos obtenidos, tres publicados y uno enviado para su publicación:

Primer trabajo:

Se realizó una selección preliminar sobre seis materiales vegetales (berenjenas, fresas, uvas, arándanos, frambuesas y ciruelas), para identificar aquellos más adecuados para ser utilizados como fuente de antocianinas. Los extractos de antocianinas obtenidos fueron utilizados para la elaboración de zumos modelo conservados durante 17 semanas en oscuridad a 20 ºC. Tras el estudio de cinética de degradación de antocianinas, estabilidad del color y capacidad antioxidante, fueron seleccionados los materiales vegetales fresa, uva y ciruela para continuar con los estudios posteriores.

Segundo trabajo:

En este segundo estudio se evaluó el efecto de la temperatura y pH sobre el color, capacidad antioxidante y contenido en fenoles totales y antocianinas de los zumos modelo elaborados a partir de extractos de fresa, uva y piel de ciruela. Los pHs empleados variaron entre 2,4 y 8,1, siendo las temperaturas de conservación aplicadas 6 y 23 ºC. La piel de ciruela fue el material vegetal que mostró los mejores resultados.

Tercer trabajo:

Una vez evaluado el efecto del pH y la temperatura sobre los zumos modelo y seleccionada la ciruela como el material vegetal idóneo, en este tercer trabajo se estudió el

efecto del ácido ascórbico y de rutín sobre la estabilidad y color de las antocianinas. Para ello, se elaboraron zumos modelo a partir de un extracto concentrado en antocianinas, ácido ascórbico y rutín que fueron conservados durante 17 semanas a 20 ºC. Los parámetros estudiados fueron la concentración de antocianinas, ácido ascórbico y rutín, el color y la capacidad antioxidante.

Cuarto trabajo:

Por último, se elaboraron yogures control y coloreados mediante la adición de zumo concentrado de ciruela. Durante la conservación frigorífica (6 ºC) fueron evaluados los parámetros acidez, pH, firmeza instrumental y color. Adicionalmente, se realizaron pruebas sensoriales para la determinación de la cremosidad e intensidad de color, así como de la aceptación de las diferentes muestras.

UNIVERSITAS Miguel Hernández

1.- Introducción

1.- INTRODUCCIÓN.

1.1.- Generalidades.

El aumento del nivel de vida ha ido acompañado de un incremento de la educación, lo que conlleva un mayor interés del consumidor por todo lo referente a la alimentación y por la influencia de ésta sobre la salud. Esta preocupación también se ha visto aumentada por la aparición de estudios que han mostrado la toxicidad de aditivos sintéticos utilizados por la industria alimentaria. Por ejemplo, la ingesta de bebidas con colorantes azoicos sintéticos (E 102, E 110, E 123, E 125, etc.) fue relacionada con el aumento de hiperactividad y déficit de atención en niños (McCann *et al.*, 2007). Así pues, tanto la regulación más estricta por parte de los organismos oficiales pertinentes como la concienciación de los consumidores, ha originado un aumento de la demanda de alimentos libres de aditivos sintéticos (Gamel y Kiritsakis, 1999; Chou *et al*., 2007), lo que ha repercutido en un mayor interés por parte de las industrias alimentarias en sustituir estos aditivos por otros de origen natural.

Los colorantes constituyen uno de los principales aditivos alimentarios, ya que el color influye en la percepción de calidad de los consumidores (Calvo, 1999; Stintzing y Carle, 2004). El interés anteriormente mencionado por sustituir aditivos alimentarios sintéticos por naturales es el que está originando la aparición de colorantes naturales como por ejemplo los basados en antocianinas. Las antocianinas están presentes en diversas frutas y bayas, tales como fresas, ciruelas y uvas entre otras, siendo esta última la principal fuente de obtención a nivel industrial (Jackman y Smith, 1996; Kammerer *et al.*, 2005). El problema de la aplicación de estos colorantes es la falta de estabilidad, pues sufren un proceso de degradación que degenera en tonos pardos poco agradables para el consumidor (Kammerer *et al.*, 2005).

El Reglamento (CE) Nº 1333/2008 del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, sobre aditivos alimentarios, aprueba las listas comunitarias de aditivos alimentarios autorizados para su uso en la elaboración de productos alimenticios, así como sus condiciones de utilización. En él se define a los colorantes como aquellas sustancias que dan color a un alimento o le devuelven su color original; pueden ser componentes naturales de los alimentos y sustancias naturales que normalmente no se consumen como

alimentos en sí mismas ni se emplean como ingredientes característicos de los alimentos. Se considerarán colorantes en el sentido del presente Reglamento los preparados obtenidos a partir de alimentos y otros materiales comestibles naturales de base mediante una extracción física, química, o físico-química, conducente a la separación de los pigmentos respecto de los componentes nutritivos o aromáticos.

Los colorantes alimentarios permitidos en la gama del rojo al azul, en la reglamentación vigente, vienen recogidos en la **Tabla 1**. En ella podemos observar que aunque la mayoría de los colorantes autorizados son sintéticos, existen diversos colorantes naturales entre los que se encuentran los carotenos, cubriendo la gama del amarillo al anaranjado; ácido carmínico, licopeno y betaninas la gama del rojo; caramelo la del marrón; los antocianos la gama del rojo al azul (Mazza *et al*., 2004); etc.

Número E	Denominación
E 110	Amarillo ocaso FCF / anaranjado S
E 120	Cochinilla, ácido carmínico, carmines
E 122	Azorrubina, carmoisina
E 123	Amaranto
E 124	Ponceau 4R, rojo cochinilla A
E 127	Eritrosina
E 129	Rojo allura AC
E 131	Azul patente V
E 132	Indigotina, carmín índigo
E 133	Azul brillante FCF
E 150a	Caramelo natural [(1)]
E 150b	Caramelo de sulfito cáustico
E 150c	Caramelo amónico
E 150d	Caramelo de sulfito amónico
E 155	Marrón HT
E 160a	Carotenos
E 160b	Annato, bixina, norbixina
E 160c	Extracto de pimentón, capsantina, capsorrubina
E 160d	Licopeno
E 160e	Beta-apo-8`-carotenal (C 30)
E 162	Rojo de remolacha, betanina
E 163	Antocianinas

[(1)] La denominación «caramelo» se refiere a productos de color pardo más o menos acentuado destinados a colorear. Es distinto del producto aromático azucarado que se obtiene calentando azúcares y que se utiliza como aromatizante (en confitería, pastelería, bebidas alcohólicas, etcétera).

Tabla 1: Lista de colorantes alimentarios permitidos, en los rangos rojo-naranja-azul (Reglamento (CE) Nº 1333/2008).

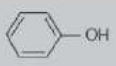
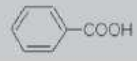
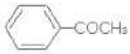
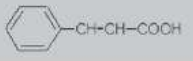
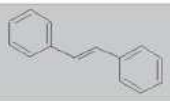
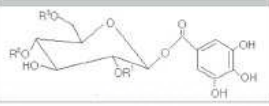
1.2.- Clasificación de los compuestos polifenólicos.

Los compuestos polifenólicos son metabolitos secundarios de origen vegetal. Varían ampliamente en estructura, desde los más simples (monómeros y oligómeros), hasta los polímeros complejos de alto peso molecular (taninos). Se han identificado más de 4000 compuestos polifenólicos individuales, los cuales se han dividido en dos grandes grupos: los flavonoides y los no flavonoides.

Los polifenoles no flavonoides incluyen a las moléculas más sencillas, como los ácidos fenólicos, con estructuras químicas de seis carbonos (C_6), ligados o no con estructuras de dos hasta cuatro carbonos (C_6-C_4). Ejemplos más complejos de compuestos no flavonoides son aquellos cuyas estructuras poseen dos anillos de seis carbonos unidos mediante dos carbonos más (C_6-C_2-C_6), como en el caso de los estilbenos, galotaninos o elagitaninos. Estos últimos son conocidos como taninos hidrolizables, que son las estructuras más complejas de este grupo. En la **Figura 1-A** se muestran los subgrupos en los que se dividen los compuestos no flavonoides. Además, los hidrógenos de los carbonos en las estructuras básicas pueden ser sustituidos por grupos hidroxilo o carboxilo, dando lugar a compuestos específicos como el ácido gálico (ácido fenólico sustituido por tres grupos hidroxilo).

Los polifenoles flavonoides poseen una estructura química que consta de tres porciones: dos anillos aromáticos y un anillo heterocíclico oxigenado (C_6-C_3-C_6). Los flavonoides conforman un grupo muy variado estructuralmente, debido a que su esqueleto base tiene numerosas posibilidades de sustitución por grupos hidroxilo (-OH), metoxilo (-OCH_3), acilo (-CO), y glucósidos. Algunos compuestos flavonoides y sus estructuras químicas básicas se muestran en la **Figura 1-B**. Los flavonoides también se pueden encontrar formando compuestos taninos de muy alto peso molecular (Marín *et al.*, 2002, Vázquez-Flores *et al.*, 2010).

A

Clase	Esqueleto químico	Estructura básica
Fenoles simples	C_6	
Benzoquinonas	C_6	
Ácidos fenólicos	C_6-C_1	
Acetofenonas	C_6-C_2	
Ácido hidroxicinámico	C_6-C_3	
Naftoquinonas	C_6-C_4	
Estilbenos	C_6-C_2-C_6	
Taninos hidrolizables (unidades de ácido gálico o elágico unidos a carbohidratos)	Estructuras variadas	

B

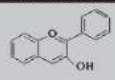
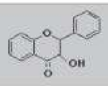
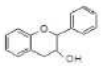
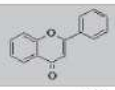
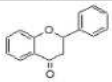
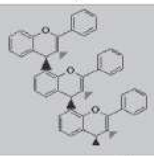
Clase	Estructura básica
Antocianidina	
Chalconas	
Dihidroflavonoles	
Flavanol	
Flavones	
Flavanonas	
Taninos condensados	

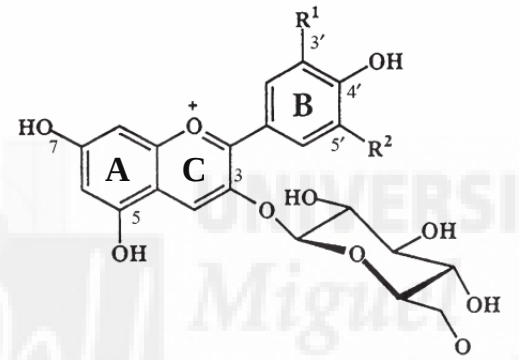
Figura 1. Estructura química de compuestos polifenólicos no flavonoides (A) y polifenólicos flavonoides (B).

1.3.- Antocianinas.

Las antocianinas (del griego *anthos* flor y *kyanos* azul) son los compuestos polifenólicos más importantes de las plantas, encontrándose principalmente en flores y frutos (Mazza *et al*., 2004). Estos pigmentos proporcionan una amplia gama de colores que va desde el naranja hasta el azul, son solubles en agua e inofensivos para la salud, lo que los convierte en unos colorantes interesantes para la industria alimentaria (Pazmiño-Durán *et al.*, 2001; Can *et al.*, 2012).

La mayoría de las antocianinas que se presentan de forma natural son glicósidos, llamándose antocianinas aglicones o antocianidinas a las que se presentan sin molécula de azúcar asociada. Las antocianidinas que normalmente se encuentran en las frutas son seis: pelargonidina, cianidina, delfinidina, peonidina, petunidina y malvidina, siendo las tres primeras las más comunes (Kong *et al.*, 2003).

Las antocianinas contienen 15 átomos de carbono configurados en una estructura básica de C_6-C_3-C_6 y constan de dos anillos aromáticos de benceno denominados A y B enlazados por tres átomos de carbono que originan un tercer anillo central C que contiene un ión oxonio con una carga positiva localizada en el átomo de oxígeno. Estos tres anillos forman la estructura básica de la antocianidina, llamada catión flavilio o 2-fenil-benzopirilio. La deficiencia en electrones del núcleo flavilio origina que sea altamente reactivo y muy sensible al pH. Los átomos de carbono de la estructura básica (C_6-C_3-C_6) son identificados mediante un sistema de numeración que utiliza números ordinarios para los anillos A y C, y números prima para el anillo B (Brouillard, 1982; Kong *et al.*, 2003; Andersen y Jordheim, 2006; Wilska-Jeszka, 2007). Los grupos ligados a las posiciones 3´ (R^1) y 5´ (R^2) del anillo B determinan las distintas antocianinas (**Figura 2**).



	R^1	R^2	Color principal	Ejemplos de fuentes
Pelargonidina 3-glicósido	-H	-H	Rojo anaranjado	Fresa
Cianidina 3-glicósido	-OH	-H	Rojo	Ciruela
Delfinidina 3-glicósido	-OH	-OH	Púrpura	Berenjena y uva
Peonidina 3-glicósido	$-OCH_3$	-H	Rojo morado	Uva y arándano
Petunidina 3-glicósido	$-OCH_3$	-OH	Púrpura	Uva
Malvidina 3-glicósido	$-OCH_3$	$-OCH_3$	Púrpura	Uva

Figura 2. Estructura química de las seis antocianidinas 3-monoglicósidos más comunes.

Las antocianinas pueden ser modificadas por diferentes mecanismos: adición o eliminación de grupos hidroxilo (-OH) o metoxilo ($-OCH_3$), polimerizaciones para producir biflavonoides u otras polimerizaciones de mayor grado, y el más importante, glicosilaciones en los grupos hidroxilo o raramente en un carbono del núcleo del flavonoide, para producir flavonoides *O*-glicósidos o *C*-glicósidos respectivamente. Las antocianinas *O*-glicósidos tienen uno o más grupos hidroxilo ligados a uno o más azúcares

mediante débiles enlaces hemiacetales. La glicosilación reduce la reactividad, y aumenta la estabilidad y la solubilidad de la molécula. Las antocianidinas son poco estables y solubles al carecer de azúcares glicosilados, por lo que no suelen encontrarse libres en la naturaleza (Brouillard, 1982; Kong *et al.*, 2003; Pior, 2004; Andersen y Jordheim, 2006).

Los oligosacáridos más comunes son dos pentosas (xilosa y arabinosa), una metil pentosa (ramnosa) y dos hexosas (glucosa y galactosa) (Goiffon *et al.*, 1999). Aunque las *O*-glicosilaciones se pueden dar en cualquier grupo hidroxilo, suelen producirse con mayor probabilidad en las posiciones 3 y 5 hidroxil de la antocianidina. El grupo libre 3-hidroxilo desestabiliza el cromóforo, por tanto siempre está glicosilado. Otras *O*-glicosilaciones se pueden producir en las posiciones 7, -3`, -4` y/o –5` hidroxilo. Las *C*-glicosilaciones, que se producen con mucha menos frecuencia, implican normalmente a las posiciones C6 y C8 (Brouillard, 1982; Ordaz-Galindo, 1999; Kong *et al.*, 2003; Andersen y Jordheim, 2006).

1.4.- Síntesis de las antocianinas.

La síntesis de las antocianinas es común a la del resto de flavonoides, formándose en primer lugar las chalconas, y a partir de éstas el resto de flavonoides. Las chalconas son inducidas bajo situaciones de estrés mediante un proceso biosintético que incorpora precursores de dos vías, la del ácido siquímico y la del acetato-malonato. El compuesto obtenido por la vía del ácido siquímico, *p*-cumaril-CoA, es luego utilizado como compuesto de partida para la vía acetato-malonato, en la cual se adicionan tres unidades de malonil-CoA, obteniéndose así las chalconas. La **Figura 3** muestra la formación de la chalcona chalcononaringenina (4,2',4',6'-tetrahidroxi chalcona) (Wong, 1976; Strack & Wray 1993; Zhang *et al*., 2002; He *et al*., 2010).

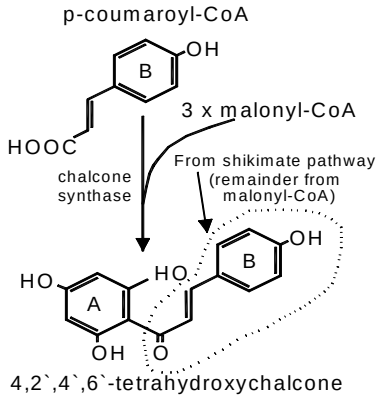


Figura 3: Diagrama de formación de las chalconas (Wong, 1976).

La chalcononaringenina es utilizada por la chalcona isomerasa (CHI) para formar naringenina. A partir de aquí, la flavanona 3-hidroxilasa (F3H) mediante la hidroxilación de la posición 3 forma dihidrocanferol. Mediante posteriores hidroxilaciones en el anillo B se pueden obtener otros dihidroflavonoles, como dihidroquercitina (hidroxilación de la posición 3`) o dihidromiricetina (hidroxilación 3` y 5`). Por medio de una reducción en la posición 4 catalizada por la dihidroflavonol reductasa (DFR) se da lugar a las leucoantocianidinas (flaván-3,4-*cis*-diol). La transformación de éstas en antocianidinas no esta muy discernida, pero en ella actúa la antocianidin sintasa (ANS) y la flavonoide 3-glucosiltransferasa (3GT) para lograr la glucosilación del grupo hidroxilo del carbono 3 (**Figura 4**) (Wong, 1976; Strack & Wray 1993; Zhang *et al*., 2002; He *et al*., 2010).

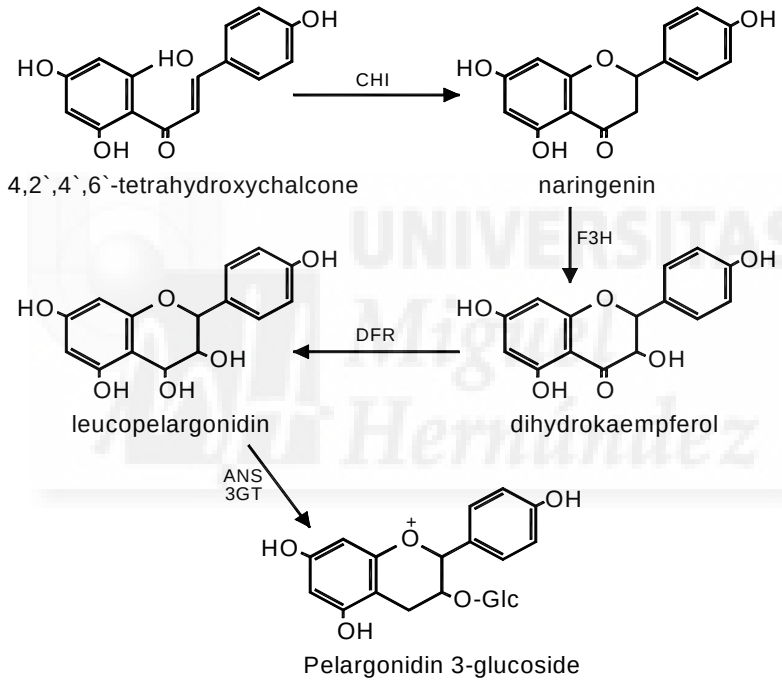


Figura 4: Esquema de formación de las antocianinas (pelargonidina 3-glucósido).

1.5.- Factores que afectan al color y estabilidad de las antocianinas.

Son numerosos los factores que afectan a la estabilidad y por tanto al color de las antocianinas, destacando entre ellos la estructura, pH, proteínas, temperatura, método de extracción, actividad de agua, dióxido de azufre, luz, iones metálicos, enzimas, azúcares y sus productos de degradación, oxígeno y ácido ascórbico, y copigmentaciones (Rein, 2005; Bobbio y Mercadante, 2008).

1.5.1- Estructura.

La localización y combinación de los grupos añadidos a los anillos de la estructura, grupos hidroxilo (-OH), metoxilo (-CH_3) o azúcares, influyen sobre el color, estabilidad y solubilidad de la antocianidina. La presencia de azúcares ligados a la estructura proporciona solubilidad en agua y estabilidad, ya que bloquea grupos hidroxilo que son altamente reactivos (Fleschhut, 2006). Las sustituciones en el anillo B dan lugar a la formación de diversas antocianinas y tonalidades de color (Durst y Wrolstad, 2001). Los grupos hidroxilo y metoxilo son grupos auxocromos donadores de electrones, es decir, grupos que por sí mismos no tienen propiedades cromóforas, pero que producen una variación del tono cuando se unen a la antocianina. La capacidad donadora de electrones de los grupos metoxilo es superior a la de los grupos hidroxilo, por lo que las hidroxilaciones incrementan la tonalidad azulada (efecto batocrómico) mientras que la metoxilación de los grupos hidroxilo aumenta la tonalidad roja (efecto hipsocrómico). Puesto que los grupos metoxilo son menos reactivos, las metoxilaciones en el anillo B conducen a la estabilización de la antocianina y del color rojo (von Elbe y Schwartz, 1996; Cuevas *et al*., 2008).

1.5.2.- pH.

Las antocianinas pueden presentar cuatro estructuras diferentes en función del pH de la solución acuosa en la que se encuentren. Las cuatro estructuras tienen diferentes colores, variando la concentración de cada una de ellas, y por tanto el color de la solución acuosa, en función del pH. A pH inferior a 2, el catión flavilio es la estructura dominante. A pH 3-6, la hidratación del C2 del catión flavilio origina dos formas hemiacetales incoloras, denominadas base carbinol y chalconas. Por último, a pHs superiores, las reacciones de desprotonación del grupo -OH del C7 originan las formas quinonoideas de color púrpura (**Figura 5**). La estructura predominante a pH < 2, catión flavilio, es la más estable y la que proporciona el característico color rojo de las antocianinas (Brouillard, 1982; Fleschhut *et al*., 2006; Hernández-Herrero y Frutos, 2014a).

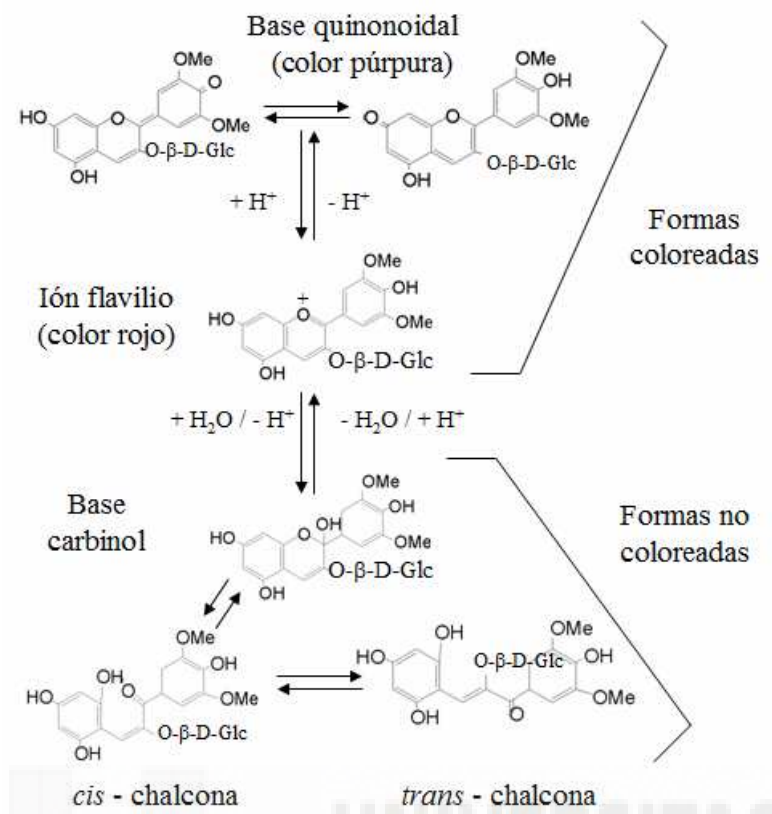


Figura 5: Transformación estructural del ión flavilio (malvidín 3-glucósido) en solución acuosa ligeramente acidificada (Malien-Aubert *et al*., 2001).

1.5.3.- Proteínas.

Las antocianinas pueden condensar con aminoácidos produciendo turbidez, pudiendo estas reacciones incrementarse por la presencia de sólidos solubles (Cemeroglu *et al*., 1994). En casos extremos, estos complejos con proteínas pueden dar lugar a precipitados (Edwards, 2001) y pérdidas de color (García-Viguera y Bridle, 1999). En otros estudios las proteínas han sido utilizadas para obtener extractos fenólicos gracias al aislamiento del complejo fenol-proteína formado (Roopchand *et al*., 2013).

1.5.4.- Temperatura.

Las altas temperaturas provocan la ruptura del enlace del azúcar de la posición 3 de la antocianina, originando antocianidinas (aglicones), y la apertura del anillo central formando chalconas incoloras (Jackman y Smith, 1996; Delgado-Vargas *et al*., 2000; Bąkowska *et al*., 2003) y otros productos de degradación, que condensan para formar

complejos poliméricos pardos conocidos como pigmentos melanoidinas (Piffaut *et al*., 1994). La degradación térmica es mayor a pH superiores a 3, coincidiendo con la simultánea conversión del ión flavilio a otras formas más vulnerables (Malien-Aubert *et al*., 2001; Matsufuji *et al.*, 2007).

1.5.5.- Método de extracción.

Disolventes. El uso de disolventes con diferentes polaridades puede causar cambios batocrómicos o hipsocrómicos en las bandas de absorción, y estos cambios dependen de la naturaleza del grupo químico y de los orbitales moleculares involucrados (Kemp, 1991; Ito *et al*., 2002; Castañeda-Ovando *et al*., 2009). Por ejemplo, en solución de metanol acidificado con ácido clorhídrico, la longitud de onda a la que pelargonidina 3-glucósido posee la máxima absorbancia es 520 nm (Mazza y Miniati, 1993); mientras que en solución de agua y acetonitrilo acidificada con ácido trifluoroacético es 502 nm (Hernández-Herrero y Frutos, 2011).

Temperatura. La temperatura de extracción juega un papel importante, mostrándose que las antocianinas de los extractos obtenidos a altas temperaturas (60 ºC) se degradan más rápidamente durante la conservación a temperatura ambiente que aquellas extraídas a 25 ºC (Kalt *et al*., 2000).

Purificación. Las antocianinas pueden verse afectadas negativamente por la presencia de compuestos no fenólicos tales como azúcares, enzimas, proteínas, ácidos orgánicos, etc. Por tanto, la eliminación de estas sustancias puede mejorar la estabilidad de las antocianinas. En el apartado *1.7.- Obtención de antocianinas* se comentan algunos de los sistemas de purificación de extractos más utilizados.

Concentración de antocianinas y otros fenoles. El incremento de la concentración de antocianinas mejora tanto la intensidad del color como la estabilidad del mismo (Ito *et al*., 2002; Giusti y Wrolstad, 2003). Estas mejoras se deben a que las altas concentraciones, tanto de antocianinas como de otros fenoles, favorecen las reacciones de copigmentación (ver apartado *1.5.14.- Copigmentación*).

1.5.6.- Actividad de agua.

Una alta actividad de agua favorece el ataque nucleofílico del agua sobre el catión flavilio, principalmente en los carbonos C2 y C4. Esta reacción conlleva la formación de base carbinol y chalconas incoloras e inestables, y por tanto de fácil degradación (Garzón y Wrolstad, 2001; Fleschhut *et al*., 2006; Lai *et al.*, 2009).

1.5.7.- Enzimas.

Las antocianinas pueden ser degradadas en los tejidos de las plantas por sistemas enzimáticos tales como glucosidasas (antocianasa o antocianin-β-glucosidasa), polifenoloxidasas (PPO) y peroxidasas (Garde-Cerdan *et al*., 2008).

Las glucosidasas rompen los enlaces glucosídicos del C3 que unen el azúcar a la aglicona, y como ya se ha comentado en el apartado *1.5.4.- Temperatura*, generan antocianidinas (aglicón) y la apertura del anillo central formando chalconas incoloras (Jackman y Smith, 1996; Delgado-Vargas et al., 2000; Bąkowska *et al.*, 2003). A su vez, las chalconas pueden acelerar el pardeamiento enzimático, pues derivan en fenoles con estructura similar al catecol (*o*-dihidroxibenceno) que podrían ser un buen substrato para la PPO (Jiang, 2000).

1.5.8.- Ión bisulfito.

El ión bisulfito (HSO_3^-) normalmente se genera a partir de la adición de bisulfito de sodio o potasio, o de gas anhídrido sulfuroso (SO_2) a una solución acuosa. El anhídrido sulfuroso o dióxido de azufre es un gas incoloro de fuerte olor que se genera por la combustión del azufre y que en contacto con agua genera ácido sulfuroso (H_2SO_3), permaneciendo en solución los iones sulfito (SO_3^{2-}) y bisulfito (HSO_3^-) en diferentes proporciones en función del pH.

El ión bisulfito (HSO_3^-) es capaz de reaccionar con el C4 de la antocianina para producir un compuesto antocianina-bisulfito incoloro (blanqueado). Para bajas concentraciones de ión bisulfito la reacción se puede revertir bajando el pH, mientras que

para altas concentraciones se provoca la decoloración irreversible de la antocianina (Wrolstad *et al*., 2005) (**Figura 6**).

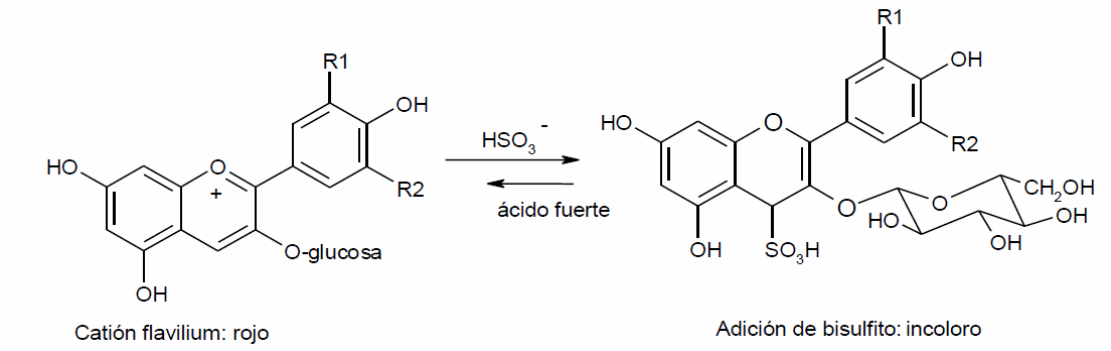


Figura 6: Reacción de las antocianinas con bisulfito para formar el compuesto antocianina-bisulfito incoloro (Wrolstad *et al*., 2005).

Aunque la adición de bisulfito supone la perdida de color de las antocianinas, su aplicación se ve justificada en algunos alimentos. En frutas y hortalizas se utilizan tratamientos por inmersión en soluciones de bisulfito en muy baja concentración (usualmente 150 ppm de bisulfito de sodio) a fin de inhibir o retardar las reacciones de pardeamiento durante el procesado y almacenamiento de frutas (Quevedo *et al*., 2011). Durante la elaboración de vino se emplea SO_2 a pequeñas concentraciones, en torno a 200 ppm, debido a sus efectos antimicrobianos, antioxidantes, y preventivos frente a pardeamientos enzimáticos (mediante la inactivación de enzimas tales como polifenoloxidasas, peroxidadas y proteasas) y no enzimáticos (inhibiendo las reacciones de Maillard) (Garde-Cerdan *et al*., 2008). En vinos tintos jóvenes, gran parte del color rojo-púrpura está generado por las antocianinas monoméricas. Con el envejecimiento del vino se producen reacciones de copigmentación y polimerización de antocianinas que originan nuevos pigmentos, predominando entonces los tonos rojo-anaranjados (He *et al*., 2012). Estos pigmentos, al no tener el C4 disponible, no son susceptibles de sufrir blanqueamientos por bisulfito (Wrolstad *et al*., 2005).

1.5.9.- Luz.

La luz, tanto ultravioleta como visible, genera un estado excitado en la antocianina vía transferencia de electrón, que conduce a una descomposición fotoquímica hacia formas chalconas (Jackman y Smith, 1996). Seguidamente se produce la rotura de éstas, originando 2,4,6-trihidroxi benzaldehído desde la parte benzopirilio de la antocianina

(anillos A y C), y ácido 3,4-dihidroxi benzoico desde el anillo fenólico (anillo B) (**Figura 2**) (Furtado *et al*., 1993; Bąkowska *et al.*, 2003). La fotoestabilidad de las antocianinas depende del pH, resultando más resistentes a la luz a bajos pHs (Matsufuji *et al.*, 2007). Los envases de alimentos coloreados con antocianinas suelen ser transparentes, para así mostrar su color, pero desde el punto de vista tecnológico estos alimentos deberían ser envasados con materiales que ejercieran barreras eficientes contra la luz UV, garantizando así durante más tiempo la aceptabilidad de su color (Carlsen y Stapelfeldt, 1997).

1.5.10.- Azúcares y sus productos de degradación.

Los azúcares a altas concentraciones, como ocurre en las conservas de frutas, estabilizan las antocianinas. Este efecto parece ser debido a la disminución de la actividad de agua (Fleschhut *et al*., 2006; Lai *et al*., 2009) y a la formación de complejos con metales y pectinas (Kopjar *et al*., 2009; Buchweitz *et al*., 2012). Cuando los azúcares están presentes en concentraciones lo suficientemente bajas como para tener poco efecto sobre la a_w, ellos o sus productos de degradación pueden a acelerar la degradación de las antocianinas. A bajas concentraciones, azúcares tales como fructosa, arabinosa, lactosa y sorbosa tienen un efecto degradante mayor sobre las antocianinas que la glucosa, sacarosa y maltosa (von Elbe y Schwartz, 1996).

Algunos aldehídos resultantes de la degradación térmica de azúcares (reacción de Maillard) o de la oxidación del ácido ascórbico, como el furfural e hidroximetilfurfural, condensan fácilmente con las antocianinas produciendo un pardeamiento oscuro (Krifi *et al*., 2000; Es-Safi *et a*l., 2000; Es-Safi *et a*l., 2002). No se conoce totalmente el mecanismo de esta reacción de polimerización, pero es muy dependiente de la temperatura, se acelera por la presencia de oxígeno, y es muy importante en los zumos de frutas (von Elbe y Schwartz, 1996).

1.5.11.- Oxígeno.

El oxígeno es capaz de generar espontáneamente formas muy reactivas e inestables conocidas como radicales libres, tales como el radical anión superóxido (O_2^-), que a su vez reaccionan con otras moléculas generando nuevos radicales, y así sucesivamente mientras que no se encuentren con sustancias antioxidantes capaces de paralizar estas reacciones.

Pues bien, las antocianinas son unos compuestos altamente antioxidantes capaces de neutralizar estos radicales libres mediante la donación de átomos de hidrogeno (Rice-Evans *et al*., 1996; Castañeda-Ovando et al., 2009), reacción que origina la destrucción de la antocianina.

La estructura de la antocianina es de gran importancia frente a la oxidación, de manera que aquellas estructuras que mejoran la capacidad antioxidante muestran una menor estabilidad frente a la oxidación. Así, las antocianidinas que poseen grupos *orto*-dihidroxilo (grupo catecol) en su anillo B (cianidina, delfinidina y petunidina) muestran más capacidad antioxidante (Noda *et al*., 2000; Kähkönen y Heinonen, 2003) pero son más sensibles a la oxidación que las que no lo poseen (malvidina, peonidina y pelargonidina) (Fleschhut *et al*., 2006; Jackson, 2008).

De forma análoga, el aumento del número de azúcares y ácidos asociados a la molécula disminuye la capacidad antioxidante (Azuma *et al.*, 2008) pero aumenta la estabilidad oxidativa. El aumento de la estabilidad se debe a que la glucosilación del grupo hidroxilo del C5 reduce el carácter nucleofílico de las posiciones C6 y C8, y a que las antocianinas 3,5-diglicósidos son menos propensas a sufrir ataques electrofílicos (Timberlake y Bridle, 1977).

La oxidación de antocianinas puede ser más importante en zumos de frutas y bebidas que en productos con bajo contenido en agua, debido a que en matrices acuosas puede disolverse mayor cantidad de oxígeno durante el procesado. Por esta razón, la eliminación del oxígeno disuelto (mediante burbujeo con nitrógeno o des-aireación por vacío) o del oxígeno del espacio de cabeza (mediante barrido con nitrógeno) pueden ser beneficiosos para la retención del color y la capacidad antioxidante en alimentos procesados (Kalt *et al*., 2000). En productos de cuarta gama, es recomendable envasar en atmósferas con bajo contenido en oxígeno para mantener el color y la capacidad antioxidante de las antocianinas (Odriozola-Serrano *et al*., 2010).

1.5.12.- Ácido ascórbico.

Las antocianinas y el ácido ascórbico se destruyen mutuamente en presencia de oxígeno, tanto por mecanismos de condensación como por reacciones de oxidación.

a.- Mecanismo de condensación.

La rápida degradación mutua entre antocianinas y ácido ascórbico puede explicarse mediante dos mecanismos de condensación:

Mecanismo directo. En presencia de oxígeno, el ácido ascórbico reacciona con el C4 de la antocianina. Este mecanismo se basa en que el ácido ascórbico es estructuralmente muy similar a la dimedona, y ésta condensa fácilmente con las sales de flavilio (Jurd, 1972; Poei-Langston y Wrolstad 1981; Garzon y Wrolstad, 2002). Sin embargo, no se ha conseguido ninguna evidencia experimental de esta condensación.

Mecanismo indirecto. Consiste en la formación de un complejo estable antocianina-metal-ácido ascórbico (Sarma *et al*., 1997).

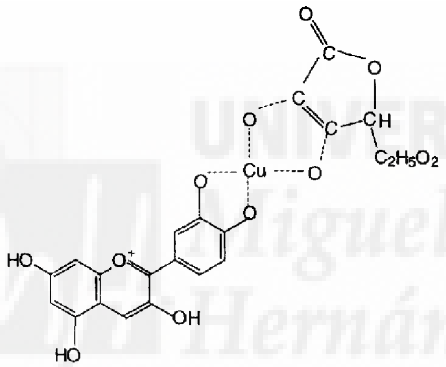


Figura 7: Ejemplo de unión entre una antocianina y el ácido ascórbico (Sarma *et al*., 1997).

b.- Reacciones de oxidación.

La degradación de la antocianina puede ser inducida por la oxidación del ácido ascórbico tanto de forma directa como indirecta. La forma directa se debe a que las antocianinas muestran una capacidad antioxidante superior a la del ácido ascórbico (Miller *et al*., 1995; Bagchi *et al*., 1998), por lo que pueden actuar como un protector del ácido ascórbico frente a la oxidación. El efecto antioxidante ejercido se debe a la reducción directa del ácido ascórbico (Pang *et al*., 2001). En cuanto a la forma indirecta, durante la oxidación del ácido ascórbico se genera peróxido de hidrógeno (H_2O_2) (Jackman y Smith, 1996) que mediante ataques nucleofílicos logra oxidar las antocianinas a estructuras quinonoideas (antocianonas) y chalconas (Lopes *et al*., 2007; Sun *et al*., 2011).

1.5.13.- Asociación con iones metálicos.

Los complejos de antocianinas con metales son muy comunes en el mundo vegetal y amplían el espectro de color de las flores. Las antocianinas con grupos *orto*-dihidroxilo en su anillo B pueden secuestrar diversos metales multivalentes (**Figura 8**) (Sarma *et al*., 1997; Yosida *et al*., 2006).

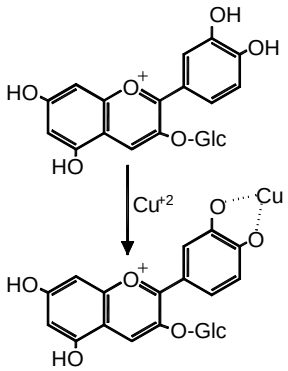


Figura 8: Quelación metálica de cianidina 3-glucósido en presencia de cobre.

La industria alimentaria se ha interesado en los últimos años por estos complejos antocianina-metales debido al efecto de estabilización (Castañeda-Ovando *et al*., 2009) y estandarización del color que originan. La formación de estos complejos produce un desplazamiento batocrómico que aumenta las tonalidades azuladas en el color percibido (Yosida *et al*., 2006; Schreiber *et al*., 2010). Para una posible aplicación en alimentos deberían utilizarse metales atóxicos y a ser posible esenciales para la dieta (Castañeda-Ovando *et al*., 2009). En otros casos, la quelación de metales puede tener otras ventajas adicionales. Por ejemplo, la quelación del hierro puede inhibir la generación de radicales ·OH (Noda *et al*., 2000), y así prevenir la peroxidación lipídica (Wang *et al*., 1999; Bąkowska-Barczak, 2005). En otras ocasiones, el complejo antocianina-metal puede interactuar con un tercer componente formando así nuevos complejos también estables antocianina-metal-copigmento, como por ejemplo antocianina-metal-vitamina C (**Figura 7**) (Sarma *et al*., 1997) y antocianina-metal-pectinas (Kopjar *et al*., 2009; Buchweitz *et al*., 2012).

1.5.14.- Copigmentación.

A pHs bajos, las antocianinas coloreadas (pigmentos) pueden formar complejos con otros compuestos orgánicos que potencien el color (copigmentos). Estos copigmentos pueden ser tres: otras antocianinas (auto-asociación o asociación entre antocianinas), compuestos no coloreados que normalmente son otros flavonoides (copigmentación

intermolecular) y ácidos hidroxicinámicos (copigmentación intramolecular o acilación) (Brouillard y Dangles, 1993; Boulton, 2001; Rein, 2005; Cavalcanti *et al*., 2011).

La interacción entre estas moléculas se lleva a cabo mediante enlaces débiles como son las fuerzas de Van der Waals, puentes de hidrógeno, interacciones iónicas e interacciones hidrofóbicas entre las nubes de electrones de los anillos aromáticos de ambas moléculas (Dangles *et al*., 1993). En este último tipo de interacción, la antocianina apila su estructura plana sobre la estructura también plana del copigmento, a modo de sándwich (**Figura 9**), compartiendo los electrones π de sus anillos aromáticos. Estas interacciones electrónicas generan fuerzas hidrofóbicas en el interior de la estructura que protegen los carbonos C2 y C4 del catión flavilio del ataque nucleofílico del agua, así como de otras sustancias tales como ión bisulfito, peróxidos, ácido ascórbico, etc. (Mazza y Miniati, 1993; Garzón y Wrolstad, 2001; Rein, 2005; Wrolstad *et al*., 2005; Fleschhut *et al*., 2006; Lai *et al.*, 2009).

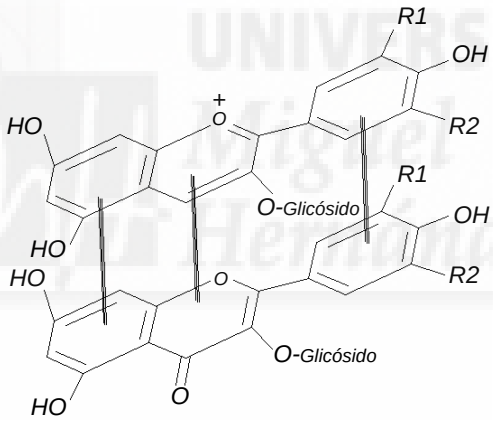


Figura 9: Ejemplo de copigmentación intermolecular, mediante interacciones hidrofóbicas, entre una antocianina (en forma de catión flavilio) y un copigmento (flavonol).

El nuevo compuesto coloreado no solamente aporta estabilidad al color, sino que también incrementa su intensidad (efecto hipercrómico) y la tonalidad azulada en el color percibido (efecto batocrómico). Este último efecto es debido al incremento de la longitud de onda donde se obtiene la máxima absorbancia del pigmento (Brouillard y Dangles, 1993).

Las copigmentaciones formadas con el catión flavilio son más estables que las formadas con la base quinonoidal. Esto indica que a bajos pHs, donde la forma catión

flavilio es predominante, se obtiene una mayor protección (Malien-Aubert *et al*., 2001; Castañeda-Ovando *et al*., 2009).

a.- Asociación entre antocianinas (auto-asociación).

Esta unión se ve favorecida por la presencia de antocianinas de alto grado de metoxilación (por ejemplo, malvidina 3-glicósido) (González-Manzano *et al*., 2008), y por una elevada concentración de éstas (> 1 mM/L) (Boulton, 2001). Al contrario que el resto de copigmentaciones, la asociación entre antocianinas causa un efecto hipsocrómico en vez de batocrómico, es decir, que aumenta las tonalidades rojas en el color percibido (Boulton, 2001; González-Manzano *et al*., 2008).

b.- Copigmentación intermolecular.

La copigmentación intermolecular consiste en la unión de una antocianina coloreada con otros compuestos no coloreados (**Figura 9**). Los flavonoides, y en especial los flavonoles, son en general los copigmentos más eficientes (Mazza y Miniati, 1993; Eiro y Heinonen, 2002; He *et al*., 2012).

c.- Copigmentación intramolecular (acilación).

La acilación consiste en la unión de uno o más azúcares de la antocianina coloreada (ión flavilio o base quinonoidal) con un ácido orgánico mediante un enlace covalente (**Figura 10**). Algunos de estos ácidos pueden ser el *p*-cumárico, acético, málico, ferúlico o cafeico (Malien-Aubert *et al*., 2001). La acilación es la copigmentación que muestra el mayor efecto estabilizador del color, efecto que aumenta con el grado de acilación. Así, las antocianinas poliaciladas destacan por la extraordinaria estabilidad frente al procesado, almacenamiento y cambios de pH (Dangles *et al*., 1993).

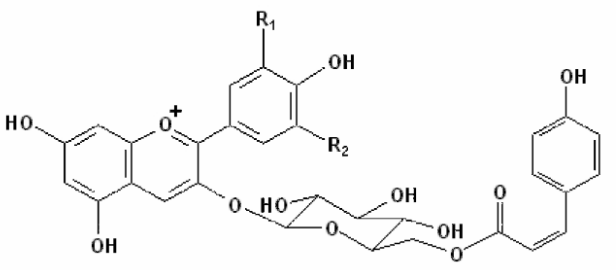


Figura 10: Ejemplo de copigmentación intramolecular: antocianidina 3-*O*-(*p*-cumárico)-glucósido (He *et al*., 2012).

1.5.15.- Polimerización.

Los pigmentos poliméricos consisten en la unión estable de una antocianina con otros compuestos, normalmente otros polifenoles como flavanoles, taninos, etc. La interacción entre estas moléculas suele producirse mediante enlaces covalentes entre los carbonos C4-C8 o en menor medida entre C4-C6. El polímero formado puede ser tanto una antocianidina-polifenol (**Figura 11**) como un polifenol-antocianidina, pero en el primer caso la estabilidad del color es superior. Ello se debe a que la posición C4 de la antocianina deja de estar libre, y por tanto la antocianina es más estable frente al ión bisulfito, cambios de pH, etc. (Boulton, 2001; Eiro y Heinonen, 2002; Salas *et al*., 2003; He *et al*., 2012).

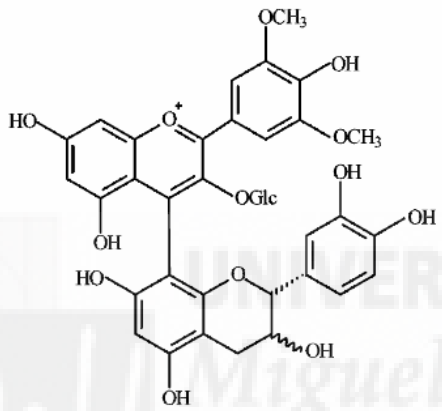


Figura 11: Ejemplo de polimerización entre una antocianina (malvidina 3-glucósido) y un flavanol (flavan-3-ol o catequina) durante la formación de taninos (Salas *et al*., 2003).

1.6.- Capacidad antioxidante.

Las antocianinas cuentan con dos mecanismos para ejercer su capacidad antioxidante. El principal se basa en su capacidad de secuestrar radicales mediante donación de hidrógenos (Rice-Evans *et al*., 1996; Castañeda-Ovando *et al*., 2009), mientras que el segundo mecanismo consistente en secuestrar metales (Yosida *et al*., 2006). Como ya se ha mencionado en el apartado *1.5.13.- Asociación con iones metálicos*, las antocianinas con grupos *orto*-dihidroxilo pueden inhibir la generación de radicales ·OH mediante la quelación del hierro (Figura 8) (Noda *et al*., 2000), y así prevenir la peroxidación lipídica (Wang *et al*., 1999; Bąkowska-Barczak, 2005).

Según el mecanismo de secuestro de radicales libres (**Figuras 12** y **13**), la antocianina (AH) cede un hidrógeno (H) al radical 2,2-difenil-1-picrilhidrazil (DPPH·), de

tal forma que ella queda oxidada (A·) mientras el radical queda reducido (DPPH-H), y por tanto estabilizado ([1]). La desprotonación (oxidación) de la antocianina origina el radical antocianina semiquinona (A·), el cual sigue reaccionando mediante interacciones radical-radical hasta originar moléculas estables ([2] y [3]). Según Auroma (1998) la segunda reacción ([2]) se produce con más facilidad que la tercera ([3]).

[1] DPPH· + AH → DPPH-H + A·

[2] DPPH· + A· → DPPH-A

[3] A· + A· → A-A

Figura 12: Esquema del mecanismo de secuestro de radicales libres (Yamaguchi *et al.*, 1998).

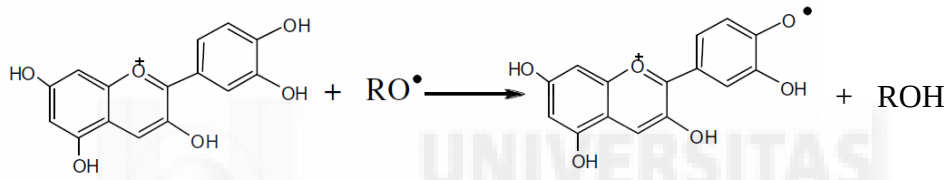


Figura 13: Mecanismo propuesto de oxidación de cianidina a cianidina semiquinona radical (Castañeda-Ovando *et al*., 2009).

La estructura de la antocianina es de gran importancia frente a la oxidación. Como se comentó en el apartado *1.5.11.- Oxígeno*, las antocianinas que poseen grupos *orto*-dihidroxilo en su anillo B (cianidina, delfinidina y petunidina) muestran más capacidad antioxidante que las que no lo poseen (malvidina, peonidina y pelargonidina) (Kähkönen y Heinonen, 2003; Jackson, 2008). El número de azúcares y ácidos orgánicos copigmentados (acilaciones) también influye, disminuyendo la capacidad antioxidante conforme aumenta el número de sustituciones (Azuma *et al.*, 2008). Diferentes estudios han mostrado que la capacidad antioxidante de las antocianinas es muy superior a las de compuestos tales como el α-tocoferol y el ácido ascórbico (Miller *et al*., 1995; Bagchi *et al*., 1998).

1.7.- Obtención de antocianinas.

1.7.1.- Métodos de extracción.

Las antocianinas son compuestos polares normalmente extraídos con mezclas acuosas de metanol, etanol o acetona débilmente acidificadas, y a temperatura ambiente (Kähkönen *et al.*, 2001). Aunque el disolvente más eficaz de estos tres es el metanol (Lee *et al.,* 2004; Kapasakalidis *et al.,* 2006), el etanol es el más habitual debido a su menor toxicidad. Es habitual realizar la extracción de antocianinas en dos etapas, primeramente se realiza una extracción con uno de estos tres disolventes acidificado, y posteriormente el residuo seco se re-extracta con una mezcla acidificada de disolvente:agua con la finalidad de extraer la porción de antocianinas más solubles en agua (Hernández-Herrero y Frutos, 2011). En la actualidad se están ensayando diferentes técnicas para mejorar el rendimiento de extracción de las antocianinas, como son las que emplean enzimas pectolíticas para hidrolizar las paredes celulares (Buchert *et al.*, 2005; Kelebek *et al.*, 2009), o ultrasonidos para favorecer tanto la rotura de las paredes como la posterior extracción de los pigmentos (Li *et al.*, 2010; Cheoka *et al.*, 2013).

La mayor fuente de antocianinas utilizada son los hollejos de uva tinta que se obtienen como subproducto en la vinificación, denominándose enocianina o antocianinas (E 163) al colorante obtenido (**Tabla 1**). La extracción en los orujos de uva suele realizarse con una solución acidificada de agua y restos de etanol procedente de la propia vinificación, utilizando un ratio hollejos:disolvente de 1:1 a 1:3,5 a temperatura ambiente durante 3 horas (Jackman y Smith, 1996; Kammerer *et al.*, 2005). Aunque el ión bisulfito provoca el blanqueamiento de las antocianinas monoméricas (**Figura 6**) (Wrolstad *et al*., 2005), el agua sulfurada suele emplearse debido al alto poder extractante del SO_2 (Cacace y Mazza, 2003).

1.7.2.- Métodos de concentración.

A nivel de laboratorio, tras la centrifugación o filtración suele realizarse una concentración por evaporación del disolvente a baja temperatura, en torno a 35 ºC, y bajo condiciones de vacío (Pazmiño-Durán *et al*., 2001; Hernández-Herrero y Frutos, 2011). Sin embargo, a nivel industrial, suele ser habitual prensar la pasta de hollejos obtenida para

posteriormente centrifugar o filtrar la solución. A continuación, ésta puede concentrarse por evaporación del agua a 70-80 ºC obteniéndose así un líquido concentrado, o por secado a spray dando lugar a un polvo rico en polifenoles.

1.7.3.- Métodos de purificación.

El alto coste económico de las técnicas de purificación limita su utilización casi exclusivamente a nivel de laboratorio, donde se realiza mediante una extracción en fase sólida. Para ello se utilizan cartuchos de C18 (Cui *et al.*, 2013), los cuales están rellenos de cadenas de C18 unidas a una matriz de sílice, o columnas de resina de intercambio iónico de Amberlita (Kammerer *et al.*, 2005; Flores *et al*., 2014).

1.8.- Aplicación en alimentos.

La aplicación de los colorantes basados en antocianinas (E 163) (**Tabla 1**) en alimentos tiene grandes limitaciones debido a su baja estabilidad (Rein, 2005; Bobbio y Mercadante, 2008). Por ser el efecto del pH el más limitador, el E 163 se ha utilizado exclusivamente para colorear o reforzar el color de diferentes alimentos acidificados como son las mermeladas, yogures, helados, bebidas refrescantes, dulces, etc. (Kopjar *et al*., 2009; Burin *et al*., 2011; Hernández-Herrero y Frutos, 2014b). En gelatinas y otros productos transparentes ricos en proteínas no se han podido utilizar por los problemas de turbidez que pueden generarse (Cemeroglu *et al.*, 1994). Actualmente se está trabajando en diversos mecanismos para aumentar la estabilidad del color de las antocianinas y así poder ampliar la variedad de alimentos en los que pueden ser aplicados. Entre ellos destacamos la quelación con iones metálicos (Castañeda-Ovando *et al*., 2009), la copigmentación con otros compuestos orgánicos (Cavalcanti *et al*., 2011), y sobre todo la micro-encapsulación mediante secado por spray (Betz y Kulozik, 2011; Flores *et al*., 2014).

1.9.- Ingesta diaria por persona de antocianinas.

La estimación de la ingesta diaria por persona de antocianinas varía de unos autores a otros y de unos países a otros. Para realizar el cálculo se deben tener en cuenta tres factores: la elección de los alimentos ricos en antocianinas (**Tabla 2**) consumidos asiduamente en un país, la estimación de la ingesta diaria de cada uno de esos alimentos, y

por último, la determinación de la concentración de antocianinas en los diferentes materiales vegetales seleccionados. Este último factor hoy en día no supone un problema puesto que los datos aportados por los diferentes autores son similares, a excepción de pequeñas diferencias debidas a las distintas variedades y condiciones de cultivo, tales como la climatología, el tipo de suelo, el abonado, etc. Por tanto, las diferencias en las estimaciones de ingesta diaria de antocianinas se deben a los dos primeros factores expuestos (Wu *et al*., 2006). Así encontramos que en Estados Unidos la media de la ingesta diaria de antocianinas por persona en 1971 fue estimada en 215 mg/día en verano y 180 mg/día en invierno (Kuhnau, 1976), mientras que en 2006 se estimó en 12,5 mg/día (Wu *et al*., 2006). Debemos destacar que datos de ingesta de antocianinas del año 1971, debido a su antigüedad, podrían contener mayores errores de medida respecto de los actuales, debido a los métodos de determinación de antocianinas utilizados en ese momento. Los 12,5 mg/día de ingesta de antocianinas determinados en Estados Unidos en 2006 son más concordantes con los 23 mg/día/persona de flavonoides totales estimados en Holanda en 1993 (Hertog *et al*., 1993). Estas cantidades podrían elevarse siguiendo las recomendaciones dadas por el Instituto Nacional del Cáncer y el Consejo Nacional de Investigación de los Estados Unidos, quienes recomiendan la ingesta diaria de 5 raciones de frutas y/u hortalizas.

Alimento	Contenido	Unidades	Referencias
Olivas negras	42-228	mg/100 g mf	Mazza y Miniati, 1993.
Cereza	2-450	mg/100 g mf	Mazza y Miniati, 1993; Gao y Mazza, 1995.
Berenjena	8-85	mg/100 g mf	Koponen *et al*., 2007; Wu *et al*., 2006.
Ciruela	2-25	mg/100 g mf	Wu *et al*., 2006.
Col lombarda	322	mg/100 g mf	Wu *et al*., 2006.
Uva tinta	30-750	mg/100 g mf	Lamikanra, 1989.
Vino tinto	16-35	mg/100 mL	Teissedre y Landrault, 2000; Frankel *et al*., 1995; Sánchez-Moreno *et al*., 2003.
Fresa	19-55	mg/100 g mf	Lopes-da-Silva *et al*., 2002.

Tabla 2: Contenido de antocianinas en varios alimentos de origen vegetal (de Pascual-Teresa *et al*., 2010). mf: materia fresca.

1.10.- Efecto de las antocianinas sobre la salud.

Son diversos los estudios que muestran el efecto positivo que la ingesta de antocianinas ejerce sobre la salud. La ingesta diaria de 500 g de fresas durante un mes redujo significativamente el colesterol total, el colesterol asociado a las lipoproteínas de baja densidad, el nivel de triglicéridos y el malondialdehído en suero; sin embargo, el colesterol asociado a las lipoproteínas de alta densidad permaneció sin cambios (Alvarez-Suarez *et al*., 2014).

Las antocianinas también han mostrado propiedades anti-trombóticas (Rechner y Kroner, 2005), antiinflamatorias debido a la inhibición de la prostaglandina EG_2 (Vuorela *et al.,* 2005) e hipoglucémicas, reduciendo complicaciones microcirculatorias tales como cataratas y retinopatías diabéticas (Ghosh y Konishi, 2007). En relación a las propiedades anticancerígenas de las antocianinas, son diversos los estudios experimentales que han demostrado esta actividad tanto en ensayos *in vitro* como en tumores *in vivo*. Sin embargo, los estudios epidemiológicos no han mostrado que el consumo de antocianinas provoque efectos protectores ante el riesgo de cáncer en humanos. Esto puede ser debido a que las cantidades de antocianinas necesarias para provocar los efectos *in vitro* exceden ampliamente las cantidades observadas en el plasma humano (Wang y Stoner, 2008), lo cual es concordante con otros estudios que muestran la baja biodisponibilidad de las antocianinas procedentes de los alimentos (Lapidot *et al*., 1998; Felgines *et al*., 2003; Galvano *et al*., 2007). Por ello, sería interesante centrar las futuras investigaciones en la mejora de la absorción de las antocianinas y/o sus metabolitos, con la finalidad de poder utilizarlas en la prevención del cáncer en humanos (Wang y Stoner, 2008). A este respecto, los recientes estudios de microencapsulación de antocianinas en matrices de grado alimenticio, como proteína de suero o goma arábica, son una opción a la hora de estabilizar las antocianinas y así favorecer su liberación en los lugares de mayor absorción (Betz y Kulozik, 2011; Flores *et al*., 2014).

1.11.- Especificaciones del colorante alimentario antocianinas (E 163).

Según el Reglamento (UE) Nº 231/2012 de la Comisión, de 9 de marzo de 2012, por el que se establecen especificaciones para los aditivos alimentarios que figuran en los anexos II y III del Reglamento (CE) Nº 1333/2008 del Parlamento Europeo y del Consejo,

los colorantes antocianinas (E 163) se clasifican según su intensidad de color y se comercializan en forma de líquido, polvo o pasta rojo púrpura. Los disolventes permitidos para su obtención son el metanol y el etanol, siendo sus residuos máximos en el colorante de 50 y 200 mg/kg respectivamente. En caso de utilizar agua sulfurada como solución extractante, el residuo máximo de SO_2 es 1000 mg/kg por porcentaje de pigmento.

1.12.- Expectativas de futuro.

La evidencia del efecto protector de las antocianinas sobre diversas enfermedades ha generado en la industria alimentaria un gran interés por este colorante natural. Como ya se ha comentado con anterioridad, el problema del uso de este colorante radica en su baja estabilidad y homogeneidad de color. Actualmente, no con la finalidad de aportar o estabilizar el color, sino de mejorar la biodisponibilidad de las antocianinas se investiga en procesos de microencapsulación. Con ello se protege la antocianina, favoreciendo así su liberación en los lugares de mayor absorción en el organismo (Betz y Kulozik, 2011; Flores *et al*., 2014). Por otro lado, la investigación en el desarrollo y aplicación de enzimas de la familia de las antocianidina *O*-metiltransferasas podría conducir a la puesta a punto de técnicas capaces de lograr la sustitución de los grupos hidroxilos de los anillos A y B por grupos metoxilos. Las metoxilaciones no solamente reducen la reactividad de las antocianinas y en consecuencia aumentan su estabilidad, sino que estandarizan su color, puesto que los grupos metoxilo aumentan la tonalidad roja (Cuevas *et al*., 2008). Cualquiera de estas dos medidas, la microencapsulación y la metoxilación, ampliaría el rango de pH de los alimentos susceptibles de ser coloreados con antocianinas.

La ingeniería genética de flavonoides está logrando variar las estructuras de las antocianinas mediante la modificación de las enzimas que las sintetizan. De esta forma, se ha conseguido variar tanto el color como la estabilidad de las mismas. Por ejemplo, ensayos genéticos en flores muestran que mediante el uso de la enzima flavonoide 3´,5´-hidroxilasa (F3`5`H) se cataliza la hidroxilación de las posiciones 3´ y 5´ del anillo B de naringenina y dihidrocanferol para así sintetizar delfinidina 3-glucósido en lugar de peonidina 3-glucósido, obteniendo así flores azules en vez de rojas (**Figura 2**) (Moreau *et al*., 2012). En ensayos *in vitro* se ha logrado sintetizar enzimáticamente malvidina 3-glucósido a partir de petunidina 3-glucósido, aumentando así las tonalidades púrpuras y la estabilidad del color. Para ello, la enzima catecol *O*-metiltransferasa metoxila la posición

5´ del anillo B de petunidina 3-glucósido (Zimman y Waterhouse, 2002). Esta tecnología que actualmente se aplica casi exclusivamente en el campo de la floricultura, en un futuro podría aplicarse en el desarrollo de colorantes alimentarios y así otorgarles una mayor homogeneidad y estabilidad.

UNIVERSITAS Miguel Hernández

2.- Objetivos

2.- OBJETIVOS.

El objetivo principal de la presente Tesis Doctoral ha consistido en el estudio de diferentes materiales vegetales ricos en antocianinas y de su potencial como fuentes de colorantes alimentarios. Para ello se realizó la caracterización de sus antocianinas y la estabilidad de éstas a diferentes condiciones de procesado, tanto en sistemas modelo como en alimentos. Con esta finalidad, se establecieron los siguientes objetivos específicos:

1. Evaluar la capacidad antioxidante, color y cinética de degradación de las antocianinas de seis extractos vegetales (berenjenas, fresas, uvas, arándanos, frambuesas y ciruelas) en soluciones acuosas, con la finalidad de identificar aquellos susceptibles de ser usados como fuente de antocianinas.

2. Evaluar la influencia del pH y temperatura de almacenamiento sobre la estabilidad del color de extractos de fresa, uva y ciruela.

3. Evaluar la influencia del ácido ascórbico y rutina sobre la estabilidad del color de un extracto colorante de ciruela purificado y altamente concentrado en antocianinas.

4. Utilizar zumo concentrado de ciruela como colorante natural en yogur, y evaluar el impacto ejercido sobre sus propiedades físico-químicas y organolépticas.

2.- Publicaciones

3.- PUBLICACIONES.

3.1.- Hernández-Herrero J.A. y Frutos M.J. (2011). Degradation kinetics of pigment, colour and stability of the antioxidant capacity in juice model systems from six anthocyanin sources. *International Journal of Food Science & Technology, 46*, 2550–2557.

International Journal of
Food Science & Technology
www.ifst.org

International Journal of Food Science and Technology 2011, **46**, 2550–2557

Original article

Degradation kinetics of pigment, colour and stability of the antioxidant capacity in juice model systems from six anthocyanin sources

José Antonio Hernández-Herrero & María José Frutos*

Department of Agro-Food Technology. Universidad Miguel Hernández. Ctra. Beniel, km 3.2. 03312 Orihuela (Alicante), Spain

(Received 18 April 2011; Accepted in revised form 1 August 2011)

Summary Coloured model juices with extracts from several species of commonly consumed vegetables rich in anthocyanins (eggplant peel, strawberry, grape, bilberry, red raspberry and plum peel) were studied in detail. The model juices prepared at pH 4.5 were stored in darkness for 17 weeks at 20 °C. The kinetics of anthocyanin degradation, colour and stability of the antioxidant capacity were measured during storage. The anthocyanins were determined identifying delphinidins, cyanidins, petunidins, pelargonidins, peonidins and malvidins. The extraction yields ranged from 2.3% to 13.3%. The level of anthocyanins in the model juices prepared with the extracts ranged between 4 and 158 mg L^{-1}. The results showed a good correlation between the anthocyanin concentration and the time of storage, with determination coefficients varying from $R^2 = 0.9470$ to $R^2 = 0.9855$. The eggplant peel, grape and plum peel anthocyanins showed the highest half-life and D values that were higher than those of 12 and 17 weeks, respectively. The antioxidant capacity showed a high stability during the time of storage for all the model juices, showing the eggplant peel model juice the highest values.

Keywords Bilberry, eggplant, grape, plum, red raspberry, strawberry, vitamin C equivalent antioxidant capacity.

Introduction

Anthocyanins are phenolic compounds that occur widely in the red to blue coloured parts of most plants. Therefore, they can be found in several fruits, such as cherries and strawberries, plums, eggplants, red cabbages and radishes, among others. They are found in nature as anthocyanidin glycosides, being the most common sugars bound to pentoses (xylose and arabinose), one methyl pentose (rhamnose) and two hexoses (glucose and galactose). The main anthocyanidins observed in red fruits are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Horbowicz *et al*., 2008).

Polyphenolic compounds are associated with prevention of diseases induced by oxidative stress, such as cardiovascular diseases, cancer and inflammation. This preventive effect is associated with their ability to act as antioxidants in biological systems as it has been reported for anthocyanins (Marin *et al*., 2002; Stintzing & Carle, 2004; Chen *et al*., 2006; Hui *et al*., 2010;). The demand for processed foodstuffs which are naturally deficient in anthocyanins has been increasing over the last years. The addition of natural extracts of anthocyanins to these foods, together with the consumption of fruits and vegetables would increase the intake of these compounds which might be interesting given their beneficial effects on health.

The aim of this study was to evaluate the antioxidant capacity, colour and anthocyanin stability of six different plant extracts in an aqueous solution at pH 4.5, to simulate their behaviour in low pH foods as yogurt, soft drinks and dressings. All the parameters were analysed during 17 weeks of storage at 20 °C in darkness to perform a comparative study among the extracts, and evaluate their potential as food colouring sources.

Materials and methods

Plant samples

The samples of eggplant (*Solanum melongena* L.) cultivar (cv.) Black bell, strawberry (*Fragaria ananassa* L.) cv. Camarosa, grape (*Vitis vinifera* L.) cv. Moravia, bilberry (*Vaccinium myrtillus*, L.), red raspberry (*Rubus idaeus* L.) cv. Zeva and plum (*Prunus salicina* L.) cv.

*Correspondent: Fax: +34 966749677;
e-mail: mj.frutos@umh.es

doi:10.1111/j.1365-2621.2011.02780.x

© 2011 The Authors. International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

Santa rosa were purchased in the same week, in local supermarkets from Orihuela (Alicante, Spain). The plant materials were stored under refrigerated conditions at the market, and strawberry, bilberry and red raspberry were packaged in perforated polyethylene boxes. The ripe fruits and eggplants were sampled selecting randomly each item and the samples were processed in the laboratory. Eggplant and plum peels were obtained by hand peeling. The fruits and peels were immediately frozen and stored at -20 °C, for 1 month before analysis.

Extraction of anthocyanins

The samples were ground in a Waring blender, and 50 g of the homogenate was extracted with 0.15% HCl in acetone for 4 h in the dark at 4 °C, in a proportion of 1:4 (plant material-solvent). The filter cake residue was re-extracted in the same proportion with aqueous acetone (30:70 v/v) acidified with 0.15% of HCl. Filtrates were combined and vacuum concentrated at 35 °C. The concentrate was resuspended with 20 mL of acidified water with HCl at 0.15% and lyophilised (Christ. Mod. ALPHA 2 4). The lyophilised extracts were stored at -84 °C prior to analysis. Extractions were repeated on three independent samples.

Preparation and storage of model juices

For the stability measurement, the lyophilised extracts were redissolved in 60 mL of buffer solution at pH 4.5 (KH phthalate, HCl 0.1 M) at a concentration of 10 g L^{-1} and were stored in darkness at 20 °C for 17 weeks. This pH is in the range of acid foods as diary fermented products and fruit juices, and does not highly affect to the colour expression of anthocyanins, that decreases at pHs near neutrality (Cabrita *et al.*, 2000). On the other hand the amount of extract used in the experiments (10 g L^{-1}), has given values above three chroma units. The 60 mL of coloured model juices were distributed as follows: for colour measurement, 45 mL were introduced in the chroma-meter tubes, and sealed with parafilm; and 15 mL were kept in 8 screw-cap vials of 2 mL for the analysis of the anthocyanin concentration and antioxidant capacity. The tubes and vials were frozen at −85 °C after their respective storage period. Samples were analysed at 0, 1, 2, 4, 6, 8, 10, 13 and 17 weeks.

Sample preparation for the anthocyanin characterisation

For the HPLC characterisation of the anthocyanins, the lyophilised extracts were dissolved in water:acetone (1:1) acidified with 0.15% HCl (15 mg mL^{-1}) and then filtered through 0.45 μm nylon membranes. The samples were analysed before and after the acid hydrolysis of anthocyanins.

Acid hydrolysis of anthocyanins

Fifteen millilitres of 2 M HCl were added to 1 mg of standard or to ca. 800 mg of extract in a screw-cap test tube, flushed with nitrogen and capped. The pigment was hydrolysed for 45 min at 100 °C, and then cooled in an ice bath (Hong & Wrolstad, 1990a). The anthocyanins were purified using a C-18 Sep-Pak cartridge (Pazmiño-Durán *et al.*, 2001).

High-performance liquid chromatography

The identification of anthocyanidins in plant extracts, and the determination of the anthocyanins in model juices have been performed according to the following HPLC method:

The analysis was performed using a Hewlett-Packard HP 1100 liquid chromatograph equipped with an Agilent Technologies G1315A photodiode array detector with a reverse phase column C18 Waters Spherisorb ODS-1 (250 × 4.6 mm, 5 μm). A mobile phase gradient was used for elution: A, acetonitrile-water (1:1) with 0.5% trifluoroacetic acid; B, acidified water with 0.5% of trifluoroacetic acid. The elution profile was 10% (A) at 0 min, 35% (A) at 10 min, 50% (A) at 20 min, 80% (A) at 21 min, 80% (A) at 23 min and 10% (A) at 25 min. The flow rate was 1 mL min^{-1} with an injection volume of 20 μL. The changes in absorbance were measured in the visible ultraviolet (vis UV) diode array, detector was set at 520 nm. The UV visible spectra of the separated compounds were recorded from 250 to 600 nm. Anthocyanin standards used were: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin 3-*O*-glucosides; aglycone malvidin, 3,5-diglucoside malvidin and cyanidin 3-rutinoside (Poly Laboratories AS, Hanavein, Norway).

Colour analysis

The L^*, a^* and b^* values (CIE 1976) were determined using an A5 Chroma-Meter Minolta CR300, with a liquid tube holder colour space CIELAB. The colour coordinates of the model juices were computed in the CIELAB scale in a CIE D65/10° illuminant/observer condition. Data were mean of nine measurements.

Determination of antioxidant capacity

The method used for the antioxidant capacity of the model juices was the vitamin C equivalent antioxidant capacity (VCEAC) that was expressed as mg of vitamin C equivalents L^{-1} of solution. The 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation was generated by the enzymatic system formed by the hydrogen peroxide (H_2O_2) and the horseradish peroxidase (HRP). The change in absorbance was measured using an UV vis spectrophotometer Hew-

© 2011 The Authors
International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

lett Packard mod. 8453, at a wavelength of 414 nm. The reaction was made mixing 1.5 mM ABTS, 1.18 mM H_2O_2 and 0.25 μM HRP in a glycin-HCl 50 mM (pH 4.5) buffer in a total volume of 2 mL. The antioxidant capacity was quantified using a standard curve of ascorbic acid (0, 1, 2, 3, 4 and 5 nM) (Cano *et al.*, 1998).

Kinetics of anthocyanin degradation

The degradation kinetics of the anthocyanins follow a first-order equation reaction: $C_t = C_0 \exp(kt)$ where C_t and C_0 are the anthocyanin concentrations (mg L^{-1}) at time t and t_0, respectively, k is the first order kinetics constant and t is the storage time (week). Furthermore half-life value ($t_{1/2}$) of total anthocyanin content was calculated as $t_{1/2} = (\ln 1/2)/k$; D value (the time required for the degradation of 90% anthocyanin) was also calculated as $D = -1/k$ (Zhang *et al.*, 2008)

Statistical analysis

Results are presented as mean ± standard deviation. Comparisons means were performed by one-way analysis of variance (ANOVA) followed by Duncan's test ($\rho < 0.05$). Statistical analyses were run using the computer SPSS 14.0 software (SPSS Institute Inc., Cary, NC, USA).

Results and discussion

Extraction yield

The amount of lyophilised extract obtained from the different plant materials varied between 2.3 g/100 g fresh matter in eggplant peel, and 13.3 g/100 g fresh matter in grape, that showed the highest extraction yield (Table 1).

Determination of anthocyanins

The identification of hydrolysed anthocyanidins (aglycones) was made by comparing their UV visible spectra and retention times with the hydrolysed standards. The identification of the anthocyanin glycosides was performed by taking into account its $\lambda_{max\ vis}$, since this depends, on a great extent, on the number of oxygen substituents (hydroxyl or methoxyl) of the anthocyanindin B-ring (Torskangerpoll *et al.*, 1998). The $\lambda_{max\ vis}$ of the six 3-glc standards used in the assays ranged from 502 nm (pelargonidin 3-glucoside) to 528 nm (malvidin 3-glucoside).

Regarding the anthocyanin profile, in strawberry it was only identified pelargonidin 3-glucoside (Table 2). Other studies in strawberry juices indicate the presence of concentrations below 5% of additional anthocyanins as cyanidin 3-glucoside, pelargonidin 3-rutinoside and pelargonidin 3-arabinoside (Kawanobu *et al.*, 2011; Buendía *et al.*, 2010).

In red raspberry extract three peaks were found in the chromatogram before hydrolysis, that were identified as cyanidin derivatives, because in the hydrolysed extract only a peak corresponding to cyanidin aglycon was identified. Two peaks were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside. According to other studies the third peak could be identified as cyanidin 3-sophoroside (Kassim *et al.*, 2009). In plum peel, two peaks were found, corresponding to cyanidin 3-glucoside and cyanidin 3-rutinoside, being the main peaks in most cases according to other research (Kim & Padilla-Zakour, 2004).

The results of the bilberry and grape showed the greatest variety, having four and five anthocyanidins, respectively. In bilberry the anthocyanins found corresponded to 3-glycosides of delphinidin, cyanidin, petunidin and malvidin, with this last anthocyanin being the most abundant form (71%) (Table 2). In other studies it has been also identified peonidin in bilberry (Lätti *et al.*, 2008) but in the lowest proportion in relation with the other anthocyanins. The five aglycones found in grape coincide with those found by other authors, the differences being the percentages of each of them depending

Table 1 Extraction yields (g of extract/100 g fresh weight)

Plant material	Extraction yields
Eggplant peel	2.3±0.3
Strawberry	5.9±0.3
Grape	13.3±1.1
Bilberry	5.0±0.2
Red raspberry	8.5±0.6
Plum peel	9.7±0.8

The data, expressed as %, are displayed with mean ± standard deviation of three replications

Table 2 Anthocyanin 3-glycoside profile, expressed as % (w/w) of the chromatogram peak area

Anthocyanin	%					
Extracts	Dp	Cy	Pt	Pg	Pn	Mv
Eggplant peel	88 + 12*					
Strawberry				100		
Grape	12	16	2		58	12
Bilberry	18	3	8			71
Red raspberry		100				
Plum peel		100				

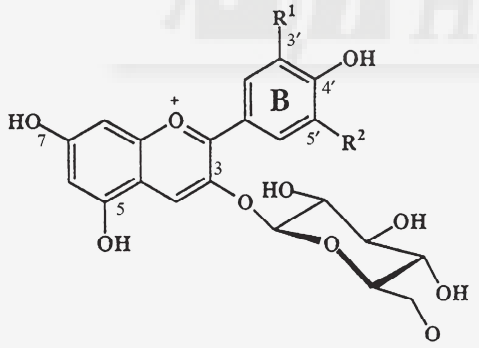
Dp, delphinidin; Cy, cyaniding; Pt, petunidin; Pg, pelargonidin; Pn, peonidin; Mv, malvidin.
*5-glycoside and monoacylated.

© 2011 The Authors
International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

on the variety used and the small amounts of pelargonindin found (Zhao *et al.*, 2010).

The anthocyanins of bilberry extract can be considered as the most stable of all those under study, since it consists mainly of malvidin, which only contains one free hydroxyl moiety in the aglycone B-ring (Fig. 1). Since the methoxyl groups are less reactive than the hydroxyl ones, the methoxylations in B-ring lead to the stabilisation of the anthocyanin (Shrikhande, 1976). The two methoxyl groups also give to the solution a higher stability at alkaline pH (Cabrita *et al.*, 2000).

In eggplant peel extract before hydrolysis, two peaks appeared in the chromatogram that were identified as delphinidin derivatives, as in the hydrolysed extract only a peak corresponding to delphinidin aglycon was found. The highest peak is a delphinidin 3-glycoside that according to the literature could be delphinidin 3-rutinoside (Sadilova *et al.*, 2006). The second one is a monoacylated 3,5-diglycoside and represents the 12% of the chromatogram peak area. These results are based on the information about anthocyanin structure provided by the spectral data. The glycosidic substitution pattern of the anthocyanins can be inferred by absorption in the 400–460 nm region, while the 3-glycosides exhibit ratios of $A_{\lambda 440nm}/A_{\lambda max\ vis}$ between 45 and 60%, an additional glycosilation in C5, lowers the ratio levels to 17–35% (Harborne, 1967; Sachse, 1973). The degree of aromatic acylation is calculated using the $A_{\lambda max\ acyl}/A_{\lambda\ max\ vis}$ ratio. Values of 50–70% indicate monoacylation and a ratio of 90–128% indicates diacylation (Harborne, 1958; Hong & Wrolstad, 1990b).



	R^1	R^2
Pg3glc:	H	H
Cy3glc:	OH	H
Pn3glc:	OCH_3	H
Dp3glc:	OH	OH
Pt3glc:	OCH_3	OH
Mv3glc:	OCH_3	OCH_3

Figure 1 Chemical structure of the six most common anthocyanidin 3-monoglucosides.

In this study all the peaks were anthocyanidins 3-glycosides except for eggplant peel where it has been found additionally a 12% of anthocyanin 3,5-glycoside (Table 2). The chromatogram corresponding to eggplant peel (Fig. 2a) shows two peaks corresponding to 3,5-glycoside monoacylated delphinidin and delphinidin 3-glycoside at retention times of 8.6 and 11.8 min, respectively. This statement is based on the following ratios obtained in this study, 17% ($A_{\lambda 440nm}/A_{\lambda max\ vis}$ ratio) and 50% ($A_{\lambda max\ acyl}/A_{\lambda max\ vis}$ ratio) (Fig. 3). After acid hydrolysis the delphinidin aglycone appears with a delay of 2.7 min compared to Dp 3-gly (Fig. 2b).

Anthocyanin stability

The results showed differences in the anthocyanin concentration after 17 weeks of storage in darkness at 20 °C and pH 4.5 (Fig. 4). The kinetic parameters for the degradation of anthocyanins are showed in Table 3. The determination coefficients obtained ranged from $R^2 = 0.9470$ to $R^2 = 0.9855$ showing a good correlation between the anthocyanin concentration and the time of storage. Similar results were observed in black carrot anthocyanins during the storage at different temperatures (Kirca *et al.*, 2006).

The extracts can be classified according to their kinetic parameters during storage ($t_{1/2}$ and D value). The highest stabilities were showed by the eggplant peel, grape and plum peel anthocyanins, that presented the highest half-life and D values, that were higher than those of 12 and 17 weeks, respectively (Table 3). The lower stability was showed by the strawberry model juice, with values of half-life and D of 2.8 and 4.0 weeks respectively. In pomegranate juice stored at 20 °C at pH 3.2 (Alighourchi & Barzegar, 2009) a half-life of 6.4 weeks and D value of 9.3 weeks, were found. These values are between those found in bilberry and red raspberry (Table 3). The eggplant peel extracts showed higher stability than expected, due to their anthocyanin composition consisting on delphinidins (Table 2), with groups –OH in the B ring (Fig. 1), and therefore less stable. However, it was found a 12% of monoacylated delphinidins with an additional glycosilation in C5 that improves the water solubility and contributes to the stabilisation of the pigment, due to the neutralisation of another hydroxyl group and the formation of sandwich-like stacking complexes (Dangles *et al.*, 1993). On the other hand, the acylation of one of its *O*-glycosides with an aromatic acid also improves its colour and stability (Malien-Aubert *et al.*, 2001).

It should be pointed out that the plum peel model juice, besides its high stability, showed the highest initial anthocyanin concentration among all the solutions under study (158 mg L^{-1}) (Fig. 4). The losses in antho-

© 2011 The Authors
International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

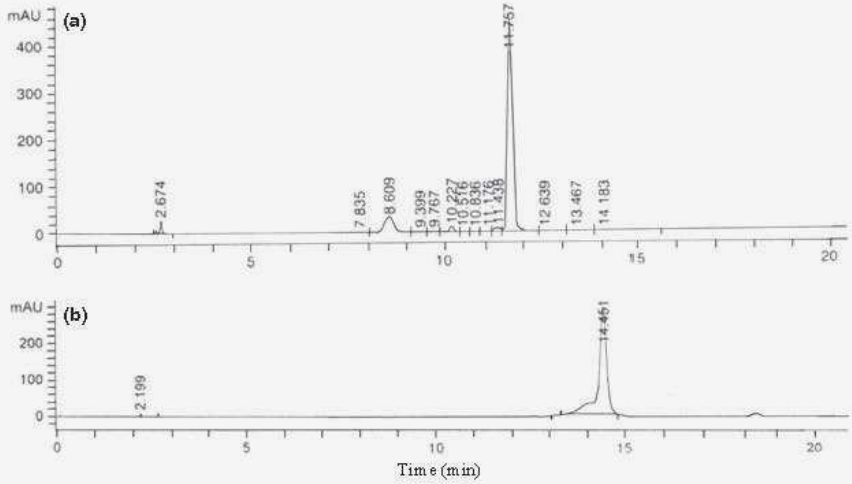


Figure 2 Representative chromatogram for eggplant peel extract. (a) Before acid hydrolysis. (b) After acid hydrolysis.

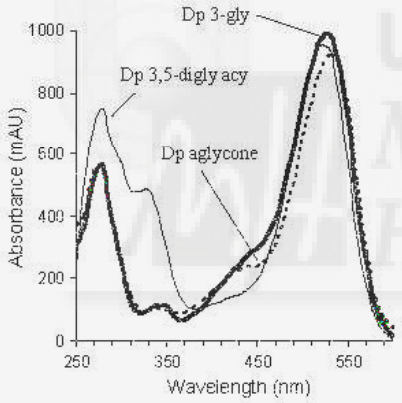


Figure 3 Spectral characteristics of eggplant peel anthocyanins, indicating glycosilation and acylation patterns. Delphinidin (Dp), 3-glycoside (3-gly), 3,5-diglycoside (3,5-digly), monoacylated (acy).

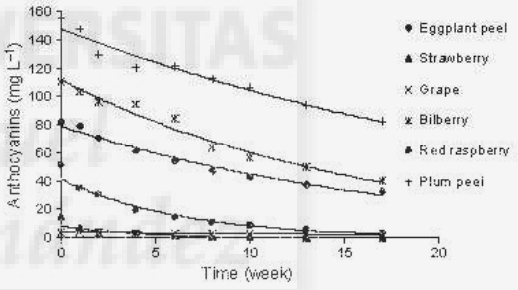


Figure 4 Monomeric anthocyanins concentrations during storage.

cyanin concentrations were of 47% in plum peel, 61% in eggplant peel and 64% in bilberry, that are similar to the reported losses of 58% in red radish anthocyanins in juice model systems at pH 3.5 after 17 weeks of storage at 25 °C (Rodríguez-Saona *et al.*, 1999). In reconstituted pomegranate juice at pH 3.2 during 17 weeks at 20 °C storage, losses of 75% were observed (Alighourchi & Barzegar, 2009). The losses in anthocyanins found in the solutions made with red raspberry extract (90%) are similar to those observed in red raspberry jam at pH 3.3 (93%), in the same storage conditions (García-Viguera *et al.*, 1998). In strawberry, the losses in anthocyanin content found were of 81% and 99% after 4 and 17 weeks of storage respectively. Those results agreed with the losses of the 90% reported in strawberry juice (pH 3.5) after 4 weeks of storage at 25 °C (Garzón & Wrolstad, 2002).

Colour stability

In the storage experiment it was used the pH 4.5 that was adequate for a good colour expression, according to previous published data on the colour and stability of six common anthocyanins in aqueous solutions (Cabrita *et al.*, 2000).

The colour data for lightness (L^*) (Fig. 5) showed a high stability of this parameter during the storage for all the solutions studied. The initial L^* was between 26.59 and 29.56 with higher values for the strawberry and grape solutions. The L^* value only showed low increases

© 2011 The Authors
International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

Table 3 Kinetic parameters for the degradation of anthocyanins during storage of model juices

Solutions	Variation kinetics	R^2	$t_{1/2}$ (weeks)	D value (weeks)
Eggplant peel	$y = 79.28 \exp(-0.057x)$	0.9791	12.2^{ab}	17.5^{ab}
Strawberry	$y = 8.28 \exp(-0.247x)$	0.9470	2.8^{d}	4.0^{d}
Grape	$y = 3.97 \exp(-0.029x)$	0.9540	23.6^{a}	34.0^{a}
Bilberry	$y = 111.97 \exp(-0.062x)$	0.9767	11.2^{b}	16.1^{b}
Red	$y = 41.48 \exp(-0.158x)$	0.9855	4.4^{c}	6.3^{c}
Plum peel	$y = 147.59 \exp(-0.035x)$	0.9624	20.0^{a}	28.9^{a}

Values with the same letter in each column are not significantly different at the level of $p > 0.05$.

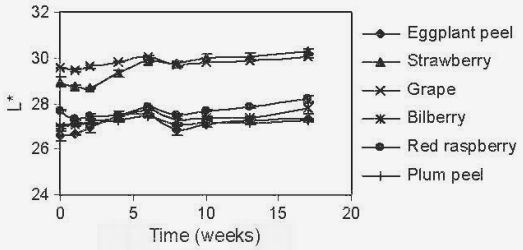


Figure 5 Lightness (L^*) of the different model juices during storage.

(1 4%) in all cases during the storage. Similar values were reported for coloured jelly (pH 3.0) with 600 mg L^{-1} of red radish anthocyanins, with increases of 9% after 12 weeks of storage at 25 °C (Cai & Corke, 1999). However, other data on model juices with 150 mg L^{-1} of purified anthocyanins from red radish (pH 3.5) showed increases of 7% after 18 weeks at 25 °C (Rodríguez-Saona *et al.*, 1999). In other cases, as in reconstituted pomegranate juice (pH 3.2) during 17 weeks at 20 °C storage (Alighourchi & Barzegar, 2009) and in canned strawberries (brine pH 3.6) (Kammerer *et al.*, 2007), losses of 10% in L^* were found.

The initial hue values (H^*) ranged between 30° (orange/red colour) and 340° (purple/red colour) (Fig. 6). The H^* value for eggplant peel varied from 340° to 355°, approaching to pure red (360°). The initial purplish colour of the eggplant peel solutions is due to its hydroxylation pattern (Fig. 1) that induces a bathochromic shift, moreover the acylation and additional 5-glycosylation of the 12% of the anthocyanins (Table 2) brings about a slight shift to red purple (Stintzing & Carle, 2004; Sadilova *et al.*, 2006). The strawberry H^* value increased from 31° to 70° during the 17 weeks of storage losing its red colour. Similar initial hue angle for strawberry was found by other authors in canned strawberries (brine pH 3.6) (Kammerer *et al.*, 2007) and pasteurised strawberry juices (pH 3.5) with increases after 4 weeks of storage at 25 °C from 38° to 70° (Garzón & Wrolstad, 2002), being this higher increase mainly due to the thermal treatment. The data for grape, bilberry, red raspberry and plum peel, showed a high stability with a maximum rate of variation for hue angle of 9°. Similar little increases were observed in the same period of storage in model juices (pH 3.5) coloured with 150 mg L^{-1} of red radish and stored at 25 °C (Rodríguez-Saona *et al.*, 1999), and in reconstituted pomegranate juice (pH 3.2) during 17 weeks at 20 °C storage (Alighourchi & Barzegar, 2009). In jelly coloured with red radish (pH 3) stored for 12 weeks the hue angle increased in 20°, and therefore showing important loses in colour (Cai & Corke, 1999).

The initial values for colour intensity (C^*) were different among the samples under study (Fig. 7). The coloured solution with plum peel showed the higher stability, with a slight decrease of a 6% during the

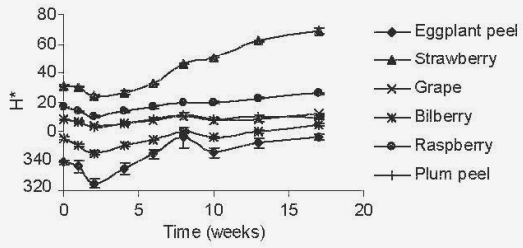


Figure 6 Hue angle (H^*) of the different model juices during storage.

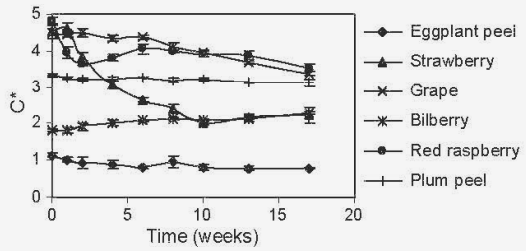


Figure 7 Colour intensity (C^*) of the different model juices during storage.

© 2011 The Authors
International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

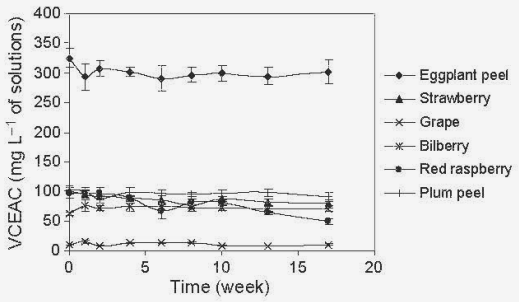


Figure 8 Antioxidant capacity of the different model juices during storage.

17 weeks of storage at 20 °C. A similar stability for colour intensity was observed in red radish coloured jelly (Cai & Corke, 1999) and coloured juices (Rodríguez-Saona *et al.*, 1999), but under storage temperatures of 4 °C (12 weeks) and 2 °C (17 weeks), respectively, while in the samples stored at 25 °C, the decrease in colour intensity was higher with loses of 70% and 40% in both cases. The higher decrease in C* (50%) during the storage was shown by the strawberry model juice. After 4 weeks it was observed a decrease of a 32%, and similar results were found in strawberry juices in the same period of storage at 25 °C with loses of a 40% (Garzón & Wrolstad, 2002). The coloured solutions with extracts of eggplant peel, grape and red raspberry, showed decreases between 25% and 30%, similar to those observed (25%) in canned strawberries (Kammerer *et al.*, 2007). Lower losses (14%) were detected in reconstituted pomegranate juice during 17 weeks at 20 °C storage (Alighourchi & Barzegar, 2009). However, the bilberry model juice showed an increase in the colour intensity of a 25%. This behaviour could be due to copigmentations with other compounds improving the colour and its stability as it has been reported by other authors (Malien-Aubert *et al.*, 2001).

Antioxidant capacity stability

The VCEAC initial values showed a wide difference among the samples studied, and ranged from 10 mg of vitamin C equivalent L^{-1} of solution in grape to 325 mg L^{-1} in eggplant peel (Fig. 8). The rest of model juices presented VCEAC values between 60 and 100 mg L^{-1}. The structural variations in anthocyanins seem to influence antioxidant activities (Stinzing & Carle, 2004). All the extracts under study showed a high stability, except for the red raspberry model juice with a VCEAC value decrease of a 50% after 17 weeks of storage at 20 °C.

The highest antioxidant capacity found in eggplant peel is in agreement with that observed in eggplant extracts that showed the most potent superoxide anion radical scavenging activity (SOD-like activity) among other vegetables. The anthocyanin found in eggplant peel was delphinidin that has three consecutive OH groups in the B-ring what confers this high antioxidant capacity (Noda *et al.*, 2000).

When the results obtained for anthocyanin content are compared to those of colour and VCEAC, it is observed that the losses in anthocyanins during the storage are much higher than those observed for colour and VCEAC. The decrease in monomeric anthocyanin concentrations could be explained as a consequence of their degradation and/or their copigmentation with other organic compounds, mainly other phenols. Copigmentations usually occur through C C links in the C-4 position of the anthocyanin structure, originating coloured complexes of higher stability, without affecting the antioxidant capacity, because the B ring is not modified by this link (Lu & Foo, 2001; Burda & Oleszek, 2001). On the other hand, the plant extracts used in this study contained anthocyanins among other polyphenols that could also contribute to the stability of the VCEAC.

Conclusion

It has been observed a high stability in the evolution of the antioxidant capacity in the six anthocyanin sources, except for red raspberry, during the 17 weeks of storage at 20 °C. It should be pointed out the high antioxidant capacity of eggplant peel, being up to three times higher than that found in plum peel. Great correlations were found between the anthocyanin concentrations and the time of storage in these conditions, showing the results of the degradation kinetics values of half-life and *D* value higher than those of 12 and 17 weeks, respectively, for the model juices of eggplant peel, grape and plum peel.

The results demonstrate higher colour stabilities and better antioxidant properties for the plum peel, bilberry and eggplant peel extracts showing a high potential for their use in the food industry as a colouring source in the red range, improving the food healthy properties of foods as yoghourts, jams and juices.

Acknowledgments

This research was supported by the Consellería de Cultura y Educación de la Generalitat Valenciana under project GV00-087-13. Assistance of Graham Arnold during manuscript preparation is also acknowledged.

References

Alighourchi, H. & Barzegar, M. (2009). Some physicochemical characteristics and degradation kinetic of anthocyanin of reconsti-

© 2011 The Authors
International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

tuted pomegranate juice during storage. *Journal of Food Engineering*, **90**, 179–185.
Buendía, B., Gil, M.I., Tudela, J.A., Gady, A.L., Medina, J.J., Soria, C., López, J.M. & Tomás-Barberán, F.A. (2010). HPLC-MS Analysis of proanthocyanidin oligomers and other phenolics in 15 strawberry cultivars. *Journal of Agricultural and Food Chemistry*, **58**, 3916–3926.
Burda, S. & Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry*, **49**, 2774–2779.
Cabrita, L., Fossen, T. & Andersen, Ø.M. (2000). Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chemistry*, **68**, 101–107.
Cai, Y. & Corke, H. (1999). *Amaranthus* betacyanin pigments applied in model food systems. *Journal of Food Science*, **64**, 869–873.
Cano, A., Hernández-Ruíz, J., García-Cánovas, F., Acosta, M. & Arnao, M.B. (1998). An end-point method for estimation of the total antioxidant activity in plant material. *Phytochemical Analysis*, **9**, 196–202.
Chen, T., Hwang, H., Rose, M.E., Nines, R.G. & Stoner, G.D. (2006). Chemopreventive properties of black raspberries in *N*-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis: down-regulation of cyclooxygenase-2, induciblenitric oxide synthase, and c-Jun. *Cancer Research*, **66**, 2853–2859.
Dangles, O., Saito, N. & Brouillard, R. (1993). Anthocyanin intramolecular copigment effect. *Phytochemistry*, **34**, 119–124.
García-Viguera, C., Zafrilla, P., Artés, F., Romero, F., Abellán, P. & Tomás-Barberán, F.A. (1998). Colour and anthocyanin stability of red raspberry jam. *Journal of the Science of Food and Agriculture*, **78**, 565–573.
Garzón, G.A. & Wrolstad, R.E. (2002). Comparison of the stability of pelargonidin-based anthocyanins in strawberry juice and concentrate. *Journal of Food Science*, **67**, 1288–1299.
Harborne, J.B. (1958). The chromatographic identification of anthocyanin pigments. *Journal of Chromatography*, **1**, 473–488.
Harborne, J.B. (1967). *Comparative Biochemistry of the Flavonoids*. London, UK: Academic Press.
Hong, V. & Wrolstad, R.E. (1990a). Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *Journal of Agricultural and Food Chemistry*, **38**, 708–715.
Hong, V. & Wrolstad, R.E. (1990b). Characterization of anthocyanin-containing colourants and fruit juices by HPLC/photodiode array detection. *Journal of Agriculture and Food Chemistry*, **38**, 698–708.
Horbowicz, M., Kosson, R., Grzesiuk, A. & Dębski, H. (2008). Anthocyanins of fruits and vegetables – their occurrence, analysis and role in human nutrition. *Vegetable Crops Research Bulletin*, **68**, 5–22.
Hui, C., Bin, Y., Xiaoping, Y., Long, Y., Chunye, C., Mantian, M. & Wenhua, L. (2010). Anticancer activities of an anthocyanin-rich extract from black rice against breast cancer cells *in vitro* and *in vivo*. *Nutrition and Cancer*, **62**, 1128–1136.
Kammerer, D.R., Schillmöller, S., Maier, O., Schieber, A. & Carle, R. (2007). Colour stability of canned strawberries using black carrot and elderberry juice concentrates as natural colourants. *European Food Research and Technology*, **224**, 667–679.
Kawanobu, S., Zushi, K., Wajima, T., Chikushi, J., Mori, T., Kondo, K. & Matsuzoe, N. (2011). The effect of dark treatment on anthocyanin composition and content of strawberry fruit. *Journal of Food Agriculture & Environment*, **9**, 325–328.
Kassim, A., Poette, J., Paterson, A., Zait, D., McCallum, S., Woodhead, M., Smith, K., Hackett, C. & Graham, J. (2009). Environmental and seasonal influences on red raspberry anthocyanin antioxidant contents and identification of quantitative traits loci (QTL). *Molecular Nutrition & Food Research*, **53**, 625–634.
Kim, D.O. & Padilla-Zakour, O.I. (2004). Jam processing effect on phenolics and antioxidant capacity in anthocyanin-rich fruits: Cherry, plum, and raspberry. *Journal of Food Science*, **69**, S395–S400.
Kirca, A., Özkan, M. & Cemeroglu, B. (2006). Stability of black carrot anthocyanins in various fruit juices and nectars. *Food Chemistry*, **97**, 598–605.
Lätti, A.K., Riihinen, K.R. & Kainulainen, P.S. (2008). Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus*, L.) in Finland. *Journal of Agricultural and Food Chemistry*, **56**, 190–196.
Lu, Y. & Foo, L.Y. (2001). Antioxidant activities of poly-phenols from sage (*Salvia officinalis*). *Food Chemistry*, **75**, 197–202.
Malien-Aubert, C., Dangles, O. & Amiot, M.J. (2001). Colour stability of commercial anthocyanin-based extracts in relation to the phenols composition. Protective effects by intra- and intermolecular copigmentation. *Journal of Agricultural and Food Chemistry*, **49**, 170–176.
Marin, F.R., Frutos, M.J., Pérez-Alvarez, J.A., Martinez-Sanchez, F. & Del Rio, J.A. (2002). Flavonoids as nutraceuticals: structural related antioxidant properties and their role on ascorbic acid preservation. In: *Studies in natural products chemistry* (edited by A. Rahman). Pp. 741–778. Amsterdam, NL: Elsevier.
Noda, Y., Kneyuki, T., Igarashi, K., Mori, A. & Packer, L. (2000). Antioxidant activity of nasunin, an anthocyanin in eggplant peels. *Toxicology*, **148**, 119–123.
Pazmiño-Durán, E.A., Giusti, M.M., Wrolstad, R.E. & Glória, M.B.A. (2001). Anthocyanins from banana bracts (*Musa X paradisiaca*) as potential food colorants. *Food Chemistry*, **73**, 327–332.
Rodríguez-Saona, L.E., Giusti, M.M. & Wrolstad, R.E. (1999). Colour and pigment stability of red redish and red-fleshed potato anthocyanins in juice model systems. *Journal of Food Science*, **64**, 451–456.
Sachse, J. (1973). Anthocyane in den kartoffelsorten Urgenta und Desiree. *Zietung für Lebensmittel Untersuhung und Foschung*, **153**, 294–300.
Sadilova, E., Stintzing, F.C. & Carle, R. (2006). Anthocyanins, colour and antioxidant properties of eggplant (*Solanum melongena* L.) and violet pepper (*Capsicum annuum* L.) peel extracts. *Zeitschrift für Naturforschung C*, **61**, 527–535.
Shrikhande, A.J. (1976). Anthocyanins in foods. *CRC Critical Reviews in Food Science & Nutrition*, **7**, 193–218.
Stintzing, F.C. & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology*, **15**, 19–38.
Torskangerpoll, K., Børve, K.J., Andersen, Ø.M. & Sæthre, L. (1998). Colour substitution pattern in anthocyanidins, a combined quantum chemical-chemometrical study. *Spectrochimica Acta A*, **55**, 761–771.
Zhang, Y., Hu, X.S., Chen, F., Wu, J.H., Liao, X.J. & Wang, Z.F. (2008). Stability and color characteristics of PEF-treated cyaniding-3-glucoside during storage. *Food Chemistry*, **106**, 669–676.
Zhao, Q., Duan, C.Q. & Wang, J. (2010). Anthocyanins profile of grape berries of *Vitis amurensis*, its hybrids and their wines. *International Journal of Molecular Sciences*, **11**, 2212–2228.

© 2011 The Authors
International Journal of Food Science and Technology © 2011 Institute of Food Science and Technology

3.2.- Hernández-Herrero J.A. y Frutos M.J. (2014). Colour and antioxidant capacity stability in grape, strawberry and plum peel model juices at different pHs and temperatures. *Food Chemistry, 154*, 199-204.

Food Chemistry 154 (2014) 199–204

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Colour and antioxidant capacity stability in grape, strawberry and plum peel model juices at different pHs and temperatures

J.A. Hernández-Herrero, M.J. Frutos *

Agro-Food Technology Department, Miguel Hernández University, Ctra. Beniel, km. 3.2, 03312 Orihuela (Alicante), Spain

ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form 31 December 2013
Accepted 3 January 2014
Available online 10 January 2014

Keywords:
Anthocyanins
Natural colouring
Antioxidant capacity
Storage
Total phenolics

ABSTRACT

The aim of the study was to evaluate the anthocyanin sources potential as natural food colouring. Model juices (MJs) of grape, strawberry and plum peel were stored during 8 weeks in darkness at 6 and 23 °C. The colour stability was evaluated at pH 2.4, 3.1, 4.0, 5.0, 6.0, 7.0 and 8.1, and the antioxidant capacity at pH 4.0. The plum peel MJ showed the best and more stable colour parameters. The grape MJs presented a high stability although the chroma values were very low. The storage of strawberry MJ demonstrated a low stability at 23 °C. The vitamin C equivalent antioxidant capacity in grape, strawberry and plum peel MJs was 7, 40 and 50 mg/L, respectively. The strawberry MJ stability was very low. Total phenolics and anthocyanins ranged between 18–101 and 2–62 mg/L of MJ, respectively. The determination coefficient was $R^2 = 0.745$ between the antioxidant capacity and total phenolics.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Anthocyanins are the biggest group of water-soluble natural pigments of plants. They are responsible for the attractive colours of flowers, fruits (particularly berries) and vegetables (Strack & Wray, 1993). Consumer concern about the safety of synthetic food colourings has increased the demand for natural colourings. Especially, there is a growing demand for natural food colourings as alternatives to the synthetic red colourings, for example FD&C Red #40 (Giusti & Wrolstad, 2003). Anthocyanins are the best-known natural red colouring used in food (Bridle & Timberlake, 1997). A major impediment to the use of these natural colourings is their inherent instability, either in simple aqueous solutions or in complex food formulations. Anthocyanins exhibit greater stability under acidic conditions, but under normal processing and storage conditions readily convert to colourless hemiketal equilibrium forms of anthocyanins and subsequently to insoluble brown pigments. A number of factors influence anthocyanin stability, including pH, heat-humidity, light, oxygen, enzymes, as well as the presence of ascorbic acid, sugars, sulfur dioxide or sulfite salts, metal ions and copigments (Francis, 1989; Marin, Frutos, Pérez-Alvarez, Martinez-Sanchez, & Del Rio, 2002). In addition to natural colouring properties, interest in anthocyanins has intensified because of their possible role in hypertension prevention and reducing the risk of coronary heart disease, cancer and stroke (Huang, Davidge, & Wu, 2013; Hui et al., 2010).

Strawberry, grape and plum are plant materials rich in phenolic compounds, whose colours are due to anthocyanin pigments. Plums may be good sources of natural food colouring, owing to their high level of polyphenolic compounds, antioxidant activity (Gallego, García-Carpintero, Sánchez-Palomo, Hermosín-Gutiérrez, & Viñas, 2012; Kim, Chun, Kim, Moon, & Lee, 2003) and colour stability, when compared with other plant materials (Hernández-Herrero & Frutos, 2011). Strawberries are a good source of ascorbic acid and anthocyanins, and they have a high antioxidant activity, but there are losses due to processing and storage, mainly at temperatures above 16 °C (Cordenunsi et al., 2005). Red grape skin is the largest natural commercial source for anthocyanins (Downham & Collins, 2000), because of the amount of anthocyanins remaining in grape skins after winemaking (García-Beneytez, Revilla, & Cabello, 2002).

The aim of the study was to evaluate the potential of strawberry, grape and plum peel extracts as natural functional food colourings in different conditions, mainly through the determination of colour and antioxidant capacity stability during storage at different pHs and temperatures.

2. Materials and methods

2.1. Plant material

The strawberry (*Fragaria ananassa* L.) cultivar (cv.) *Camarosa*, grape (*Vitis vinifera* L.) cv. *Moravia* and plum (*Prunus salicina* L.) cv. *Santa rosa* were purchased in local supermarkets from Orihuela (Alicante, Spain). The plant materials were stored under

* Corresponding author. Tel.: +34 966749744; fax: +34 966749677.
E-mail address: mj.frutos@umh.es (M.J. Frutos).

0308-8146/$ - see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2014.01.007

refrigerated conditions at the market. Strawberry was packaged in perforated polyethylene boxes. Plum peels were obtained by hand peeling. The fruits and peels were immediately frozen and stored at −20 °C, for 1 month before analysis.

2.2. Extract preparation

The samples were ground in a Waring blender, and then 50 g of the homogenate were extracted with 0.15% HCl in acetone for 4 h in the dark at 4 °C, in a proportion of 1:4 (plant material–solvent). The filter cake residue was re-extracted in the same proportion with aqueous acetone (30:70 v/v) acidified with 0.15% of HCl. Filtrates were combined and vacuum concentrated at 35 °C. The concentrate was resuspended with 20 mL of acidified water with HCl at 0.15% and lyophilised (Christ. Mod. ALPHA 2–4). The lyophilised extracts were stored at −84 °C prior to analysis (Hernández-Herrero & Frutos, 2011). Extractions were repeated on three independent samples.

2.3. Preparation and storage of model juices (MJ)

The lyophilised extracts were dissolved in buffer solution at different pHs (Table 1) at a concentration of 4 g/L. Each buffer solution was kept in parafilm sealed tubes in darkness at 6 and 23 °C. The 65 mL of each MJ were distributed as follows: for colour measurement, 45 mL were introduced in the chroma-meter tubes, and sealed with parafilm; and 18 mL were kept in 9 screw-cap vials of 2 mL for the analysis of the anthocyanin contents and antioxidant capacity. The tubes and vials were frozen at −85 °C after their respective storage period. The measurement of colour stability were analysed at 0, 1, 2, 5, 7 days, 2, 4, 6 and 8 weeks, at the 2 temperatures (6 and 23 °C) and at the 7 different pHs. The antioxidant capacity stability was analysed at 0, 2, 4, 6 and 8 weeks at 2 temperatures and at pH 4.0.

2.4. Total phenolics and anthocyanin analysis

The identification of total phenolics and anthocyanins in model juices have been performed using a Hewlett–Packard HP 1100 liquid chromatograph equipped with an Agilent Technologies G1315A photodiode array detector with a reverse phase column C18 Waters Spherisorb ODS-1 (250 mM × 4.6 mM i.d., 5 μm particle size). Four pumps (A, B, C, and D) were used for mixing the mobile phase to avoid pressure fluctuations due to the mixing of methanol (MeOH) in water. Formic acid (5%) was added to both water and methanol to increase peak resolution before preparing the following mobile phases: 95% water + 5% methanol (A); 88% water + 12% MeOH (B); 20% water + 80% MeOH (C); and MeOH (D). All solvents were high performance liquid chromatography (HPLC) grade. Elution started with 100% A, which remained isocratic until 5 min. A gradient was then used to reach 100% B at 10 min, held isocratic for 3 more minutes. From 13 to 35 min a linear gradient was used to reach 75% B and 25% C, and then 50% B and 50% C at 50 min, and 100% C at 52 min, then maintained isocratic until 57 min. The column was then washed with 100% D at 60 min. The flow rate was 1 mL/min with an injection volume of 20 μL. The chromatograms were recorded at 520, 340 and 280 nm (Tomás-Barberán et al., 2001). The UV spectra of the different compounds were recorded with a diode array detector. The standards used were cyanidin 3-*O*-glucoside (Polyphenols Laboratories AS, Sandness, Norway), quercetin 3-rutinoside (Merck, Darmstadt, Germany) and chlorogenic acid (HPLC grade, Sigma–Aldrich, Poole, Dorset, UK).

Table 1
Solvent proportions (v/v) used for the seven different buffer solutions in the pH region 2.4–8.1.

pH	KH ftalato 0.1 M	KH_2PO_4 0.1 M	HCl 0.1 M	NaOH 0.1 M
2.4	100		103.7	
3.1	100		46.0	
4.0	100		0.3	
5.0	100			45.6
6.0		100		13.9
7.0		100		59.3
8.1		100		97.1

2.5. Determination of colour stability

Colour measurements were determined using an A5 Chroma-Meter Minolta CR300 (Minolta Co. Ltd., Osaka, Japan), with a liquid tube holder colour space CIELAB. The colour coordinates of the model juices were computed in the CIELAB scale in a CIE D65/10° illuminant/observer condition. Colour results were expressed as tristimulus parameters (L^*, a^*, b^*, H^*, C^*). Hue angle ($H^* = \tan^{-1} b^*/a^*$) indicates sample colour (0° or 360° = red, 90° = yellow, 180° = green, 270° = blue), and chroma ($C^* = [a^{*2} + b^{*2}]^{1/2}$) indicates colour purity or saturation (high values are more vivid). a^* and b^* chromaticity coordinates indicate colour directions green ($-a^*$)/red ($+a^*$) and blue ($-b^*$)/yellow ($+b^*$) (Bakker, Bridle, & Timberlake, 1986). For each sample 10 measurements were made.

2.6. Determination of antioxidant capacity stability

The method used for the antioxidant capacity of the model juices was the vitamin C equivalent antioxidant capacity (VCEAC) that was expressed as mg of vitamin C equivalents L^{-1} of solution. The 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation was generated by the enzymatic system formed by the hydrogen peroxide (H_2O_2) and the horseradish peroxidase (HRP). The change in absorbance was measured using an UV–vis spectrophotometer Hewlett–Packard mod. 8453, at a wavelength of 414 nm. The reaction was made mixing 1.5 mM ABTS, 1.18 mM H_2O_2 and 0.25 μM HRP in a glycin–HCl 50 mM (pH 4.5) buffer in a total volume of 2 mL. The antioxidant capacity was quantified using a standard curve of ascorbic acid (0, 1, 2, 3, 4 and 5 nM) (Cano, Hernández-Ruíz, García-Cánovas, Acosta, & Arnao, 1998).

2.7. Statistical analysis

Results are presented as mean ± standard deviation. Means comparisons were performed by one-way analysis of variance (ANOVA) followed by Duncan's test ($P \leqslant 0.05$). Statistical analyses were run using the computer SPSS 20.0 software (SPSS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Total phenolics and anthocyanins

Total phenolics and anthocyanin values ranged between 18–101 and 2–62 mg/L of MJ, respectively (Table 2). The grape MJ are the ones with the lower total phenolic and anthocyanin contents. Other previous studies with several table grapes varieties demonstrated that the *Moravia* cv. also showed the lower anthocyanin contents (Carreño, Almela, Martínez, & Fernández-López, 1997).

In previous studies with the same vegetable materials, the extraction yields were of 13.3, 5.9 and 9.7 g/100 g fresh weight

Table 2
Total phenolics and anthocyanin contents in model juices (mg/L). The data are displayed as mean ± standard deviation of three replications.

Extract	Total phenolics	Anthocyanins
Strawberry	42 ± 2	6 ± 0
Grape	18 ± 1	2 ± 0
Plum peel	101 ± 6	62 ± 4

for grape, strawberry and plum peel extracts, respectively (Hernández-Herrero & Frutos, 2011). Considering the above mentioned extraction yield (EY) and the amount of plum peel extract used to elaborate the MJ (4 g/L, as it is described in Section 2), the phenolic and anthocyanin contents in plant material on a fresh weight basis (X) can be calculated:

$$X(\text{mg}/100\,\text{g}) = \text{Concentration in MJ}\,(\text{mg}/100\,\text{g})\,\text{EY}\,(\text{g}/100\,\text{g})\,4\,(\text{g/L})^{-1}.$$

The phenolic content in plum peel MJ (101 mg/L) (Table 2) corresponds to a concentration of 246 mg/100 g of fresh plums peels. Other authors have reported phenolic concentrations in fresh plum from 136 to 372 mg/100 g (Kim et al., 2003). For strawberry, the anthocyanin concentration in MJ corresponds to 9 mg/100 g fresh weight. This anthocyanin concentration has been lower than the 32–48 mg/100 g found by other authors in the same variety of strawberry (Lopes-da-Silva, Escribano-Bailón, Perez-Alonso, Rivas-Gonzalo, & Santos-Buelga, 2005). In previous research on strawberry juices, it has been found that there was a variation in anthocyanin composition and content depending on the strawberry cultivar (Bakker, Bridle, & Bellworthy, 1994).

The total phenolics content in grape MJ (18 mg/L) corresponds to 60 mg/100 g in fresh grape, that is similar to the values determined in other studies for the same variety (Gallego et al., 2012).

As it can be stated from the results, the plum peel MJ were those with higher total phenolics and anthocyanins respect to those of grape and strawberry.

3.2. Colour stability

3.2.1. Effect of pH on colour parameters

The grape MJ showed the H^* values closest to 0°, and therefore to red colour followed by plum peel MJ and strawberry. Nevertheless the C^* value in grape MJ is the lowest among the three MJ (Fig. 1). The MJ elaborated with plum peel showed the highest C^* value. The results indicate that plum peel extracts have the best colouring properties.

As it has been determined in many studies, the colour and the anthocyanin structure depends on the pH of the solution (Brouillard, 1988; Malien-Aubert, Dangles, & Amiot, 2001). At pH values of 2.4 and 3.1, the colour showed by the strawberry MJ was orange, while for the grape and plum peel MJ was red. This is due to the fact that at low pHs the anthocyanins exist as a flavylium ion. As the pH is raised to values between 4 and 6, anthocyanins exist as colourless hemiketal forms (Brouillard, 1988; Malien-Aubert et al., 2001). At pH 4.0, 5.0 and 6.0 the 3 MJ studied showed a deviation of the H^* to purplish colour (270°), an important decrease in C^* and an increase in L^*. At pH 5.0 and 6.0 it has been detected the highest colour losses, so that at pH 5.0 the H^* decrease to 10°, 0° and 327° for the strawberry, plum peel and grape MJ, respectively.

According to the literature, at pHs higher than 6.0 the anthocyanin exists as purplish quinoid bases (Brouillard, 1988; Malien-Aubert et al., 2001). The results at pH 7.0 and 8.1 showed, respect to pH 5.0 and 6.0, a slight increase in C^* and a change to H^* values corresponding to orange and red colour. It is observed that the C^* values (Fig. 1) in model juices increase as the anthocyanin concentrations raise (Table 2). It has also been reported that colour

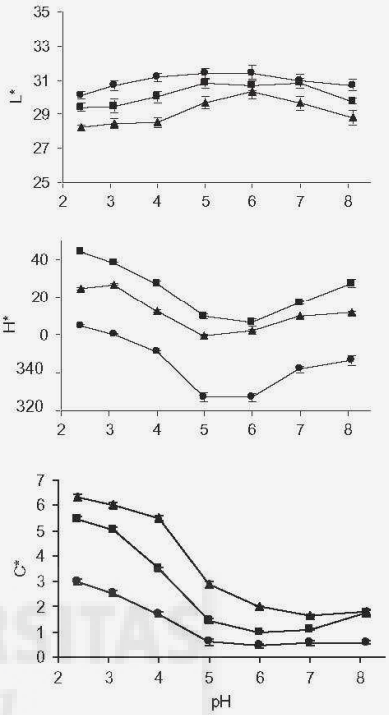


Fig. 1. Changes in lightness (L^*), hue angle (H^*) and chroma (C^*) in different pHs of model juices. Strawberry (■), grape (●) and plum peel juices (▲). Bars represent the standard deviations.

stability in strawberry juices depend on the different anthocyanin patterns of each cultivar or breeding line (Bakker et al., 1994).

The results obtained in this assay are similar to those obtained by other authors in studies of pure anthocyanins at different pHs (Cabrita, Fossen, & Andersen, 2000; Torskangerpoll & Andersen, 2005).

3.2.2. Effect of storage at different pHs and temperatures on H^* colour parameter

Fig. 2 shows the H^* results obtained at different pHs during the 8 weeks of storage in darkness at 6 and 23 °C. The H^* values decrease at pHs near neutrality as it has been also observed in the literature (Cabrita et al., 2000). The results with more stability and nearer to 0° (red colour) at both storage temperatures were shown by the plum peel, strawberry and grape MJ. The highest stability of the plum peel MJ was also found in model juices at pH 4.5 during storage in darkness at 20 °C (Hernández-Herrero & Frutos, 2011). The highest H^* stability was showed at the lowest pHs (2.4 and 3.1). The initial values of plum peel MJ are the ones nearer to 0°, and ranged between 26.89° (pH 3.1) and 0.1° (pH 5.0). It should be pointed out the high stability to temperature showed by the plum peel MJ at pH 3.1, as the increase in H^* at the end of the 8 weeks of storage were of a 5% and 8% at 6 and 23 °C, respectively. These variations are in agreement with the results in reconstituted pomegranate juice (pH 3.1) during 60 days of storage with increases of 0% at 4 °C and 8% at 20 °C (Alighourchi & Barzegar, 2009). It has also been reported that plum pulps lose their colour as a result of thermal treatment, but only at higher temperatures and times (Lozano & Ibarz, 1997). In strawberry beverages, it has also been observed an increase in total colour difference after heating at 85 °C that was higher for longer treatment times (Mollov,

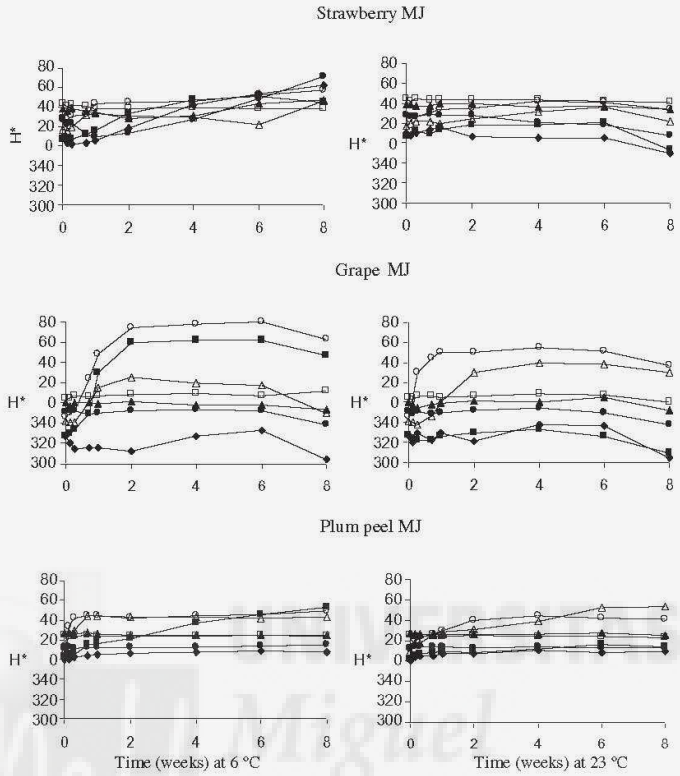


Fig. 2. Changes in hue angle (H^*) in model juices during 8 weeks of storage in darkness at 6 and 23 °C, and different pHs: 2.4 (□), 3.1 (▲), 4.0 (●), 5.0 (♦), 6.0 (■), 7.0 (Δ) and 8.1 (○).

Mihalev, Shikov, Yoncheva, & Karagyozov, 2007). Therefore the mechanisms involved in colour changes during storage temperatures in anthocyanin rich MJs are different to those involved during high temperature thermal processing.

3.2.3. Effect of storage at different temperatures on colour parameters at pH 4.0

At pH 4.0, the L^* value shows a high stability of all the MJ and temperatures (Fig. 3). The highest values were found in grape and strawberry stored at 23 °C while plum peel MJ presented the lowest ones. The stability for all the MJs during the 8 weeks of storage at both temperatures (6 and 23 °C) was very high, with variations of ca. 2%. The samples stored at 6 °C showed the lower L^* values according to the higher C^* values. The stability of the results at 6 °C are similar to those found in model juices coloured with potato and radish anthocyanins and pH adjusted to 3.5 during storage a 2 °C. In this study, when the storage temperature was of 25 °C, the increases in L^* were of a 7–8% and the C^* value decreased in the same way (Rodríguez-Saona, Giusti, & Wrolstad, 1999). In other studies with model juices at pH 4.5 and stored at 20 °C it was also found a similar stability in L^* with variations lower than 3% (Hernández-Herrero & Frutos, 2011). In reconstituted pomegranate juice (pH 3.1) during 60 days of storage at 4 and 20 °C the losses in L^* were of a 5% and 10%, respectively (Alighourchi & Barzegar, 2009).

The H^* values of grape and plum peel MJ stored at 6 °C showed the highest stabilities with variations lower than the 4%. At this temperature the strawberry MJ showed a decrease from 27° to 7° (74%). This change to values closer to red ($H^* = 0°$) occurs at the same time of a decrease in C^*, and therefore this means a colour deterioration. The stability results at 23 °C, show that the grape MJ is the more stable with variations of 3%. The H^* values of plum peel MJ present a 21% of increase up to 15°. The strawberry MJ suffers a variation of 161%, reaching values of $H^* = 72°$, near to yellow ($H^* = 90°$). This H^* value is similar to the 71.1° determined in strawberry juices (pH 3.5) after 103 days (14.7 weeks) of storage at room temperature (Rein & Heinonen, 2004).

The C^* losses at 6 °C in grape, strawberry and plum peel MJ were 18%, 24% and 0%, while at 23 °C were 32%, 75% and 15%, respectively. The results in plum peel MJ are similar to those found in reconstituted pomegranate juice (pH 3.1) during 60 days of storage at 4 and 20 °C with losses of 0% and 11%, respectively (Alighourchi & Barzegar, 2009).

These results show the deleterious effect of temperature and anthocyanins content on the colour of MJs (Figs. 2 and 3 and Table 2). Other authors have also found a good correlation between colour and anthocyanin content at different temperatures, indicating that total visual colour from CIELAB parameters can be used as an index of product quality (Ahmed, Shivhare, & Raghavan, 2004). In storage studies of plum juices, it was observed higher anthocyanin losses after 90 days of storage at 20 °C than after 300 days at 4 °C (Will & Dietrich, 2006). These results corroborate the importance of the storage temperature on the colour stability in anthocyanin rich juices.

3.3. Antioxidant capacity

The grape MJ clearly showed the lower antioxidant capacity with an initial value of 7 mg/L of VCEAC, while strawberry and plum peel MJs showed 40 and 50 mg/L, respectively (Fig. 4).

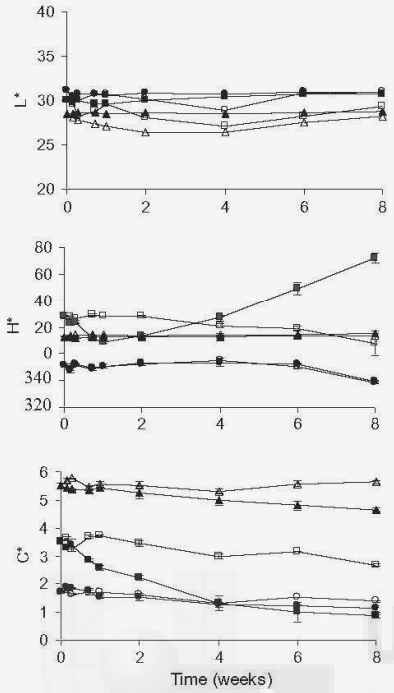


Fig. 3. Changes in lightness (L^*), hue angle (H^*) and chroma (C^*) in model juices at pH 4.0 during 8 weeks of storage in darkness at 6 and 23 °C. Strawberry (■), grape (●) and plum peel model juices (▲). Filled symbols represent room temperature (23 °C) treatments, open symbols represent refrigerated (6 °C) treatments. Bars represent the standard deviations.

However, the antioxidant capacity in the grape MJ showed the highest stability, without significative differences ($P \leqslant 0.05$) between 23 and 6 °C during the 8 weeks of storage. The strawberry MJ showed a high antioxidant capacity decrease (23%) during the first 2 weeks of storage at 23 °C. This result is significatively different ($P \leqslant 0.05$) when compared with the value at 6 °C after this storage period. The high losses in colour and antioxidant capacity showed in strawberry MJ at 23 °C, are in agreement with the data reported by other authors that reported loses in strawberry anthocyanin and antioxidant activities due to processing and storage mainly at temperatures above 16 °C (Cordenunsi et al., 2005). The plum skin MJ showed the highest antioxidant capacity

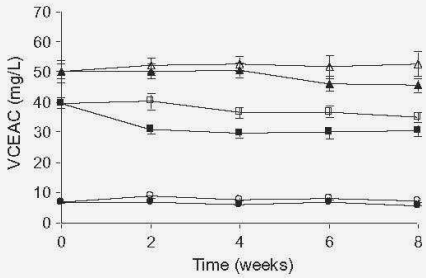


Fig. 4. Antioxidant capacity of model juices at pH 4.0 during 8 weeks of storage in darkness at 6 and 23 °C and pH 4.0. Strawberry (■), grape (●) and plum peel model juices (▲). Filled symbols represent room temperature (23 °C) treatments, open symbols represent refrigerated (6 °C) treatments. Bars represent the standard deviations.

together with high temperature stability, because the differences among temperatures are observed after 6 weeks of storage.

The VCEAC values in plum peel, grape and strawberry are similar to those found in other storage studies in darkness with model juices (20 °C and pH 4.5), taking into consideration that the concentration in that case was higher (10 g of extract/L) (Hernández-Herrero & Frutos, 2011). The extraction yield in fresh plum peel was 9.7/100 g (Hernández-Herrero & Frutos, 2011). Therefore from this data, the 50 mg of CVEAC/L of MJ correspond to 118 mg of VCE/100 g fresh plum peel. This amount is lower to that observed in fresh plum, with values ranging from 205 to 567 mg of VCE/100 g fresh plum depending on the cultivar (Kim, Chun, Kim, Moon, & Lee, 2003).

The antioxidant capacity in fruits and vegetables originates mainly from phenolic compounds and ascorbic acid (Miller & Rice-Evans, 1997). It has been observed a low correlation of the VCEAC and total phenolics results in MJs, with the equation $y = 0.4535x + 7.8725$, and a determination coefficient of $R^2 = 0.745$ (where y = total phenolics and x = antioxidant capacity).

The antioxidant capacity depends in a great extent on the polyphenolic composition, but also on other compounds, therefore when different plant materials are used the determination coefficient resulting from the correlation is not necessarily high. However when only one plant material is used, higher determination coefficients have usually been found. In previous studies, with different plum cultivars, the correlations of total phenols and antioxidant capacity found $R^2 = 0.977$ (Kim, Chun, Kim, Moon, & Lee, 2003). It has also been reported, that the correlation of total phenols and antioxidant capacity in several anthocyanin-rich materials (blueberries, cherries, red onion scales, purple sunflower hulls and purple potatoes) was $R^2 = 0.462$ (Velioglu, Mazza, Gao, & Oomah, 1998).

4. Conclusions

The colour of model juices remained stable during the 8 weeks of storage in darkness at refrigerated (6 °C) and room temperature (23 °C) conditions. Therefore the storage temperature was not a decisive factor for the colour preservation, except for the strawberry model juice that suffered extensive losses at 23 °C. The plum peel model juices showed the higher contents in total phenolics, anthocyanins and antioxidant capacity and can be stored during 4 weeks at room temperature without significative decreases in the antioxidant capacity. The colour of plum peel model juices was stable up to pH 5.0 during the 8 weeks of storage at 6 and 23 °C.

It should be highlighted that the plum peel extract could be an interesting functional natural colouring considering the colour stability to pH and temperature, as well as its antioxidant capacity.

Acknowledgement

This research was supported by the Conselleria de Cultura y Educación de la Generalitat Valenciana under Project GV00-087-13.

References

Ahmed, J., Shivhare, U. S., & Raghavan, G. S. V. (2004). Thermal degradation kinetics of anthocyanin and visual colour of plum puree. *European Food Research and Technology, 218*, 525–528.

Alighourchi, H., & Barzegar, M. (2009). Some physicochemical characteristics and degradation kinetic of anthocyanin of reconstituted pomegranate juice during storage. *Journal of Food Engineering, 90*, 179–185.

Bakker, J., Bridle, P., & Bellworthy, S. J. (1994). Strawberry juice color – A study of the quantitative and qualitative pigment composition of juices from 39 genotypes. *Journal of the Science of Food and Agriculture, 64*, 31–37.

Bakker, J., Bridle, P., & Timberlake, C. F. (1986). Tristimulus measurements (CIELAB 76) of port wines. *Vitis, 25*, 67–78.

Bridle, P., & Timberlake, C. F. (1997). Anthocyanins as natural food colours-selected aspects. *Food Chemistry, 58*(12), 103–109.
Brouillard, R. (1988). Flavonoids and flower colour. In J. B. Harborne (Ed.), *The flavonoids. Advances in research since 1980* (pp. 525). London: Chapman and Hall Inc.
Cabrita, L., Fossen, T., & Andersen, Ø. M. (2000). Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chemistry, 68*, 101–107.
Cano, A., Hernández-Ruíz, J., García-Cánovas, F., Acosta, M., & Arnao, M. B. (1998). An end-point method for estimation of the total antioxidant activity in plant material. *Phytochemical Analysis, 9*, 196–202.
Carreño, J., Almela, L., Martínez, A., & Fernández-López, J. A. (1997). Chemotaxonomical classification of red table grapes based on anthocyanin profile and external colour. *Lebensmittel Wissenschaft und Technologie, 30*, 259–265.
Cordenunsi, B. R., Genovese, M. I., Nascimento, J. R. O., Hassimotto, N. M. A., Santos, R. J., & Lajolo, F. M. (2005). Effects of temperature on the chemical composition and antioxidant activity of three strawberry cultivars. *Food Chemistry, 91*, 113–121.
Downham, A., & Collins, P. (2000). Colouring our foods in the last and next millennium. *International Journal of Food Science and Technology, 35*(1), 5–22.
Francis, F. J. (1989). Food colorants: Anthocyanins. *Critical Reviews in Food Science and Nutrition, 28*, 273–314.
Gallego, M. A. G., García-Carpintero, E. G., Sánchez-Palomo, E., Hermosín-Gutiérrez, I., & Viñas, M. A. G. (2012). Study of phenolic composition and sensory properties of red grape varieties in danger of extinction from the Spanish region of Castilla-La Mancha. *European Food Research and Technology A, 234*, 295–303.
García-Beneytez, E., Revilla, E., & Cabello, F. (2002). Anthocyanin pattern of several red grape cultivars and wines made from them. *European Food Research and Technology, 215*, 32–37.
Giusti, M. M., & Wrolstad, R. E. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal, 14*(3), 217–225.
Hernández-Herrero, J. A., & Frutos, M. J. (2011). Degradation kinetics of pigment, colour and stability of the antioxidant capacity in juice model systems from six anthocyanin sources. *International Journal of Food Science & Technology, 46*, 2550–2557.
Huang, W. Y., Davidge, S. T., & Wu, J. P. (2013). Bioactive natural constituents from food sources – Potential use in hypertension prevention and treatment. *Critical Reviews in Food Science and Nutrition, 53*(6), 615–630.
Hui, C., Bin, Y., Xiaoping, Y., Long, Y., Chunye, C., Mantian, M., et al. (2010). Anticancer activities of an anthocyanin-rich extract from black rice against breast cancer cells *in vitro* and *in vivo*. *Nutrition and Cancer, 62*, 1128–1136.
Kim, D.-O., Chun, O. K., Kim, Y. J., Moon, H.-Y., & Lee, C. Y. (2003). Quantification of polyphenolics and their antioxidant capacity in fresh plums. *Journal of Agricultural and Food Chemistry, 51*, 6509–6515.
Lopes-da-Silva, F., Escribano-Bailón, M. T., Perez-Alonso, J. J., Rivas-Gonzalo, J. C., & Santos-Buelga, C. (2005). Anthocyanin pigments in strawberry. *Food Science and Technology, 40*(2), 374–382.
Lozano, J. E., & Ibarz, A. (1997). Colour changes in concentrated fruit pulp during heating at high temperatures. *Journal of Food Engineering, 31*, 365–373.
Malien-Aubert, C., Dangles, O., & Amiot, M. J. (2001). Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 49*, 170–176.
Marin, F. R., Frutos, M. J., Pérez-Alvarez, J. A., Martinez-Sanchez, F., & Del Rio, J. A. (2002). Flavonoids as nutraceuticals: Structural related antioxidant properties and their role on ascorbic acid preservation. In Atta-ur-Rahman (Ed.), *Studies in natural products chemistry* (pp. 741–778). Amsterdam, NL: Elsevier Inc.
Miller, N. J., & Rice Evans, C. A. (1997). Factors influencing the antioxidant activity determined by the ABTS^{+} radical cation assay. *Free Radical Research, 26*, 195–199.
Mollov, P., Mihalev, K., Shikov, V., Yoncheva, N., & Karagyozov, V. (2007). Colour stability improvement of strawberry beverage by fortification with polyphenolic copigments naturally occurring in rose petals. *Innovative Food Science and Emerging Technologies, 8*, 318–321.
Rein, M. J., & Heinonen, M. (2004). Stability and enhancement of berry juice color. *Journal of Agricultural and Food Chemistry, 52*, 3106–3114.
Rodríguez-Saona, L. E., Giusti, M. M., & Wrolstad, R. E. (1999). Color and pigment stability of red radish and red-fleshed potato anthocyanins in juice model systems. *Journal of Food Science, 64*(3), 451–456.
Strack, D., & Wray, V. (1993). The anthocyanins. In J. B. Harborne (Ed.), *The flavonoids: Advances in research since 1986* (pp. 1–22). London: Chapman and Hall Inc.
Tomás-Barberán, F. A., Gil, M. A., Cremin, P., Waterhouse, A. L., Hess-Pierce, B., & Kader, A. A. (2001). HPLC–DAD–ESIMS analysis of phenolic compounds in nectarines, peaches, and plums. *Journal of Agricultural and Food Chemistry, 49*, 4748–4760.
Torskangerpoll, K., & Andersen, Ø. M. (2005). Colour stability of anthocyanins in aqueous solutions at various pH values. *Food Chemistry, 89*, 427–440.
Velioglu, Y. S., Mazza, G., Gao, L., & Oomah, B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry, 46*, 4113–4117.
Will, F., & Dietrich, H. (2006). Optimised processing technique for colour- and cloud-stable plum juices and stability of bioactive substances. *European Food Research and Technology, 223*, 419–425.

3.3.- Hernández-Herrero J.A. y Frutos M.J. (2014). Influence of rutin and ascorbic acid in colour, plum anthocyanins and antioxidant capacity stability in model juices. *Food Chemistry* **(enviado)**.

UNIVERSITAS Miguel Hernández

Elsevier Editorial System(tm) for Food Chemistry
Manuscript Draft

Manuscript Number: FOODCHEM-D-14-01983

Title: Influence of rutin and ascorbic acid in colour, plum anthocyanins and antioxidant capacity stability in model juices

Article Type: Research Article (max 7,500 words)

Keywords: VCEAC; Prunus salicina L., Quercetin 3-rutinoside, Copigmentation, Cyanidin 3-glucoside.

Corresponding Author: Dr. MARIA JOSE FRUTOS, PhD in Biology

Corresponding Author's Institution: MIGUEL HERNANDEZ UNIVERSITY

First Author: JOSE ANTONIO HERNÁNDEZ-HERRERO, Ing. Sc. Agronomy

Order of Authors: JOSE ANTONIO HERNÁNDEZ-HERRERO, Ing. Sc. Agronomy; MARIA JOSE FRUTOS, PhD in Biology

Abstract: Model juices at pH 3.7 were prepared with different combinations of ascorbic acid, rutin (quercetin 3-rutinoside) and anthocyanin purified extract of plums (cv. Black Gold). The anthocyanins in the purified extract were cyanidin 3-glucoside and cyanidin 3-rutinoside, in a proportion of 76 and 24% respectively. The model juices were stored during 17 weeks in darkness at 20 ºC. The colour stability was improved by the presence of rutin and strongly damaged by the ascorbic acid. The fortification of anthocyanin model juices with ascorbic acid originated the degradation of most of anthocyanins. However, anthocyanins improved ascorbic acid stability during storage. The copigmentation of anthocyanin and rutin showed a beneficial effect on colour stability from the five weeks of storage. In model juices prepared exclusively with purified plum extract it was found a positive relationship (R2= 0,881) between anthocyanins and vitamin C equivalent antioxidant capacity (VCEAC).

*Manuscript
Click here to view linked References

Influence of rutin and ascorbic acid in colour, plum anthocyanins and antioxidant capacity stability in model juices

J.A. Hernández-Herrero, M.J. Frutos*

Department of Agro-Food Technology, Universidad Miguel Hernández. Ctra. Beniel, km. 3.2. CP 03.312 Orihuela (Alicante), Spain.

***Corresponding Author**

Dr. María José Frutos Fernández
Department of Agro-Food Technology, Universidad Miguel Hernández, Ctra. Beniel, km. 3.2. CP. 03.312 Orihuela (Alicante) Spain.
e-mail: mj.frutos@umh.es
Tel. +34 966749744
Fax. +34 966749677

Running title: Effect of rutin and vitamin C on plum anthocyanins stability

21

22 **ABSTRACT**

23

24 Model juices at pH 3.7 were prepared with different combinations of ascorbic acid, rutin
25 (quercetin 3-rutinoside) and anthocyanin purified extract of plums (cv. Black Gold). The
26 anthocyanins in the purified extract were cyanidin 3-glucoside and cyanidin 3-rutinoside,
27 in a proportion of 76 and 24% respectively. The model juices were stored during 17 weeks
28 in darkness at 20 ºC. The colour stability was improved by the presence of rutin and
29 strongly damaged by the ascorbic acid. The fortification of anthocyanin model juices with
30 ascorbic acid originated the degradation of most of anthocyanins. However, anthocyanins
31 improved ascorbic acid stability during storage. The copigmentation of anthocyanin and
32 rutin showed a beneficial effect on colour stability from the five weeks of storage. In
33 model juices prepared exclusively with purified plum extract it was found a positive
34 relationship (R^2= 0,881) between anthocyanins and vitamin C equivalent antioxidant
35 capacity (VCEAC).

36

37 **KEYWORDS**

38 VCEAC; *Prunus salicina* L., Quercetin 3-rutinoside, Copigmentation, Cyanidin 3-
39 glucoside.

40

41

42 **1. Introduction**

43

44 Nowadays, consumers prefer natural food additives against the synthetic ones,
45 including food colourings. Therefore, it is interesting to evaluate their behavior in different
46 food matrix. Anthocyanins are a natural colouring in the red range that have a high
47 antioxidant capacity, that contributes to beneficial effects in reducing the risk of heart
48 disease, cancer and stroke (Heo, Kim, Chung & Kim, 2007; Hui *et al.*, 2010; Huang,
49 Davidge & Wu, 2013). Plums may be good sources of food colourings, due to the high
50 level of anthocyanins and other phenolic compounds (Kim, Chun, Kim, Moon & Lee,
51 2003). Previous studies have reported that plums show higher colour levels and stability
52 compared to other plant sources as grape, bilberry, eggplant, strawberry and red raspberry
53 (Hernández-Herrero & Frutos, 2011).

54 Anthocyanins are unstable to different environmental conditions, as pH, light, vitamin
55 C, oxygen and water activity among others (Malien-Aubert, Dangles & Amiot, 2001;
56 Wrolstad, Durst & Lee, 2005; Sun, Bai, Zhang, Liao & Hu, 2011). The most limiting factor
57 is pH and for this reason, anthocyanin based food colourings are been exclusively used in
58 low pH foods, as juices, yoghurts and soft drinks (Aryana, Barnes, Emmick, Mcgrew &
59 Moser, 2006; De Rosso & Mercadante, 2007; Hernández-Herrero & Frutos, 2014a).
60 Ascorbic acid is other limiting factor, and can be found in many food products, including
61 fruit juices, either naturally or as an antioxidant additive (Elliott, 1999), preventing
62 browning and improving the nutritional value (Freedman & Francis, 1984). Therefore, the
63 presence of ascorbic acid could also reduce the number of foods where anthocyanins can
64 be applied as food colouring.

It has been previously reported that the colour of anthocyanins can be stabilized by the presence of other phenolic compounds through intermolecular copigmentation (Malien-Aubert, Dangles & Amiot, 2001; Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez & Galan-Vidal, 2009; Cavalcanti, Santos & Meireles, 2011; He *et al.*, 2012). Flavonols as quercetin-3-rutinoside are the most efficient copigments (Mazza & Miniati, 1993).

The main aim of this study was to investigate, in model juices with plum anthocyanin extract as colouring, the influence of ascorbic acid and quercetin 3-rutinoside (rutin) on the colour and antioxidant capacity stability.

2. Materials and methods

2.1. Plant material

Plums (*Prunus salicina* L.) cv. Black Gold, were purchased in local supermarkets from Orihuela (Alicante, Spain). The plant materials were stored under refrigerated conditions at the market.

2.2. Extract preparation

The samples were ground in a Waring blender, and then 14 g of the homogenate were extracted with 0.15% HCl in acetone for 4 h in the dark at 4 ºC, in a proportion of 1:4 (plant material-solvent). The filter cake residue was re-extracted in the same proportion with aqueous acetone (30:70 v/v) acidified with 0.15% of HCl. Filtrates were combined and vacuum concentrated at 35 ºC. The concentrate was resuspended with 20 mL of

90 acidified water with HCl at 0.15% and lyophilised (Christ. Mod. ALPHA 2–4). The
91 lyophilised extracts were stored at -84 ºC prior to analysis. Extractions were repeated on
92 three independent samples.
93
94 *2.3. Purification of phenolic compounds*
95
96 The extract was purified by an extraction in solid phase. The extract (aproxx. 1 g) was
97 resuspended with 10 mL of acidified water with HCl at 0.15%. The C-18 Sep-Pack
98 cartridge (Agilent Technologies) was activated passing through 10 mL of acidified
99 methanol (0.15% of HCl), followed by 10 mL of acidified water (0.15% of HCl). The
100 resuspended extract (10 mL) was also passed through and the phenolic compounds were
101 absorbed onto cartridge. Sugars, organic acids and other water-soluble compounds were
102 washed with 20 mL of acidified water (0.15% of HCl). The phenolic compounds were
103 recovered with 20 mL acidified methanol (0.15% of HCl). The methanolic extract was
104 vacuum concentrated at 35 ºC to dryness leaving a red residue.
105
106 *2.4. Concentrated anthocyanin extract (CAE) preparation*
107
108 The red residue was resuspended with 30 mL of dietil ether and centrifugated at 15,000
109 x g for 15 min at 4 ºC. The red precipitate contained the anthocyanins due to its insolubility
110 in this solvent. The red precipitate obtained was liquid nitrogen powdered, obtaining
111 aproxx. 20 mg of CAE powder. This extract was used for the preparation of model juices.
112
113 *2.5. Preparation and storage of model juices (MJ)*
114

115 The plum CAE powder, ascorbic acid (AA) and rutin (R) standard were dissolved in
116 buffer solution (KH Ftalato-HCl 0.1 M) at pH 3.7 containing 0.1% (w/v) of sorbic acid
117 (Sigma-Aldrich Co., St. Louis, MO). Therefore, the following MJs were prepared:
118 concentrated anthocyanin extract (CAE) juice, concentrated anthocyanin extract plus
119 ascorbic acid (CAE+AA) juice, concentrated anthocyanin extract plus rutin (CAE+R)
120 juice, ascorbic acid (AA) juice and rutin (R) juice (Table 1).
121 The 65 mL of each MJ were distributed as follows: for colour measurement, 45 mL
122 were introduced in the chroma-meter tubes, and sealed with parafilm; and 18 mL were kept
123 in 9 screw-cap vials of 2 mL for the analysis of the anthocyanin, rutin and ascorbic acid
124 contents, and antioxidant capacity. MJs were stored during 17 weeks in darkness at 20 ºC.
125 The sample vials were frozen at -85 ºC after their respective storage period. The analyses
126 of colour, rutin, anthocyanin and antioxidant capacity were performed at 0, 1, 3, 5, 7, 9, 11,
127 14 and 17 weeks of storage.
128
129 *2.6. Determination of colour stability*
130
131 Colour measurements were determined using an A5 Chroma-Meter Minolta CR300
132 (Minolta Co. Ltd., Osaka, Japan), with a liquid tube holder colour space CIELAB. The
133 colour coordinates of the model juices were computed in the CIELAB scale in a CIE
134 D65/10º illuminant/observer condition. Colour results were expressed as tristimulus
135 parameters (L*, a*, b*, H*, C*). Hue angle ($H^* = \tan^{-1} b^*/a^*$) indicates sample colour (0º
136 or 360º= red, 90º= yellow, 180º= green, 270º= blue), and chroma ($C^* = [a^{*2} + b^{*2}]^{1/2}$)
137 indicates colour purity or saturation (high values are more vivid). a* and b*chromaticity
138 coordinates indicate colour directions green (-a*)/red (+a*) and blue (-b*)/yellow (+b*)
139 (Baker, Bridle & Timberlake, 1986). For each sample 10 measurements were made.

140

141 *2.7. Anthocyanin and rutin analysis*

142

143 The identification of anthocyanins and quercetin 3-rutinoside in MJ has been
144 performed according to the following high performance liquid chromatography (HPLC)
145 method:

146 The analysis was performed using a Hewlett-Packard HP 1100 liquid chromatograph
147 equipped with an Agilent Technologies G1315A photodiode array detector with a reverse
148 phase column C18 Waters Spherisorb ODS-1 (250 mm x 4.6 mm, 5 μm). A mobile phase
149 gradient was used for elution: A, acetonitrile-water (1:1) with 0.5% trifluoroacetic acid; B,
150 acidified water with 0.5% of trifluoroacetic acid. The elution profile was 10% (A) at 0 min,
151 35% (A) at 10 min, 50% (A) at 20 min, 80% (A) at 21 min, 80% (A) at 23 min and 10%
152 (A) at 25 min. The flow rate was 1 mL/min with an injection volume of 20 μL. The
153 changes in absorbance were measured in the visible–ultraviolet (vis–UV) diode array. The
154 anthocyanins were analyzed at 520 nm and rutin at 360 nm. The UV–visible spectra of the
155 separated compounds were recorded from 250 to 600 nm. Anthocyanins were quantified as
156 cyanidin 3-glucoside and cyanidin 3-rutinoside and rutin was quantified as quercetin 3-
157 rutinoside (Polyphenols Laboratories, AS, Sandness, Norway). The standards were
158 dissolved in methanol acidified with 0.15% of HCl.

159

160 *2.8. Determination of antioxidant capacity stability*

161

162 The method used for the antioxidant capacity of the plum MJs was the vitamin C
163 equivalent antioxidant capacity (VCEAC) that was expressed as mg of vitamin C
164 equivalents (VCE)/100 mL of solution. The 2,2′-azino-bis (3-ethylbenzthiazoline-6-

165 sulphonic acid) (ABTS) radical cation was generated by the enzymatic system formed by
166 the hydrogen peroxide (H_2O_2) and the horseradish peroxidase (HRP). The change in
167 absorbance was measured using an UV–vis spectrophotometer Hewlett-Packard mod.
168 8453, at a wavelength of 414 nm. The reaction was made mixing 1.5 mM ABTS, 1.18 mM
169 H_2O_2 and 0.25 μM HRP in a glycin-HCl 50 mM (pH 4.5) buffer in a total volume of 2 mL.
170 The antioxidant capacity was quantified using a standard curve of ascorbic acid (0, 1, 2, 3,
171 4 and 5 nM) (Cano, Hernández-Ruíz, García-Cánovas, Acosta & Arnao, 1998).
172
173 *2.9. Statistical analysis*
174
175 Results are presented as mean ± standard deviation. Means comparisons were
176 performed by one-way analysis of variance (ANOVA) followed by Duncan's test ($P \leq$
177 0.05). Statistical analyses were run using the computer SPSS 20.0 software (SPSS Institute
178 Inc., Cary, NC, USA).
179
180 **3. Results and discussion**
181
182 *3.1. Determination of colour stability*
183
184 The lightness (L*) of the three samples had an stable behaviour, because they presented
185 a variation lower than 3.5% after 17 weeks of storage (Fig. 1). Other anthocyanin rich
186 juices (at pH 4.5) have showed similar L* losses after 17 weeks at 20 ºC (Hernández-
187 Herrero & Frutos, 2011).
188 The evolution of the hue angle (H*) during the first seven weeks of storage in CAE
189 juice is similar to the CAE+R (Fig. 1). From the week seven, it can be observed a high

190 increase in the H* value of CAE MJ against the CAE+R juices. This result indicates that
191 the effects of copigmentation on the hue angle are shown from this storage period.
192 Moreover, other studies have shown that copigmentation take place in a progressive way
193 (Gómez-Miguez, González-Manzano, Escribano-Bailón, Heredia & Santos-Buelga, 2006;
194 González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón & Santos-Buelga, 2009).
195 The CAE+AA juices did not show the stability of the CAE and CAE+R MJs in the first
196 weeks, and suffered high colour losses from the third week reaching a value of H*= 56º.
197 These results demonstrate a deleterious immediate effect of ascorbic acid on the colour.
198 Many previous studies have demonstrated the deleterious effect of ascorbic acid on the
199 anthocyanins and therefore on the juice colour (Garzon y Wrolstad, 2002; Lopes, Richard,
200 Saucier, Teissedre, Monti & Glories, 2007; Sun, Bai, Zhang, Liao & Hu, 2011). The initial
201 H* values of the three samples (CAE, CAE+R, CAE+AA) are similar to those showed by
202 solutions of elderberry purified extracts (cyanidin 3-glucoside) at pH 3.5, and lower than
203 the blackberry ones (cyanidin 3-glucoside) at pH 3.5 (Stintzing, Stintzing, Carle, Frei &
204 Wrolstad, 2002) and strawberry juices (pelargonidin 3-glucoside) at pH 3.4 (Garzón &
205 Wrolstad, 2002). It has been previously reported that the colouring power in the red range
206 due to the pelargonidin 3-glucoside is lower than cyanidin 3-glucoside (Cabrita, Fossen &
207 Andersen, 2000). The H* increases in CAE juices are higher to those observed for the
208 same storage period in other anthocyanin rich products as strawberry marmelades (García-
209 Viguera, Zafrilla, Artés, Romero, Abellán & Tomás-Barberán, 1998). The higher stability
210 observed in marmelades can be due to other factors that preserve the anthocyanin colour
211 expression such as lower acidification with citric acid, low water activity, pectins, other
212 phenols, etc. (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez &
213 Galan-Vidal, 2009; Cavalcanti, Santos & Meireles, 2011).

214 The behavior of the C* parameter of the MJs was similar to that observed in its H*
215 parameter (Fig. 1). The CAE+R juice showed stable C* values during the 17 weeks of
216 storage in darkness at 20 ºC, while the CAE juice showed stability during the first five
217 weeks and colour losses from the week seven. The causes of this behavior are due to the
218 copigmentation effect that takes place progressively. The CAE+AA sample showed big
219 losses from the first weeks of storage, making evident the deleterious effect of the ascorbic
220 acid on the anthocyanins. Strawberry juices (pH 3.4) showed losses in C* of the 30% after
221 25 days (aproxx. 4 week) of storage at 25 ºC (Garzón & Wrolstad, 2002), while the
222 CAE+R and CAE samples were stable during the first six weeks of storage at 20 ºC. The
223 higher losses in this study could be due to several factors, such as the higher storage
224 temperature (25 ºC) and the higher stability of anthocyanin present in plum juice (cyanidin
225 3-glycoside) against anthocyanins in strawberry juice (pelargonidin 3-glycoside) (Cabrita,
226 Fossen & Andersen, 2000).
227
228 *3.2. Anthocyanin analysis*
229
230 The HPLC results showed the presence of two compounds in the CAE juices. The first
231 one, cyanidin 3-glucoside, appears at 11.47 min. with a maximum absorbance at 520 nm
232 representing the 76% of the chromatographic area. The second one, cyanidin 3-rutinoside,
233 represents the 24% of chromatographic area, appears at 11.81 min. with a maximum
234 absorbance at 518 nm (Fig. 2). Other studies confirm the presence of those cyanins in
235 several plum cultivars different to the one used in this study (cv. Black Gold) (Tomás-
236 Barberán, Gil, Cremin, Waterhouse, Hess-Pierce & Kader, 2001; Kim & Padilla- Zakour,
237 2004).
238

239 *3.3. Anthocyanins and rutin stability*

240

241 The anthocyanin concentration in the CAE juices showed a marked decrease (60%)
242 during the first five weeks of storage, and remained constant until the end of the assay (Fig.
243 3). This pattern is similar to that reported previously with plum extracts (cv. Santa Rosa)
244 (Hernández-Herrero & Frutos, 2011). The anthocyanin concentration in the CAE+R juices
245 was stable during the first five weeks of storage with values around 0.5 mg/100 mL. At the
246 end of storage, the anthocyanin concentration in CAE and CAE+R was similar. The
247 evolution of rutin concentration followed a behavior similar to that of the anthocyanin
248 content. While the rutin concentration in the R juices decreased a 50% during the first three
249 weeks of storage, the rutin concentration in the CAE+R juice remained constant with
250 values around 4.5 mg/100 mL. The initial low anthocyanin and rutin levels in the CAE+R
251 juices indicate that the copigmentation between anthocyanin and rutin takes place just at
252 the beginning of the storage. These results are similar to those reported by other authors,
253 where copigmentations between anthocyanins and different phenolic acids also occur at the
254 beginning (Eiro & Heinonen, 2002). The high initial ratio rutin/anthocyanin in CAE+R
255 juices (7.8) promotes the fast copigmentation (Malien-Aubert, Dangles & Amiot, 2001).
256 The HPLC analysis showed a concentration of 9.5% of anthocyanins in the CAE. The
257 initial anthocyanin and rutin concentrations in CAE and R juices was of 1.3 and 10.1
258 mg/100 mL respectively (Fig. 3). These amounts of anthocyanins and rutin were lower
259 than the expected (1.9 and 15 mg/100 mL respectively) due to the lower solubility of
260 anthocyanins and rutin in aqueous media respect to acidified methanol.
261 The deleterious effect of the ascorbic acid can be observed from the beginning of the
262 storage, with an initial degradation of the 90% of anthocyanins in the CAE+AA (Fig. 3).
263 The mutual degradation of anthocyanins and ascorbic acid in the presence of oxygen has

264 been already demonstrated. There are three main hypothesis for the mechanisms involved
265 in this degradation. The condensation of the two molecules (Jurd, 1972; Poei-Langston &
266 Wrolstad, 1981; Garzon & Wrolstad, 2002), the degradation of the anthocyanin preventing
267 the oxidation of ascorbic acid (García-Viguera & Bridle, 1999; Pang, Zhang, Duan & Ji,
268 2001) and the degradation of anthocyanins by the hydrogen peroxide generated from the
269 ascorbic acid oxidation (Lopes, Richard, Saucier, Teissedre, Monti & Glories, 2007; Sun,
270 Bai, Zhang, Liao & Hu, 2011; Jackman & Smith, 1992).
271
272 *3.4. Antioxidant capacity stability*
273
274 The initial antioxidant capacity in CAE juices was of 2.4 mg of VCE/100 mL (Fig. 4).
275 The estimated contribution of the anthocyanins to the total antioxidant capacity of CAE
276 juice was of 1.7 mg of VCE/100 mL, according to the following equation:
277
278 $VCEAC\ (mg\ VCE/100\ mL) = \Sigma[CC\ (mg/100\ mL) \cdot Ra \cdot PC\ (\%) \cdot 100^{-1}]$
279
280 where CC: cyanin content in the CAE juice (1.3 mg/100mL) (Fig. 3), Ra: ratio
281 VCE/cyanin for the two cyanins present (1.4 for cyanidin 3-glucoside and 1.1 for cyanidin
282 3-rutinoside in 50% (v/v) acidified aqueous metanol) (Heo, Kim, Chung & Kim, 2007) and
283 PC: proportions of the two cyanins in the CAE (76% for cyanidin 3-glucoside and 24% for
284 cyanidin 3-rutinoside) (Fig. 2).
285 The estimated antioxidant capacity due to anthocyanins (1.7 mg of VCE/100 mL)
286 respect to the total antioxidant capacity of CAE juice (2.4 mg of VCE/100 mL) represents
287 the 71% of the antioxidant capacity in the CAE juice. Considering that the anthocyanin

288 concentration in the CAE is of 9.5%, it should be highlighted the important contribution of
289 anthocyanins to the antioxidant capacity generated by the CAE in the juices.

290 The results indicate that rutin has not showed any antioxidant capacity. Other studies in
291 50% (v/v) aqueous methanol, have reported a ratio VCE/rutin of 0.9-1.0 (Heo, Kim, Chung
292 & Kim, 2007; Kim, Lee, Lee & Lee, 2002). In this study the solution was not acidified and
293 therefore the pH was higher than the pH in the R juices (3.7). Other authors have reported
294 that the maximum antioxidant capacity for rutin due to the deprotonation, was observed at
295 pH 7.0 (Ignjatovic, Jasmina, Markovic, Markovic & Baranac, 2002; Gomathi, Gopinath,
296 Rafiuddin-Ahmed & Jayakumar, 2003). The lack of antioxidant capacity of rutin at this pH
297 (3.7) originated that the antioxidant capacities of CAE and CAE+R juices were the same.
298 Due to this fact any synergic effects between anthocyanins and rutin have been
299 investigated at this pH. However other previous antioxidant capacity studies with
300 individual and combined phenolics (chlorogenic acid, cyanidin 3-glucoside, quercetin 3-
301 glucoside, catechin, …) in a model system, reported the lack of a synergistic effect (Heo,
302 Kim, Chung & Kim, 2007).

303 The juices with vitamin C (AA and CAE+AA) showed the higher antioxidant capacity
304 (Fig. 4). Other studies demonstrated a correlation between antioxidant capacity and
305 ascorbate concentration (R^2= 0.903), so that the vitamin C fortified juices implies an
306 increase in the antioxidant capacity (Miller, Diplock & Rice-Evans, 1995). It should be
307 highlighted that from the 11th week of storage at 20 ºC in darkness, the CAE+AA juices
308 showed a higher antioxidant capacity than AA juice. This could be due to the degradation
309 of the anthocyanin preventing the oxidation of ascorbic acid (García-Viguera & Bridle,
310 1999; Pang, Zhang, Duan & Ji, 2001), according to one of the three mechanisms described
311 previously.

312 The samples CAE and CAE+R showed a similar antioxidant capacity, that was stable
313 during 17 weeks of storage at 20 ºC in darkness. This evolution in antioxidant capacity is
314 in agreement with that observed in plum peel model juices at pH 4.5 in the same storage
315 conditions (Hernández-Herrero & Frutos, 2011), and at pH 4.0 and 23 ºC in darkness
316 (Hernández-Herrero & Frutos, 2014b). There was a correlation of the VCEAC and the
317 anthocyanin concentration in CAE juices, with a determination coefficient of R^2= 0.881
318 (Fig. 5). The correlation of antioxidant activity and anthocyanin content has been already
319 reported by other authors (Sariburun, Şahin, Demir, Türkben & Uylaşer, 2010).
320
321 **4. Conclusions**
322
323 The copigmentation of anthocyanins and rutin takes place inmediately from the plum
324 model juice preparation. The effect of the copigmentation on the juice colour stability was
325 observed from the five weeks of storage. The copigmentation promotes higher colour
326 stability during 17 weeks of storage at 20 ºC in darkness.
327 The ascorbic acid promotes the degradation of anthocyanins just after its addition to
328 plum model juices. From this result, the fortification of anthocyanin juices with ascorbic
329 acid is not recommended. On the contrary, the anthocyanins prevent ascorbic acid from
330 oxidation.
331 Most of the antioxidant capacity of plum model juices is due to the anthocyanins.
332 These model juices showed a high correlation between antioxidant capacity and
333 anthocyanin content. The antioxidant capacity of rutin in acidified model juices (pH 3.7)
334 was extremely low.
335

Acknowledgements

This research was supported by the Conselleria de Cultura y Educación de la Generalitat Valenciana under Project GV00-087-13.

References

Aryana, K. J., Barnes, H. T., Emmick, T. K., Mcgrew, P., & Moser, B. (2006). Lutein is stable in strawberry yogurt and does not affect its characteristics. *Journal of Food Science, 71*(6), S467-S472.

Baker, J., Bridle, P., & Timberlake, C. F. (1986). Tristimulus measurements (CIELAB 76) of port wines. *Vitis, 25*, 67-78.

Cabrita, L., Fossen, T., & Andersen, Ø. M. (2000). Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chemistry, 68*, 101-107.

Cano, A., Hernández-Ruíz, J., García-Cánovas, F., Acosta, M., & Arnao, M. B. (1998). An end-point method for estimation of the total antioxidant activity in plant material. *Phytochemical Analysis, 9*, 196-202.

Castañeda-Ovando, A., Pacheco-Hernández, M. d -L., Páez-Hernández, M. E., Rodríguez, J. A., & Galan-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry, 113,* 859–871.

Cavalcanti, R. N., Santos, D. T., & Meireles, M. A. A. (2011). Non-thermal stabilization mechanisms of anthocyanins in model and food systems - An overview. *Food Research Internacional, 44*, 499-509.

359 De Rosso, V. V., & Mercadante, A. Z. (2007). Evaluation of colour and stability of
360 anthocyanins from tropical fruits in an isotonic soft drink system. *Innovative Food*
361 *Science and Emerging Technologies, 8*, 347–352.
362 Eiro, M. J., & Heinonen, M. (2002). Anthocyanin color behavior and stability Turing
363 storage: effect of intermolecular copigmentation. *Journal of Agricultural and Food*
364 *Chemistry, 50*, 7461-7466.
365 Elliott, J. G. (1999). Application of antioxidant vitamins in foods and beverages. *Food*
366 *Technology, 53*(2), 46-48.
367 Freedman, L., & Francis, F. J. (1984). Effect of ascórbico acido n color of jellies. *Journal*
368 *of Food Science, 49*(4), 1212-1213.
369 García-Viguera, C., Zafrilla, P., Artés, F., Romero, F., Abellán, P., & Tomás-Barberán, F.
370 A. (1998). Colour and anthocyanin stability of red raspberry jam. *Journal of the Science*
371 *of Food and Agriculture, 78*, 565-573.
372 García-Viguera, C., & Bridle, P. (1999). Influence of structure on colour stability of
373 anthocyanins and flavylium salts with ascorbic acid. *Food Chemistry, 64*, 21-26.
374 Garzón, G.A., & Wrolstad, R.E. (2002). Comparison of the stability of pelargonidin-based
375 anthocyanins in strawberry juice and concentrate. *Journal of Food Science, 67*(4),
376 1288-1299.
377 Gomathi, K., Gopinath, D., Rafiuddin-Ahmed, M., & Jayakumar, R. (2003). Quercetin
378 incorporated collagen matrices for dermal wound healing processes in rat.
379 *Biomaterials, 24*, 2767-2772.
380 Gómez-Miguez, M., González-Manzano, S., Escribano-Bailón, M.T., Heredia, F. J., &
381 Santos-Buelga, C. (2006). Influence of different phenolic copigments on the color of
382 malvidin 3-glucoside. *Journal of Agricultural and Food Chemistry*, *54*, 5422–5429.

González-Manzano, S., Dueñas, M., Rivas-Gonzalo, J. C., Escribano-Bailón, M. T., & Santos-Buelga C. (2009). Studies on the copigmentation between anthocyanins and flavan-3-ols and their influence in the colour expression of red wine. *Food Chemistry*, *114*, 649–656.

He, F., Liang, N.-N., Mu, L., Pan, Q.-P., Wang J., Reeves, M. J., & Duan, C.-Q. (2012). Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expresión. Review. *Molecules*, *17*, 1571-1601.

Heo, H. J., Kim, Y. J., Chung, D., & Kim, D.-O. (2007). Antioxidant capacities of individual and combined phenolics in a model system. *Food Chemistry, 104*, 87-92.

Hernández-Herrero, J. A., & Frutos, M. J. (2011). Degradation kinetics of pigment, colour and stability of the antioxidant capacity in juice model systems from six anthocyanin sources. *International Journal of Food Science & Technology*, *46*, 2550-2557.

Hernández-Herrero, J. A., & Frutos, M. J. (2014a). Effect of concentrated plum juice on physicochemical and sensory properties of yoghurt made at bench top scale. *International Journal of Dairy Technology, 67*, 123-128.

Hernández-Herrero, J. A., & Frutos, M. J. (2014b). Colour and antioxidant capacity stability in grape, strawberry and plum peel model juices at different pHs and temperatures. *Food Chemistry, 154*, 199-204.

Huang, W. Y., Davidge, S. T., & Wu, J. P. (2013). Bioactive natural constituents from food sources – potential use in hypertension prevention and treatment. *Critical Reviews in Food Science and Nutrition, 53*(6), 615-630.

Hui, C., Bin, Y., Xiaoping, Y., Long, Y., Chunye, C., Mantian, M., & Wenhua, L. (2010). Anticancer activities of an anthocyanin-rich extract from black rice against breast cancer cells *in vitro* and *in vivo*. *Nutrition and Cancer*, *62*, 1128-1136.

Ignjatovic, L. M., Jasmina, M., Markovic, D., Markovic, D. A., & Baranac, J. M. (2002). The spectroabsorphotometric and voltammetric behavior of malvin in buffered solutions and its antioxidant properties. *Journal of the Serbian chemical Society, 67(1)*, 53–60.

Jackman, R. L., & Smith, J. L. (1992). Anthocyanins and betalaines. In Hendry G. A. F. & Houghton J. D. (Eds.), *Natural food colorants* (pp. 183-231). New Jork: Van Norstrand Reinhold Inc.

Jurd, L. (1972). Some advances in the chemistry of anthocyanin-type plant pibments. In Chichester C.O. (Ed.), *The chemistry of plant pigments* (pp. 123-142). London and New York: Academic Press.

Lopes, P., Richard, T., Saucier, C., Teissedre, P. L., Monti, J. P., & Glories, Y. (2007). Anthocyanone A: A quinone methide derivative resulting from malvidin 3-O-glucoside degradation. *Journal of Agricultural and Food Chemistry, 55*, 2698–2704.

Kim, D.-O., Chun, O. K., Kim, Y. J., Moon, H.-Y., & Lee, C. Y. (2003). Quantification of polyphenolics and their antioxidant capacity in fresh plums. *Journal of Agricultural and Food Chemistry, 51*, 6509–6515.

Kim, D. O., Lee, K. W., Lee, H. J., & Lee, C. Y. (2002). Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *Journal of Agricultural and Food Chemistry, 50,* 3713-3717.

Kim, D. O., & Padilla-Zakour, O. I. (2004). Jam processing effect on phenolics and antioxidant capacity in anthocyanin-rich fruits: Cherry, plum, and raspberry, *Journal of Food Science, 69*, S395-S400.

Malien-Aubert, C., Dangles, O., & Amiot, M. J. (2001). Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 49*, 170-176.

Mazza, G., & Miniati, E. (1993). In *Anthocyanins in fruits, vegetables y grains*, CRC Press, London.

Miller, N. J., Diplock, A. T., & Rice-Evans, C. A. (1995). Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *Journal of Agricultural and Food Chemistry, 43*, 1794-1801.

Pang, X. Q., Zhang, Z. Q., Duan, X. W., & Ji, Z. L. (2001). The influence of oxidizing and reducing agents on the stability of anthocyanin in pericarp of lychee fruits. *Journal of South China Agricultural University, 22*(2), 15-17.

Poei-Langston, M. S., & Wrolstad, R. E. (1981). Color degradation in an ascorbic acid-anthocyanin-flavonol model system. *Journal of Food Science, 46*, 1218-1236.

Sariburun, E., Şahin, S., Demir, C., Türkben, C., & Uylaşer, V. (2010). Phenolic content and antioxidant activity of raspberry and blackberry cultivars. *Journal of Food Science, 75*(4), C328-C335.

Stintzing, F. C., Stintzing, A. S., Carle, R., Frei, B., & Wrolstad, R. E. (2002). Color and antioxidant properties of cyaniding-based anthocyanin pigments. *Journal of Agricultural and Food Chemistry, 50(21)*, 6172-6181.

Sun, J., Bai, W., Zhang, Y., Liao, X., & Hu, X. (2011). Identification of degradation pathways and products of cyanidin-3-sophoroside exposed to pulsed electric field. *Food Chemistry, 126(3)*, 1203–1210.

Tomás-Barberán, F. A., Gil, M. A., Cremin, P., Waterhouse, A. L., Hess-Pierce, B., & Kader, A. A. (2001). HPLC-DAD-ESIMS analysis of phenolic compounds in nectarines, peaches, and plums. *Journal of Agricultural and Food Chemistry, 49,* 4748-4760.

455 Wrolstad R.E., Durst R.W. y Lee J. (2005). Tracking color and pigment changes in
456 anthocyanin products. A review. *Trends in Food Science and Technology, 16,* 423-
457 428.
458
459

UNIVERSITAS Miguel Hernández

460

461 **Figure legends**

462 **Fig. 1.** Changes in lightness (L*), hue angle (H*) and chroma (C*) of plum model juices
463 elaborated with concentrated anthocyanin extract (♦), concentrated anthocyanin extract
464 plus ascorbic acid (□) and concentrated anthocyanin extract plus rutin (Δ) during 17 weeks
465 of storage in darkness at 20 ºC. Bars represent the standard deviations.

466

467 **Fig. 2.** Chromatogram of the plum concentrated anthocyanin extract.

468

469 **Fig. 3.** Changes in anthocyanin and rutin concentration of plum model juices elaborated
470 with concentrated anthocyanin extract (♦), concentrated anthocyanin extract plus ascorbic
471 acid (□) and concentrated anthocyanin extract plus rutin (Δ) during 17 weeks of storage in
472 darkness at 20 ºC. Bars represent the standard deviations.

473

474 **Fig. 4.** Vitamin C equivalent antioxidant capacity (VCEAC) of plum model juices
475 elaborated with concentrated anthocyanin extract (♦),concentrated anthocyanin extract
476 plus ascorbic acid (□),concentrated anthocyanin extract plus rutin (Δ), ascorbic acid (■) and
477 rutin (▲) during 17 weeks of storage in darkness at 20 ºC. VCE: vitamin C equivalents.
478 Bars represent the standard deviations.

479

480 **Fig. 5.** Correlation between antioxidant capacity and anthocyanins concentration in plum
481 model juices elaborated with concentrated anthocyanin extract during 17 weeks of storage
482 in darkness at 20 ºC. Bars represent the standard deviations.

483

484

485

486 **Table 1**

487 Composition (mg/100 mL) of the different plum model juices elaborated with concentrated
488 anthocyanin extract (CAE), concentrated anthocyanin extract plus ascorbic acid
489 (CAE+AA), concentrated anthocyanin extract plus rutin (CAE+R), ascorbic acid (AA) and
490 rutin (R).

491

Model juices	Anthocyanin	Ascorbic acid	Rutin
CAE	20	---	---
CAE+AA	20	60	---
CAE+R	20	---	15
AA	---	60	---
R	---	---	15

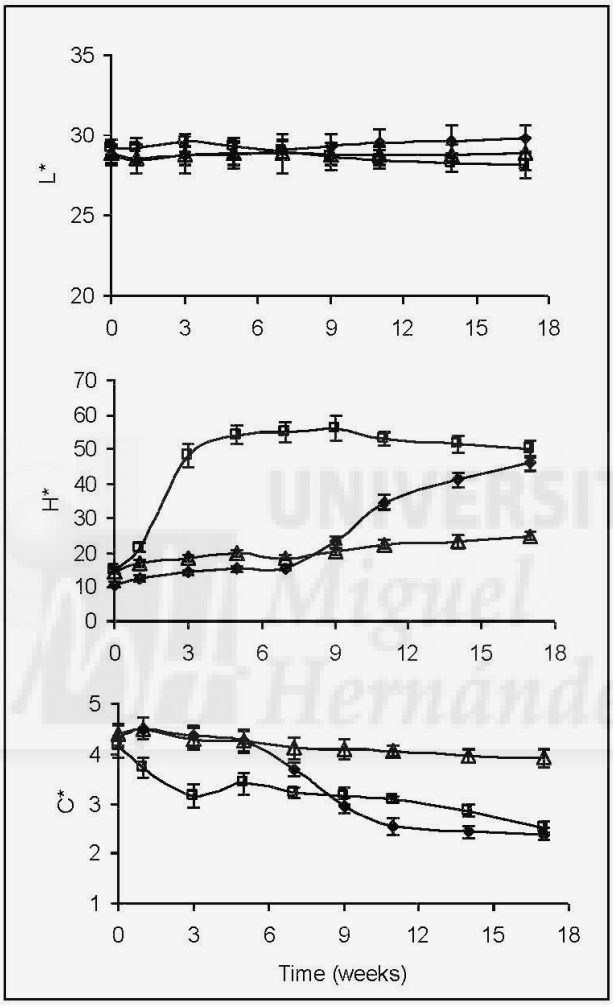
492

493

UNIVERSITAS Miguel Hernández

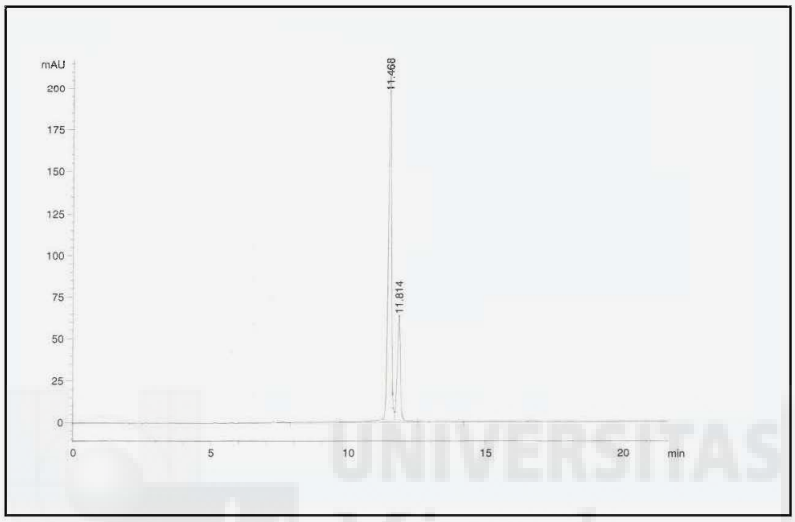
Figure(s)

Fig. 1



Figure(s)

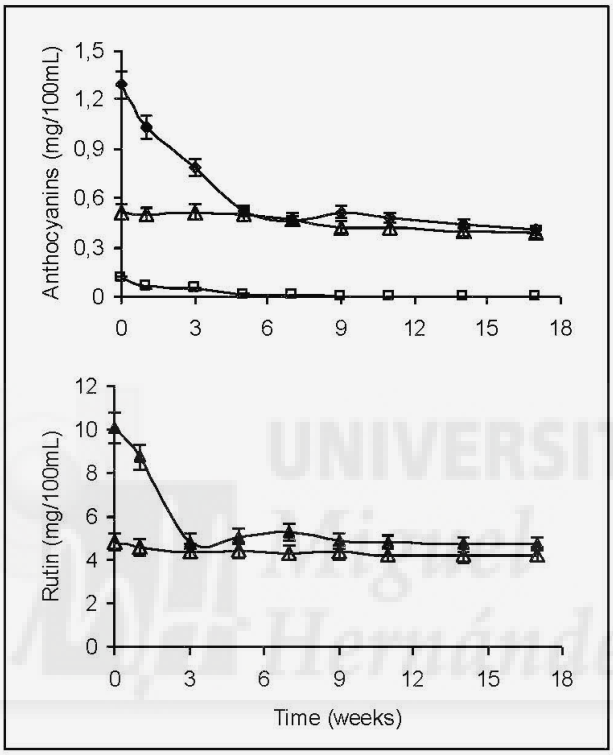
Fig. 2



UNIVERSITAS Miguel Hernández

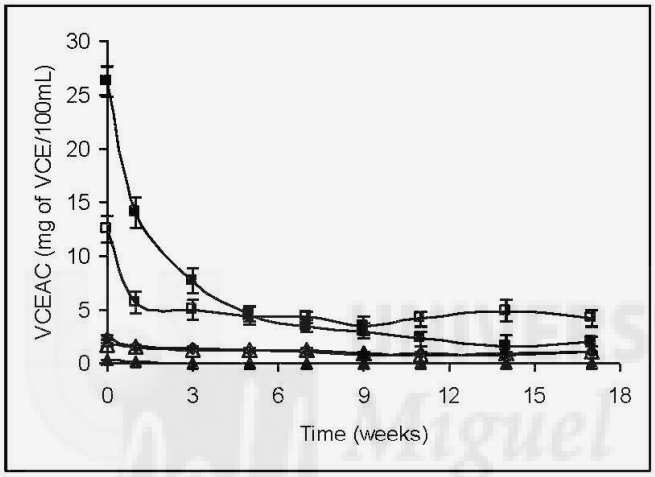
Figure(s)

Fig. 3



Figure(s)

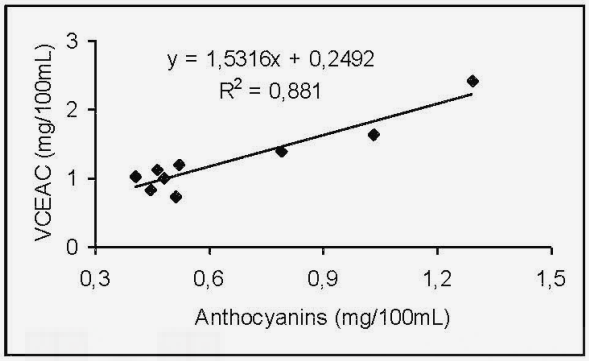
Fig. 4



UNIVERSITAS Miguel Hernández

Figure(s)

Fig. 5



UNIVERSITAS Miguel Hernández

3.4.- Hernández-Herrero J.A. y Frutos M.J. (2014). Effect of concentrated plum juice on physicochemical and sensory properties of yoghurt made at bench top scale. *International Journal of Dairy Technology, 67(1)*, 123–128.

International Journal of Dairy Technology SDT SOCIETY OF DAIRY TECHNOLOGY

doi: 10.1111/1471-0307.12101

ORIGINAL RESEARCH

Effect of concentrated plum juice on physicochemical and sensory properties of yoghurt made at bench top scale

JOSÉ A HERNÁNDEZ-HERRERO and MARÍA J FRUTOS*

Department of Food Technology, Universidad Miguel Hernández, Ctra. Beniel, km. 3.2, 03.312 Orihuela, Alicante Spain

Yoghurt with 9% (v/v) of plum juice concentrate (PJC) was stored for 24 days in darkness at 6 °C. Titratable acidity and pH were similar in all samples during storage. The firmness was in the range of 1.7–2.3 N and correlated with total solids of the mixes (R^2 = 0.982). The sample with PJC and 5.33% (w/v) nonfat dry milk had the highest firmness and the weakest creaminess. The colour in PJC yoghurt was stable during the first seven days of storage. In the ranking test for acceptability, there were no significant differences ($P \leq 0.05$) between the yoghurts.

Keywords Creaminess, Sensory analysis, Texture, Azorubine, *Prunus salicina* L., Colouring.

INTRODUCTION

Colour is an important attribute for the evaluation and acceptance of foods, and for many years, it has been enhanced or modified through the addition of food colourings (Calvo 1999). Since the 1990s, consumers have shown an increasing tendency to reject synthetic additives in foods (Gamel and Kiritsakis 1999). Anthocyanins have great potential as red food colourings, together with high antioxidant capacity and healthy properties (Wang *et al.* 2012), and offer an alternative to current synthetic food colourings (Giusti *et al.* 1998).

The traditional sources of anthocyanins investigated by researchers include red grape skin extracts and juice (Karaaslan *et al.* 2011), strawberry (Aryana *et al.* 2006) and blueberry (Cinbas and Yazici 2008).

Yoghurt products have gained considerable economic importance worldwide because of their high nutritional content that can be increased further by adding nutraceutical ingredients (Guggisberg *et al.* 2007). The incorporation of fruits in yoghurt has significantly contributed to the healthy image of yoghurt and the consumption of yoghurt by people of all ages. Fruits may be added to yoghurt formulae as single fruits or blends in the form of refrigerated, frozen or canned fruit and as a juice or syrup. The most common fruits used in yoghurt formulae are peach, cherry, orange, lemon, purple plum, boysenberry, spiced apple, apricot, pineapple, strawberry, raspberry and blueberry (Chandan and Shahani 1993).

In previous research, plums have been found to have a high concentration of anthocyanins, showing a high stability during storage in model systems compared with other anthocyanin sources (Frutos *et al.* 2002; Kim *et al.* 2003; Hernández-Herrero and Frutos 2011). The effect of the addition of plum juice on the physicochemical and organoleptic properties of yoghurt has not been studied in the literature. Therefore, the main objective of this research was to use plum juice as a natural colouring in yoghurt and evaluate its impact on the physicochemical and organoleptic properties of the yoghurt.

MATERIALS AND METHODS

Plant material

Plum samples (*Prunus salicina* L., cv. Black diamond) were obtained from a local market in Orihuela (Alicante, Spain). For the assays, plums with a uniform surface red colour were selected, with hue (H^*) and chroma (C^*) values of 6.44 ± 0.38° and 6.68 ± 0.51, respectively.

Concentrated plum juice preparation

The samples were ground in a Waring blender, and 100 g of the homogenate was extracted with

*Author for correspondence. E-mail: mj.frutos@umh.es

© 2013 Society of Dairy Technology

citric acid (8 mM) in water for 4 h in the dark at 4 °C, in a proportion of 1:3 (plant material-solvent). Filtrates were centrifuged for 10 min at 1800 *g* and vacuum concentrated (Heidolph VV2000) at 55 °C. The concentrate was resuspended with acidified water with citric acid (8 mM) up to a final volume of 40 mL. The samples of concentrated plum juice (PJC) were kept in a freezer (−84 °C) until their use.

Anthocyanin analysis

The anthocyanin concentration of PJC was measured by UV–Visible Spectroscopy (Hewlett-Packard mod. 8453) with the pH differential method (Giusti and Wrolstad 2001). The absorbance was measured in the visible wavelength, where the maximum absorbance is at 520 and 700 nm. Three readings were taken per replication.

Yoghurt manufacture

The following yoghurt treatments were performed at bench top scale, uncoloured yoghurt (W-Control) with 4.00% (w/v) of nonfat dry milk (NFDM), yoghurt with 2.5 mg/kg of E122 synthetic colouring (C-Control) and 4.00% (w/v) of NFDM, yoghurt with 9% (v/v) of plum juice concentrate (PJC) and 4.85% (w/v) of NFDM (Y1) and yoghurt with 9% (v/v) of PJC and 5.33% (w/v) of NFDM (Y2). The batch size for the yoghurt production was 3 L per treatment for acceptability tests, 1 L for sensory evaluation of creaminess and colour, and 2 L for the instrumental analysis. Subsamples of approx. 50 mL were taken for analysis.

Yoghurt mixes comprised the following: 1 L whole fat (3.6 %) homogenised milk (WM) (El Prado, Valencia, Spain), 8% (w/v) sucrose (Azucarera, AB sugar company, Zamora, Spain) 4.00, 4.85 or 5.33% (w/v) of NFDM (Svelteesse, Nestlé, Vevey, Switzerland) and 0 or 9% (v/v) PJC preparation, or 2.5 mg/kg azorubine synthetic colouring (E122) (Coralim aditivos, Valencia, Spain). All ingredients (batch size 3 L per treatment) were manually mixed in an 8-L stainless steel container and poured into three 1 L Pyrex® (Corning Incorporated, Corning, NY, USA) bottles and pasteurised at 85 °C for 30 min. under agitation in a water bath. The mixes were cooled to 43 °C in an ice water bath and were then inoculated with commercial yoghurt cultures consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (MY900 Ezal; TEXAL, Sassenage, France). Cultures were added directly to the mix, according to the manufacturers' recommendations (10 DCU/100 L of milk). The inoculated mix was incubated at 43 °C to a final pH of 4.5 (5–6 h).The bottles were cooled to 20 °C in an ice bath. Yoghurt samples were stored at 6 °C in darkness for 24 days.

The addition of PJC caused a decrease in protein concentration of the mix. Treatment Y1 was supplemented with an additional amount of 0.85% (w/v) of NFDM with the aim of standardising the protein content with respect to the control. Sample Y2 was supplemented with a higher NFDM concentration to obtain an increase in the total solids level (Table 1).

Table 1 Composition of the treatments (1 L of yoghurt). Whole fat homogenised milk (WM), nonfat dry milk (NFDM), plum juice concentrate (PJC) and E122 synthetic colouring. Uncoloured yoghurt (W-Control), E122 coloured yoghurt (C-Control), yoghurt with PJC (Y1 and Y2). TS: total solids

Sample	*WM (L)*	*E122 (mg)*	*PJC (L)*	*NFDM (%)*	*TS (%)*
Y1	0.91	0	0.09	4.85	16.9
Y2	0.91	0	0.09	5.33	17.3
C-Control	1	2.5	0	4	17.2
W-Control	1	0	0	4	17.2

The results of samples Y1 and Y2 were compared with those of W-Control, except for the sensory and instrumental colour assays, where the C-Control was used. Sensory tests were performed with yoghurt samples refrigerated at 6 °C for 24 h before analysis. The titratable acidity, pH, firmness and colour were measured in yoghurt samples stored at 6 °C in darkness during 24 days. The analyses were performed at 1, 3, 7, 10, 14, 17, 21 and 24 days of storage.

Titratable Acidity, pH and total solids

The pH was measured using a Crison GLP 21 pH meter (CRISON, Alella, Barcelona, Spain). Titratable acidity was determined as lactic acid percentage by titrating with 0.1 N NaOH, using phenolphthalein as indicator. Total solids were determined with a moisture analyser (LJ16; Mettler Toledo, Barcelona, Spain). Three readings were taken per replication.

Firmness

Firmness of the yoghurt samples was measured with an instrumental compression/penetration test (Carson *et al.* 2002), using a texturometer (TA-XT2, Stable Micro Systems, Godalming, UK) equipped with an extrusion cell (A/BE) of 35 mm diameter and 49 N load cell. The pretest, test and post-test speed was 1.0 mm/s. All yoghurt was measured at 6 °C. The determinations were carried out in crystal cylindrical containers (47 mm diameter and 68 mm height). The depth of the yoghurt was 25 mm. Firmness was expressed as the maximum force as the test cell penetrated to a depth of 23 mm into the sample, as described by Mohamed and Morris (1987). The mean of five determinations was calculated for each yoghurt sample.

Colour analysis

Colour measurements were taken with a reflectance spectrophotometer (chroma meter CM-2600d; Minolta Co. Ltd., Osaka, Japan), using a CIE D65/10° illuminant/observer condition and an illumination area of 11 mm diameter.

© 2013 Society of Dairy Technology

Colour results were expressed as tristimulus parameters (L^*, a^*, b^*, H^*, C^*). Hue angle ($H^* = \tan^{-1} b^*/a^*$) indicates sample colour (0° or 360° = red, 90° = yellow, 180° = green, 270° = blue), and chroma ($C^* = [a^{*2} + b^{*2}]^{1/2}$) indicates colour purity or saturation (high values are more vivid) (Baker *et al.* 1986). For each sample, 15 measurements were taken.

Sensory analysis

For the determination of creaminess (evaluated by mouthfeel) and colour (relative to a commercial sample), a 7-point scale was used in descriptive tests with respect to creaminess and colour (7-strong, 1-weak) (ISO 4121 2003). During the creaminess evaluation tests, monochromatic illumination was used to hide colour differences.

The samples were evaluated by a trained panel of 15 judges, aged between 22 and 35 years, at a temperature of 6 °C. The tests were performed in a standardised test room according to ISO 8589 (2007).

The acceptability of yoghurt was evaluated with a ranking test (ISO 8587 2006). This test was performed by 60 consumers among the students at the High Polytechnical School (aged 22–26 years). The samples were ranked from 1 (least preferred) to 3 (most preferred).

Statistical analysis

Results are presented as mean ± SD. Comparisons of means were performed by one-way analysis of variance (ANOVA) followed by Duncan's test ($P < 0.05$). The ranking test data were analysed by Fisher's test ($P < 0.05$). Statistical analyses were run using the computer SPSS 14.0 software (SPSS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Concentrated plum juice

The °Brix value and pH of the concentrated juice were 10.4 and 2.9, respectively. At this pH value, the anthocyanins exist mostly as the flavylium cation, which is the most colourful form (Brouillard and Delaporte 1977). The anthocyanin concentration in the PJC used in yoghurt manufacture was of 15 ± 1 mg/L. The anthocyanin composition of the plum fruit (*P. salicina* L.) exists mainly in the cyanidin 3-glucoside and cyanidin 3-rutinoside forms, with a high colour stability, antioxidant capacity and anthocyanins concentration during storage at 20 °C when compared with other anthocyanin sources (Hernández-Herrero and Frutos 2011).

Titratable Acidity and pH

The Y1 and W-Control yoghurt showed a high increase in acidity during the first 10 days (Figure 1), maintaining similar values until day 17 and increased again to the end of storage. When comparing the acidity of Y1 and W-Control for the same storage period, the differences observed were not significant ($P \leq 0.05$). These results are in agreement with those reported for yoghurt supplemented with 10% concentrated grape juice by Öztürk and Öner (1999).

With acidity development, the pH decreased during the first 10 days, reaching in both samples a value of 4.36, without further changes until the end of storage (Figure 2). The pH differences between samples were not significant ($P \leq 0.05$) during storage; therefore, the addition of a 9% (v/v) of PJC (pH 2.9) to yoghurt did not significantly affect the pH of the yoghurt. In contrast, in yoghurt supplemented with rectified grape juice and blueberry pulp by others, the yoghurt showed a lower pH relative to a control during the first days of storage of the samples where the fruit ingredient was added (Calvo *et al.* 2002; Cinbas and Yazici 2008).

Firmness

Firmness was stable during storage at 6 °C, but significant increases ($P \leq 0.05$) of about 15% occurred after 24 days of storage for the three yoghurt samples (Y1, Y2 and W-Control) (Figure 3). When the different samples are compared on the same day of analysis, significant differences

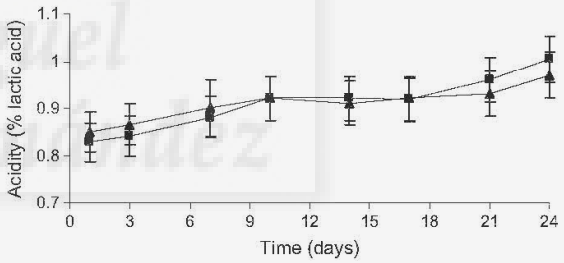


Figure 1 Titratable acidity (% lactic acid) of yoghurt Y1 (▲) and W-Control (■) during 24 days of storage at 6 °C. Y1: sample with 9% plum juice concentrate (PJC) and 4.85% nonfat dry milk (NFDM). W-Control: uncoloured yoghurt.

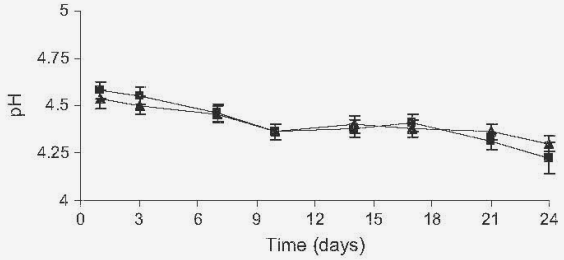


Figure 2 The pH of yoghurt Y1 (▲) and W-Control (■) during the 24 days of storage at 6 °C. Y1: sample with a 9% of PJC and 4.85% NFDM. W-Control: uncoloured yoghurt.

© 2013 Society of Dairy Technology

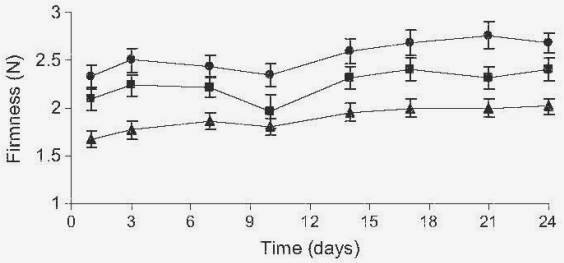


Figure 3 Firmness of yoghurt Y1 (▲), Y2 (●) and W-Control (■) during 24 days of storage at 6 °C. Y1: sample with a 9% of PJC and 4.85% NFDM. Y2: sample with 9% of PJC and 5.33% of NFDM. W-Control: uncoloured yoghurt.

($P \leq 0.05$) are observed among the different yoghurt treatments, except on day 10 between the samples W-Control and Y1. The average firmness of the Y2 samples showed values 13% higher than W-Control yoghurt samples. At the same time, W-Control samples are 10% higher than the Y1 yoghurt. The results reported here for Y2 are similar to those obtained in yoghurt with a 3.5% of fat level with firmness values of 2.57 N by Guggisberg *et al.* (2007).

The total solids level was 17.2% in W-Control and C-Control, 16.9% in Y1 and 17.3% in sample Y2 (Table 1). Comparing the total solids level (%) of the mixes with the initial firmness values, a correlation with a first-order equation of:

$$\text{Firmness } (N) = 1.5904\ \text{TS}(\%) - 25.2160$$

was found.

The coefficient of determination was $R^2 = 0.982$ showing a good correlation between both parameters. Higher firmness values resulting from an increase in total solids have been previously reported by other authors (Mahdian and Tehrani 2007). In this research, the TS variations are due to the dilution of the solids in the samples supplemented with a 9% (v/v) of PJC. In the same manner, the supplementation with PJC resulted in fat dilution. This fact could explain the lower texture values of the Y1 sample. Other studies have reported that fat globules coated with proteins reinforce yoghurt texture (Lucey *et al.* 1998; Sandoval-Castilla *et al.* 2004). Similarly, when the protein content is increased in the Y2 sample, with additional NFDM, a corresponding increase in its texture value is observed (Figure 3). Firmness is a very important quality parameter in yoghurt. The addition of PJC at 9% (v/v) would reduce the yoghurt firmness, due to the dilution effect of the fat and protein in mixes. In the present work, the firmness was standardised by the addition of total solids, in skimmed milk powder form.

Colour

The anthocyanin concentration in the PJC used in yoghurt manufacture was 15 ± 1 mg/L. This concentration was

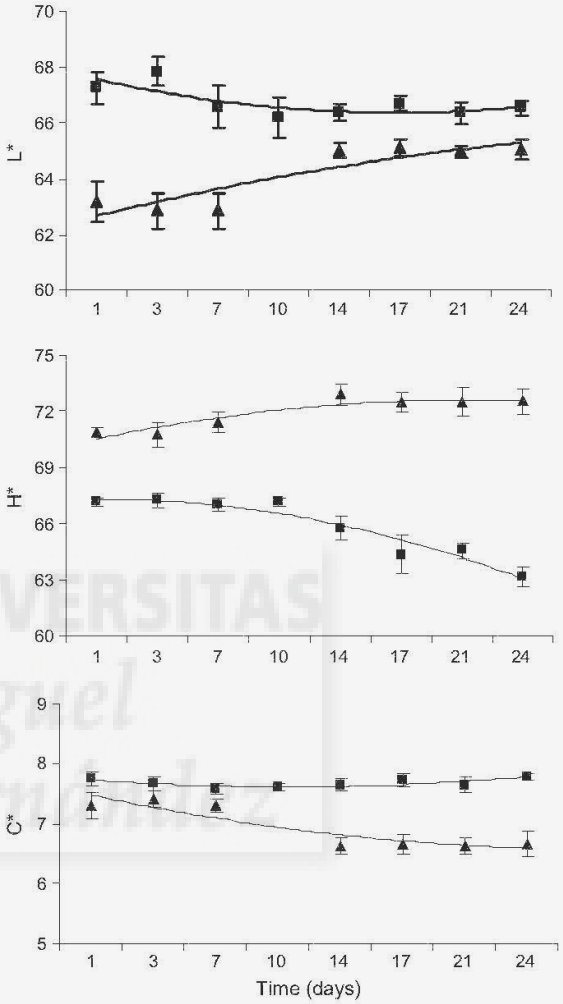


Figure 4 Colour lightness (L^*), hue angle (H^*) and chroma (C^*) of yoghurt Y1 (▲) and C-Control (■) during 24 days of storage at 6 °C. Y1: sample with a 9% of PJC and 4.85% NFDM. C-Control: E122 coloured yoghurt.

adequate to achieve the desired colour ($L^* = 63$, $H^* = 71$ and $C^* = 7.3$) when added at a level of 9% (v/v) in the yoghurt (Figure 4). This level of addition resulted in good scores in the sensory tests.

The lightness (L^*) of the C-Control yoghurt was 6% higher than Y1 during the first 7 days of storage and subsequently decreased back to a differential of only 3% higher after 14 days (Figure 4). The similarity of L^* values is mainly due to the significant increase ($P \leq 0.05$) in Y1 samples (3%) from day 7 to 14, while remaining constant until the end of storage. The hue angle results (H^*) indicate that

© 2013 Society of Dairy Technology

the pinkish colour in yoghurt was stable during the first 7 days of storage, with an H^* value of 71° in Y1 and 67° in C-Control samples (5% lower) (Figure 4). From day 10, the H^* value of C-Control starts to decrease significantly ($P \leq 0.05$) from 67° to 63° at the end of storage (24 days). The Y1 yoghurt showed only a 2% increase between days 7 and 14, keeping a stable H^* value (72–73°) until the end of storage. The colour intensity or chroma (C^*) in C-Control yoghurt was stable with values about 7.7 during the 24 days of storage at 6 °C (Figure 4). The Y1 yoghurt showed values slightly lower ($C^* = 7.3$) than C-Control during the first 7 days of storage. From day 7 to 14, the C^* values in Y1 showed a significant decrease ($P \leq 0.05$) of 12%. From day 14 until the end of storage, the C^* values were stable around 6.6.

The results obtained show that the colour of the yoghurt with PJC was stable during the first 7 days of storage at 6 °C. This storage period where the colour is stable is higher than that reported in strawberry yoghurt, where after a week of storage at 5°C significant increases ($P \leq 0.05$) in H^* values and significant decreases in C^* values were observed (Aryana *et al.* 2006). Other studies reported that the colour stability of the plum anthocyanins is higher than that found in strawberry anthocyanins (Hernández-Herrero and Frutos 2011). The colour stability (H^* and C^* values) observed in Y1 yoghurt (Figure 4) is similar to that reported in strawberry yoghurt from the beginning to the end of storage (five weeks) (Aryana *et al.* 2006).

According to the literature (Hernández-Herrero and Frutos 2011), the colour stability is higher than anthocyanin stability. Taking into consideration this observation and the fact that from the seventh day, the samples showed a decrease in colour (Figure 4), anthocyanin stability in the yoghurt matrix appears limited. This behaviour could be due to the thermal treatment during processing, particularly the pasteurisation treatment of yoghurt (85 °C for 30 min) and the incubation temperature of the mix (43 °C for 5–6 h).

Sensory analysis

Regarding the results of creaminess (evaluated by mouthfeel), significant differences ($P \leq 0.05$) were observed among the three samples (Table 2). The results show that samples W-Control and Y1 obtained higher scores with respect to creaminess, with values of 5.80 and 5.53, respectively. The Y2 yoghurt received a score of 5.00. This pattern reflects the pattern found for firmness (Figure 3). As has been indicated previously in the discussion of the firmness results, the higher total solids levels in Y2 yoghurt lead to a higher firmness in yoghurt (Mahdian and Tehrani 2007). Taking into account the lower fat content of yoghurt supplemented with PJC (samples Y1 and Y2) and the firmness results, a higher creaminess would be expected in Y1 when compared with W-Control samples in the sensory analysis (Guven *et al.* 2005; Cinbas and Yazici 2008). However, no significant differences were found ($P \leq 0.05$).

Table 2 Sensory evaluation of creaminess and colour intensity in yoghurt and acceptability ranking test (ranking sums). Y1: sample with a 9% (v/v) of PJC and 4.85% (w/v) of NFDM. Y2: sample with 9% (v/v) of PJC and 5.33% (w/v) of NFDM. C-Control: E122 coloured yoghurt. W-Control: uncoloured yoghurt.

	Y1	*Y2*	*W-Control*	*C-Control*
Creaminess	5.53[a]	5.00[b]	5.80[a]	–
Colour	4.07[b]	4.00[b]	–	5.87[a]
Acceptability	144	116	–	100

Values with different letters in each row are significantly different at the level of $P \leq 0.05$.

Sensory perception of colour also showed significant differences ($P \leq 0.05$) (Table 2), with the C-Control yoghurt receiving the best score value of 5.87. No significant differences ($P \leq 0.05$) were detected between Y1 and Y2 samples. Both yoghurts were manufactured with a 9% (v/v) of PJC and obtained scores of 4.07 and 4.00, respectively, as would be expected.

In the ranking test for acceptability, the ranking test sums were 144, 116 and 100 for Y1, Y2 and C-Control, respectively (Table 2). These values were not different ($P \leq 0.05$), indicating that the perceived creaminess by the panellists was the same.

CONCLUSIONS

The addition of concentrated plum juice as natural colouring in yoghurt did not appear to affect the titratable acidity and pH. Texture parameters are very important in yoghurt quality. Therefore, it should be highlighted that a decrease in yoghurt firmness resulting from the addition of concentrated plum juice can be compensated for by the addition of NFDM to standardise the total solids of the formulation. The yoghurt sample with 9% (v/v) PJC and 5.33% (w/v) NFDM had the highest firmness value and the weakest creaminess. The colour parameters measured in yoghurt manufactured with concentrated plum juice were stable during 7 days of storage at 6 °C. According to sensory results for colour, the azorubine (E122) coloured yoghurt showed higher colour intensity than plum juice coloured ones. Nevertheless, it should be pointed out that yoghurt with concentrated plum juice obtained an intermediate colour score of four on a scale of one (weak) to seven (strong) and in terms of overall acceptability compared favourably with the yoghurt coloured with azorubine (E122).

From the results of this preliminary study, we can conclude that concentrated plum juice has potential use as a natural food colouring source for yoghurt. Further sensory analysis of colour and acceptability during storage is required to establish the influence of colour changes on yoghurt shelf life.

© 2013 Society of Dairy Technology

ACKNOWLEDGEMENTS

This research was supported by the Conselleria de Cultura y Educación de la Generalitat Valenciana under Project GV00-087-13. Assistance of Graham Arnold during manuscript preparation is also acknowledged.

REFERENCES

Aryana K J, Barnes H T, Emmick T K, Mcgrew P and Moser B (2006) Lutein is stable in strawberry yogurt and does not affect its characteristics. *Journal of Food Science* **71** S467–S472.

Baker J, Bridle P and Timberlake C F (1986) Tristimulus measurements (CIELAB 76) of port wines. *Vitis* **25** 67–78.

Brouillard R and Delaporte B (1977) Chemistry of anthocyanin pigments. II. Kinetic and thermodynamic study of proton transfer, hydration, and tautomeric reactions of malvidin 3-glucoside. *Journal of the American Chemical Society* **99** 8461–8468.

Calvo C (1999) Use of colourings in foods. *Alimentación, Equipos y Tecnologőa* **3** 79–88.

Calvo M M, Diez O and Cobos A (2002) Use of rectified grape juice in yogurt edulcoration. *Journal of Food Science* **67** 3140–3143.

Carson K, Meullenet J F C and Reische D W (2002) Spectral stress strain analysis and partial least squares regression to predict sensory texture of yogurt using a compression/penetration instrumental method. *Journal of Food Science* **67** 1124–1128.

Chandan R C and Shahani K M (1993) Yogurt. In *Dairy Science and Technology Handbook: Product Manufacturing*, pp 1–56. Hui Y H, ed. New York: Wiley-VCH.

Cinbas A and Yazici F (2008) Effect of the addition of blueberries on selected physicochemical and sensory properties of yoghurts. *Food Technology and Biotechnology* **46** 434–441.

Frutos M J, Hernández-Herrero J A, Sayas E, Uribesalgo T and Marőn F R (2002) Effect of drying operation on the qualitative anthocyanin composition of different plant materials as potential sources of anthocyanins as natural colourants. In *Functionalities of Pigments in Food*, pp 347–350. Empis J A, ed. Lisbón: Sociedade Portuguesa de Quőmica.

Gamel T H and Kiritsakis A (1999) Effect of methanol extracts of rosemary and olive vegetable water on the stability of olive oil and sunflower oil. *Grasas y Aceites* **50** 345–350.

Giusti M M and Wrolstad R E (2001) Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*, units F1.2.1–F1.2.13. Wrolstad R E, ed. New York: John Wiley & Sons.

Giusti M M, Rodrőguez-Saona L E, Baggett J R, Reed G L, Durst R W and Wrolstad R E (1998) Anthocyanin pigment composition of red radish cultivars as potential food colorants. *Journal of Food Science* **63** 219–224.

Guggisberg D, Eberhard P and Albrecht B (2007) Rheological characterization of set yogurt produced with additives of native whey proteins. *International Dairy Journal* **17** 1353–1359.

Guven M, Yasar K, Karaca O B and Hayaloglu A A (2005) The effect of inulin as a fat replacer on the quality of set-type low-fat yogurt manufacture. *International Journal of Dairy Technology* **58** 180–184.

Hernández-Herrero J A and Frutos M J (2011) Degradation kinetics of pigment, colour and stability of the antioxidant capacity in juice model systems from six anthocyanin sources. *International Journal of Food Science & Technology* **46** 2550–2557.

ISO 4121 (2003) *Sensory analysis – Guidelines for the Use of Quantitative Response Scales*. Geneva: International Organization for Standardization.

ISO 8587 (2006). *Sensory Analysis – Methodology – Ranking*. Geneva: International Organization for Standardization.

ISO 8589 (2007) *Sensory Analysis – General Guidance for the Design of Test Rooms*. Geneva: International Organization for Standardization.

Karaaslan M, Ozden M, Vardin H and Turkoglu H (2011) Phenolic fortification of yogurt using grape and callus extracts. *LWT - Food Science and Technology* **44** 1065–1072.

Kim D-O, Chum O K, Kim Y J, Moon H–Y and Lee C Y (2003) Quantification of polyphenolics and their antioxidant capacity in fresh plums. *Journal of Agricultural and Food Chemistry* **51** 6509–6515.

Lucey J A, Munro P A and Singh H (1998) Rheological properties and microstructure of acid milk gels as affected by fat content and heat treatment. *Journal of Food Science* **63** 660–664.

Mahdian E and Tehrani M M (2007) Evaluation the effect of milk total solids on the relationship between growth and activity of starter cultures and quality of concentrated yoghurt. *American-Eurasian Journal of Agricultural & Environmental Sciences* **2** 587–592.

Mohamed M O and Morris H A (1987) Textural and microstructural properties of rennet-induced milk coagulum as affected by the addition of soy protein isolate. *Journal of Texture Studies* **18** 137–155.

Öztürk B A and Öner M D (1999) Production and evaluation of yogurt with concentrate grape juice. *Journal of Food Science* **3** 530–532.

Sandoval-Castilla O, Lobato-Calleros C, Aguirre-Mandujano E and Vernon-Carter E J (2004) Microstructure and texture of yogurt as influenced by fat replacers. *International Dairy Journal* **14** 151–159.

Wang Y, Zhang Y, Wang X, Liu Y and Xia M (2012) Supplementation with cyanidin-3-O-β-glucoside protects against hypercholesterolemia-mediated endothelial dysfunction and attenuates atherosclerosis in apolipoprotein E-deficient mice. *The Journal of Nutrition* **142** 1033–1037.

© 2013 Society of Dairy Technology

4.- Resultados y Discusión

4.- RESULTADOS Y DISCUSIÓN.

La totalidad de los resultados y discusión de la presente tesis se encuentra en las diferentes publicaciones adjuntas, incluyéndose en este apartado únicamente aquellos más relevantes, de modo que se puedan enlazar los diferentes trabajos realizados y ofrecer así una visión global de los mismos.

4.1.- Caracterización de antocianinas y selección del material vegetal más adecuado.

Los materiales vegetales utilizados para la realización de este ensayo fueron:

- Piel de berenjena (*Solanum melongena,* L.) cultivar (cv.) Black bell.
- Fresa (*Fragaria ananassa,* L.) cv. Camarosa.
- Uva (*Vitis vinifera,* L.) cv. Moravia.
- Arándano (*Vaccinium myrtillus,* L.).
- Frambuesa (*Rubus idaeus,* L.) cv. Zeva.
- Piel de ciruela (*Prunus salicina,* L.) cv. Santa rosa.

A partir de los extractos de estos materiales vegetales se elaboraron zumos modelo a pH 4,5 que fueron conservados durante 17 semanas a 20 ºC en oscuridad. La concentración de extracto utilizada fue 10 g/L.

4.1.1- Rendimiento de extracción.

La cantidad de extracto liofilizado obtenido varió entre los 2,3 g/100 g de peso fresco (pf) de piel de berenjena a los 13,3 g/100 g (pf) de uva (**Tabla 3**).

Material vegetal	Rendimiento de extracción (%)
Piel de berenjena	2,3 ± 0,3
Fresa	5,9 ± 0,3
Uva	13,3 ± 1,1
Arándano	5,0 ± 0,2
Frambuesa	8,5 ± 0,6
Piel de ciruela	9,7 ± 0,8

Tabla 3: Rendimiento de extracción (g de extracto/100 g de material vegetal en peso fresco). Los datos expresan el valor medio ± la desviación estándar de tres repeticiones.

4.1.2.- Determinación de antocianinas.

La identificación de las antocianidinas hidrolizadas (aglicones) se realizó por comparación de su espectro UV-visible y tiempo de retención con los patrones hidrolizados. La identificación de las antocianinas glicósidos se realizó teniendo en cuenta su $\lambda_{max\ vis}$, la cual depende principalmente del número de grupos hidroxilo o metoxilo presentes en el anillo B de la antocianidina (Torskangerpoll *et al*., 1998). La $\lambda_{max\ vis}$ de los seis patrones 3-glc utilizados varió entre los 502 nm (pelargonidina 3-glucósido) y los 528 nm (malvidina 3-glucósido).

El perfil de antocianinas presente en los diferentes materiales vegetales se muestra en la **Tabla 4**. En el extracto de frambuesa fueron encontrados tres picos en el cromatograma previo a la hidrólisis, correspondientes a tres cianidinas glicósido, puesto que en el cromatograma posterior a la hidrólisis solamente fue identificado un pico, cianidina aglicón. Dos de las tres cianidinas glicósido encontradas fueron identificadas como cianidina 3-glucósido y cianidina 3-rutinósido. Según la bibliografía consultada, la tercera podría ser cianidina 3-soforósido (Kassim *et al*., 2009). En piel de ciruela se detectaron dos picos, cianidina 3-glucósido y cianidina 3-rutinósido. En fresa únicamente se identificó pelargonidina 3-glucósido.

Extracto de antocianina	**% (peso/peso)**					
	Dp	**Cy**	**Pt**	**Pg**	**Pn**	**Mv**
Piel de berenjena	88+12*					
Fresa				100		
Uva	12	16	2		58	12
Arándano	18	3	8			71
Frambuesa		100				
Piel de ciruela		100				

Tabla 4: Perfil de antocianinas 3-glicósido, expresado como % de area cromatografica de picos. Delfinidina (Dp), cianidina (Cy), petunidina (Pt), pelargonidina (Pg), peonidina (Pn), malvidina (Mv). *5-glicósido y monoacilado.

Los resultados en arándano y uva muestran la mayor variedad de antocianinas, ya que poseen cuatro y cinco antocianidinas respectivamente. Las antocianinas encontradas en arándano se corresponden con 3-glicósidos de delfinidina, cianidina, petunidina y

malvidina, siendo esta última la mas abundante con un 71% de área cromatográfica (**Tabla 4**). Otros estudios han mostrado la presencia de un sexto aglicón en uva, concretamente pequeñas cantidades de pelargonindina 3-glicósido (Zhao *et al*., 2010). Por otro lado, este último estudio también muestra diferencias en los porcentajes de aglicones, pudiendo depender estas diferencias de la variedad utilizada.

Las antocianidinas del extracto de arándano pueden ser consideradas *a priori* las más estables de todas las ensayadas en este estudio, puesto que consisten principalmente en malvidina 3-glicósido (**Tabla 4**). Esta estabilidad se explica por la presencia de dos grupos metoxilo en el anillo B (**Figura 2**), que son menos reactivos que los hidroxilo (Shrikhande, 1976) y por tanto menos vulnerables a los pHs alcalinos (Cabrita *et al*., 2000).

En piel de berenjena fueron detectados dos picos antes de la hidrólisis, correspondientes con delfinidina 3-glicósido, puesto que tras la hidrólisis solamente se detectó delfinidina aglicón. El pico con mayor área cromatográfica fue una delfinidina 3-glicósido, que según bibliografía podría ser delfinidina 3-rutinósido (Sadilova *et al.*, 2006). El segundo pico es un monoacilado 3,5-diglicósido y representa el 12% del área cromatográfica de los picos. Esta deducción se basa en los ratios $A_{\lambda\ 440\ nm}/A_{\lambda\ max\ vis}$= 17% y $A_{\lambda\ max\ acyl}/A_{\lambda\ max\ vis}$= 50% (**Figura 14**).

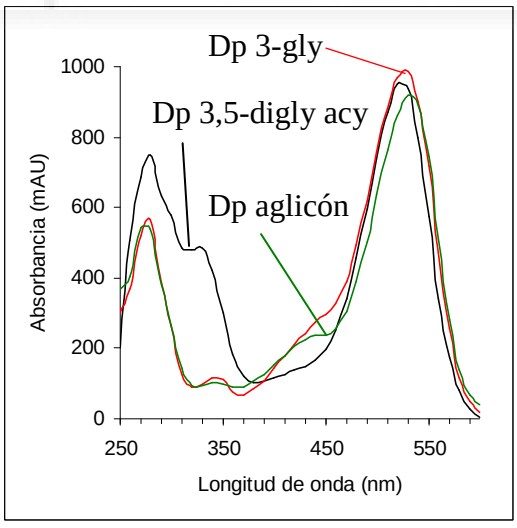


Figura 14: Espectro de absorción de las antocianinas de piel de berenjena en el que se indican las zonas representativas de glicosilación y acilación. Dp: delfinidina. 3-gly: 3-glicósido. 3,5-digly: 3,5-diglicósido. acy: monoacilada.

4.1.3.- Estabilidad de las antocianinas.

Durante toda la conservación, el zumo de piel de ciruela fue el que presentó los valores más altos de concentración, mientras que el de fresa y uva mostraron los más bajos (**Figura 15**).

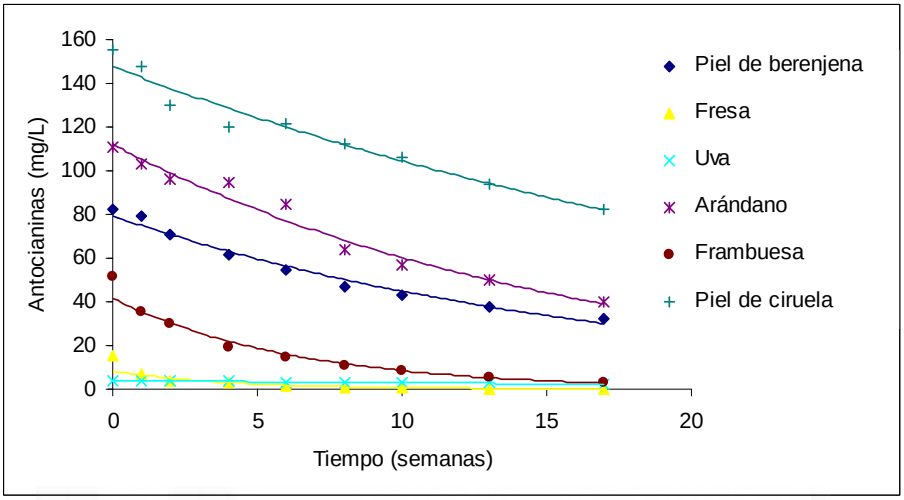


Figura 15: Concentración de las antocianinas monoméricas en los zumos modelo durante la conservación.

Zumos modelo	$t_{1/2}$ (semanas)	Valor D (semanas)
Piel de berenjena	12,2 ^{ab}	17,5 ^{ab}
Fresa	2,8 ^{d}	4,0 ^{d}
Uva	23,6 ^{a}	34,0 ^{a}
Arándano	11,2 ^{b}	16,1 ^{b}
Frambuesa	4,4 ^{c}	6,3 ^{c}
Piel de ciruela	20,0 ^{a}	28,9 ^{a}

Tabla 5: Parámetros de cinética de degradación de antocianinas durante la conservación de los zumos modelo. Para una misma columna, los valores seguidos de una misma letra no son significativamente diferentes ($P \leq 0,05$). $t_{1/2}$: tiempo que tarda en degradarse el 50% de la concentración de antocianinas. Valor D: tiempo necesario para que la concentración de antocianinas descienda hasta el 10% de la concentración inicial.

Los extractos pueden ser clasificados de acuerdo a sus parámetros de cinética de degradación ($t_{1/2}$ y valor D) (**Tabla 5**). La mayor estabilidad fue mostrada por las antocianinas de uva, piel de ciruela y piel de berenjena, cuyos tiempos de vida media y

valor D fueron superiores a 12 y 17 semanas respectivamente (Tabla 5). El extracto de arándano muestra una estabilidad inferior a la que cabría esperar en base a su composición en antocianinas (ver apartado *4.1.2.- Determinación de antocianinas*), mientras que para el extracto de piel de berenjena sucede lo contrario. En este último extracto, la única antocianidina encontrada fue delfinidina aglicón, la cual posee tres grupos –OH en su anillo B (**Figura 2**), y por eso se prevé la menos estable. El motivo de esta alta estabilidad radica en el 12% de delfinidina 3,5-diglicósido monoacilada (Dangles *et al*., 1993; Malien-Aubert *et al*., 2001).

4.1.4.- Estabilidad del color.

El tono (H*) inicial de los zumos modelo osciló entre los 30º correspondientes al color naranja/rojo anaranjado y los 340º correspondientes al color púrpura/rojo (**Figura 16**). El tono de los zumos de fresa asciende de 31º a 71º durante la conservación, lo que indica las grandes pérdidas de color sufridas en estos zumos. Resultados similares se han encontrado en fresas en conserva (a pH 3,6) (Kammerer *et al.*, 2007) y zumos de fresa pasteurizados (pH 3,5) (Garzón y Wrolstad, 2002). Los resultados de uva y piel de ciruela son los que muestran la mayor estabilidad, con unos incrementos de tono inferiores a 4º.

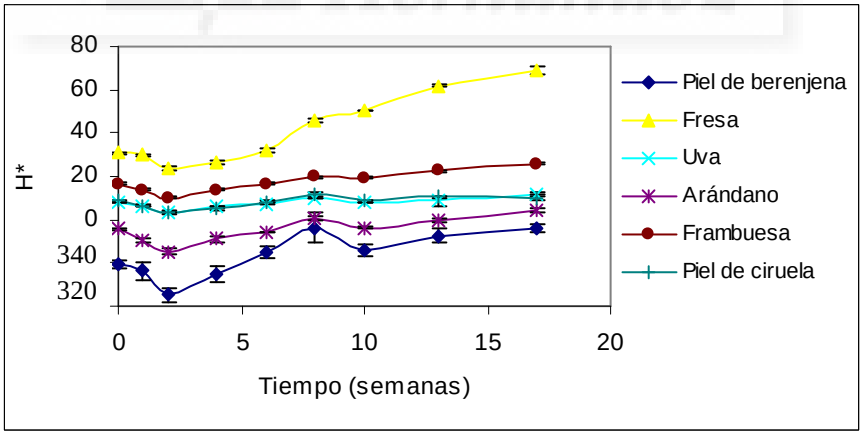


Figura 16: Evolución del tono (H*) de los zumos modelo durante la conservación.

El color del zumo de piel de ciruela mostró la menor pérdida de C* (6%) durante las 17 semanas de conservación en oscuridad a 20 ºC, mientras que el zumo de fresa, con

un 50% mostró la mayor (**Figura 17**). Los zumos de piel de berenjena, uva y frambuesa mostraron pérdidas comprendidas entre el 25 y el 30%.

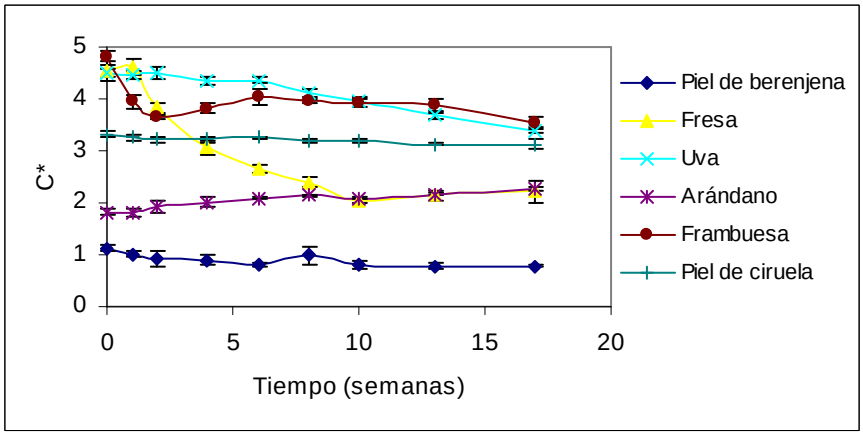


Figura 17: Evolución de la intensidad de color (C*) de los zumos modelo durante la conservación.

4.1.5.- Estabilidad de la capacidad antioxidante.

Los valores iniciales de capacidad antioxidante equivalente a vitamina C (VCEAC) muestran grandes variaciones entre los diferentes zumos modelo, oscilando entre los 10 mg de equivalencia a vitamina C (VCE)/L de zumo en uva y los 325 mg/L en piel de berenjena (**Figura 18**). El resto de zumos muestra valores que oscilan entre 60 y 100 mg/L. La alta capacidad antioxidante mostrada por piel de berenjena concuerda con el tipo de antocianinas presentes en ella, siendo delfinidina la antocianidina con mayor número de grupos –OH presentes en su anillo B (Stinzing y Carle, 2004; Noda *et al.*, 2000).

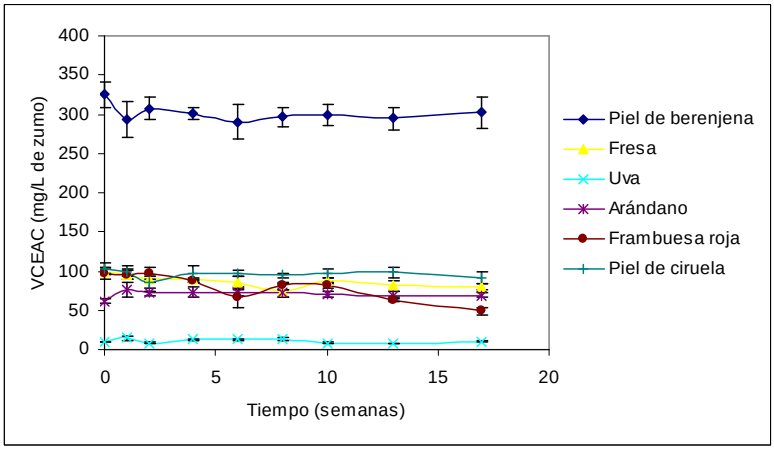


Figura 18: Evolución de la capacidad antioxidante (VCEAC) de los zumos modelo durante la conservación.

Comparando los resultados de concentración de antocianinas monoméricas con los de color y capacidad antioxidante, observamos que durante la conservación las pérdidas en antocianinas son superiores a las de color y capacidad antioxidante. La disminución de la concentración de antocianinas monoméricas puede deberse tanto a la degradación de éstas como a su unión con otros compuestos presentes en el zumo, principalmente otros fenoles. Las uniones de las antocianinas con otros fenoles suelen realizarse por enlaces covalentes (a través de su carbono 4), fuerzas de Van der Waals, interacciones iónicas e interacciones hidrofóbicas. Estas uniones originan complejos de mayor estabilidad sin afectar a la capacidad antioxidante, puesto que el anillo B de la estructura no se ve afectado (**Figura 2**) (Lu y Foo, 2001; Burda y Oleszek, 2001). Por otro lado, la presencia en el extracto de otros fenoles también puede contribuir al valor y estabilidad de la capacidad antioxidante.

4.2.- Efecto del pH y temperatura sobre el color de las antocianinas.

Para este ensayo fueron seleccionados tres materiales vegetales:

- Piel de ciruela (*Prunus salicina*, L.) cv. Santa rosa, por sus excelentes resultados mostradas en el primer apartado "4.1.- Caracterización y selección de la fuente de antocianinas más adecuada".
- Uva (*Vitis vinifera*, L.) cv. Moravia, el uso de este material vegetal se hace indispensable a modo de control, puesto que hoy día es el único material vegetal utilizado para la obtención de antocianinas como colorante alimentario (E 163).
- Fresa (*Fragaria ananassa*, L.) cv. Camarosa, por ser el material vegetal más común de entre los cuatro restantes en el mercado nacional.

A partir de los extractos de estos materiales vegetales se elaboraron zumos modelo a siete pHs diferentes que oscilaron entre los 2,4 y los 8,1. Los zumos fueron conservados durante 8 semanas a 6 y 23 ºC en oscuridad. La concentración de extracto utilizada fue 4 g/L.

4.2.1.- Estabilidad del color.

4.2.1.1.- Efecto del pH sobre los parámetros de color.

La **Figura 19** muestra cómo el color de las diferentes antocianinas se mantiene relativamente estable entre pH 2,4 y 4,0, observándose una gran pérdida de color a partir de pH 5,0. El zumo de uva es el que muestra en este primer rango de pH (2,4 a 4,0) los resultados de tono más cercanos a 0º y por tanto al rojo. Sin embargo, el valor de intensidad de color del zumo de uva es muy inferior al mostrado por el zumo de piel de ciruela, que además es el que muestra la menor pérdida de C* en este rango de pH. El zumo de fresa a esta concentración de 4 g de extracto/L no es capaz de aportar color rojo, mostrando un valor H* de 43,9º correspondiente con el color naranja.

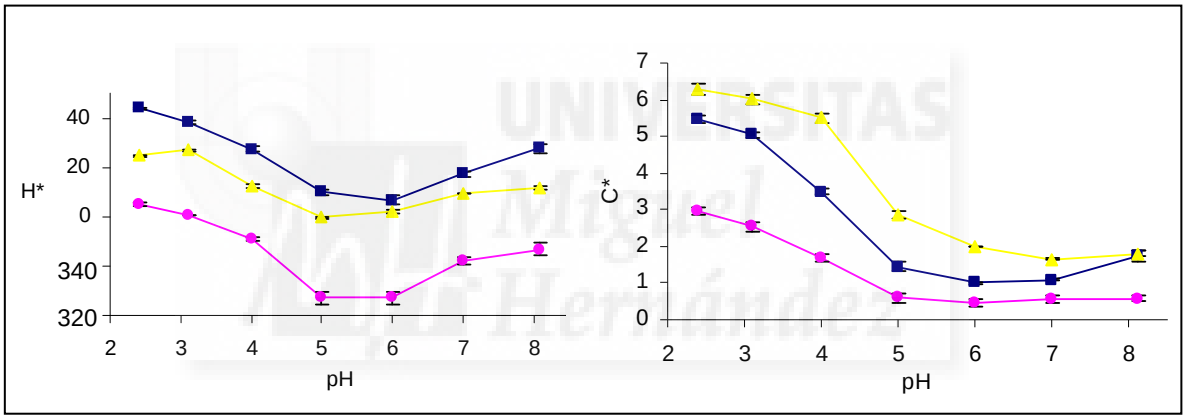


Figura 19: Cambios en tono (H*) e intensidad de color (C*) en función del pH de los zumos modelo. Zumos de fresa (■), uva (●) y piel de ciruela (▲). Las barras representan la desviación estándar.

4.2.1.2.- Efecto de la conservación a diferentes pHs y temperaturas sobre el parámetro de color tono (H).*

La **Figura 20** muestra los resultados de tono obtenidos a los diferentes pHs durante las 8 semanas de conservación en oscuridad a 6 y 23 ºC. En todos los casos, los resultados más estables y cercanos a H*= 0º (color rojo) son los mostrados por los zumos de piel de ciruela. La mayor estabilidad del tono se observó a los pH más bajos, 2,4 y 3,1. Destacamos, que el zumo de piel de naranja elaborado a pH 3,1 tan solo incrementó su valor H* un 5 y 8% a 6 y 23 ºC respectivamente.

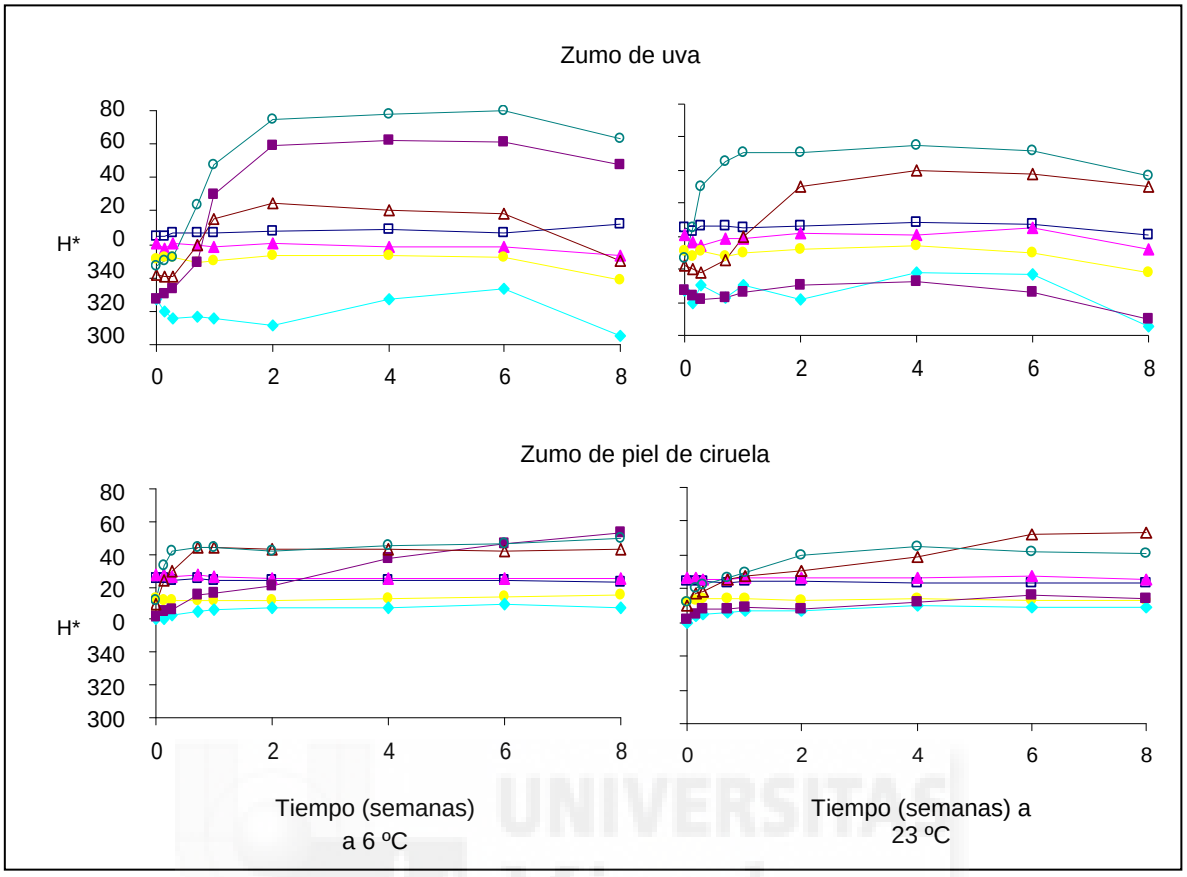


Figura 20: Evolución del tono (H*) de los zumos modelo en función del pH y temperatura de conservación. pHs: 2,4 (□), 3,1 (▲), 4,0 (●), 5,0 (♦), 6,0 (■), 7,0 (Δ) y 8,1 (○).

4.2.2.- Capacidad antioxidante.

Los zumos de uva claramente muestraron la menor capacidad antioxidante de las tres muestras ensayadas, sin embargo, ésta no se ve afectada por el aumento de la temperatura de conservación (**Figura 21**). El zumo de piel de ciruela muestra la mayor capacidad antioxidante, con unos valores muy estables a 6 ºC. La capacidad antioxidante de los zumos conservados a 23 ºC comienza a descender ($P \leq 0,05$) a partir de la cuarta semana de conservación.

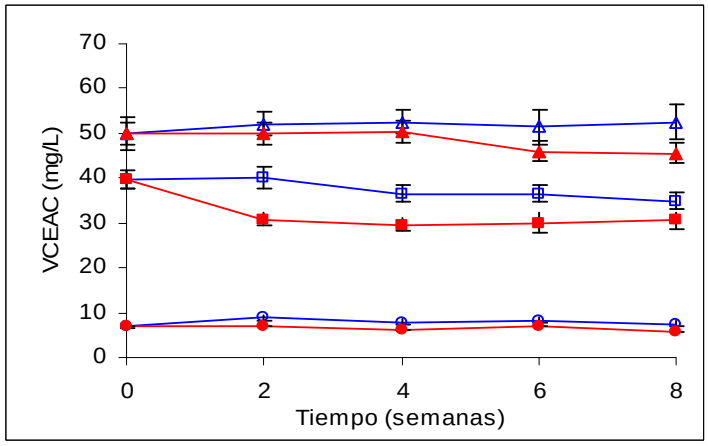


Figura 21: Evolución de la capacidad antioxidante de los zumos modelo a pH 4,0 durante la conservación. Zumo modelo de fresa (■), uva (●) y piel de ciruela (▲). Los símbolos rellenos representan el tratamiento a 23 ºC, y los símbolos huecos representan el tratamiento a 6 ºC. Las barras representan la desviación estándar.

4.3.- Efecto de la presencia de rutina y ácido ascórbico sobre el color de las antocianinas.

Este ensayo se realizó exclusivamente con ciruela (*Prunus salicina*, L.) cv. Black gold, a partir de la cual se obtuvo un extracto que se sometió a un proceso de concentración de antocianinas.

El extracto concentrado en antocianinas (9,5% p/p), en forma de polvo rojo, se empleó en la elaboración de zumos modelos a pH 3,7 que fueron conservados en oscuridad durante 17 semanas a 20 ºC. Los zumos modelo elaborados fueron cinco:

1. Zumo modelo con extracto concentrado de antocianinas (CAE).
2. Zumo modelo con extracto concentrado de antocianinas y rutina (CAE+R).
3. Zumo modelo con extracto concentrado de antocianinas y ácido ascórbico (CAE+AA).
4. Zumo modelo con ácido ascórbico (AA).
5. Zumo modelo con rutina (R).

4.3.1.- Determinación de la estabilidad del color.

En este estudio, los efectos sobre la estabilidad del color comienzan a ser observables a partir de la quinta y novena semana de conservación para los valores C* y H* respectivamente (**Figura 22**). Por otro lado, los resultados muestran el efecto degradante inmediato del ácido ascórbico sobre el color aportado por las antocianinas, que ya ha sido descrito en otros estudios (Garzon y Wrolstad, 2002; Lopes *et al*., 2007; Sun *et al*., 2011).

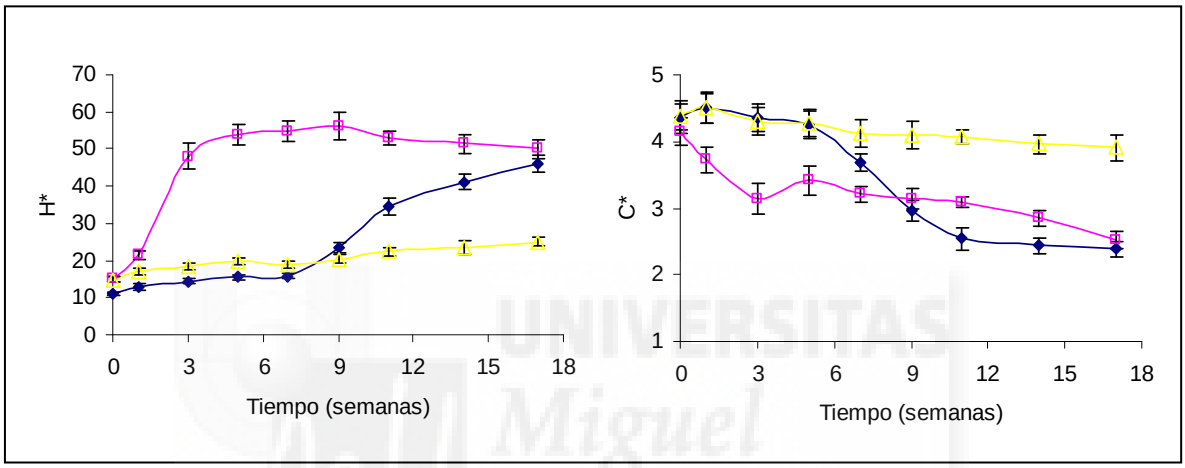


Figura 22: Evolución del tono (H*) e intensidad de color (C*) de los zumos modelo durante la conservación. Zumos CAE (♦), CAE+AA (□) y CAE+R (Δ). Las barras representan la desviación estándar.

4.3.2.- Estabilidad de las antocianinas y rutina.

En la **Figura 23** se puede observar que la concentración inicial de antocianinas y rutina en los zumos CAE+R es respectivamente un 60 y 50% inferior a la determinada en los zumos CAE y R. Estos bajos valores iniciales de antocianinas y rutina en los zumos CAE+R indican que la copigmentación entre los dos compuestos acontece de inmediato, lo que concuerda con los resultados encontrados por otros autores (Eiro y Heinonen, 2002). Por otro lado, el alto ratio inicial copigmento (rutina)/antocianina (7,8/1) en los zumos CAE+R favorece la rápida copigmentación (Malien-Aubert *et al*., 2001). La baja concentración de antocianinas en el zumo CAE+AA corrobora el efecto degradante del ácido ascórbico comentado anteriormente.

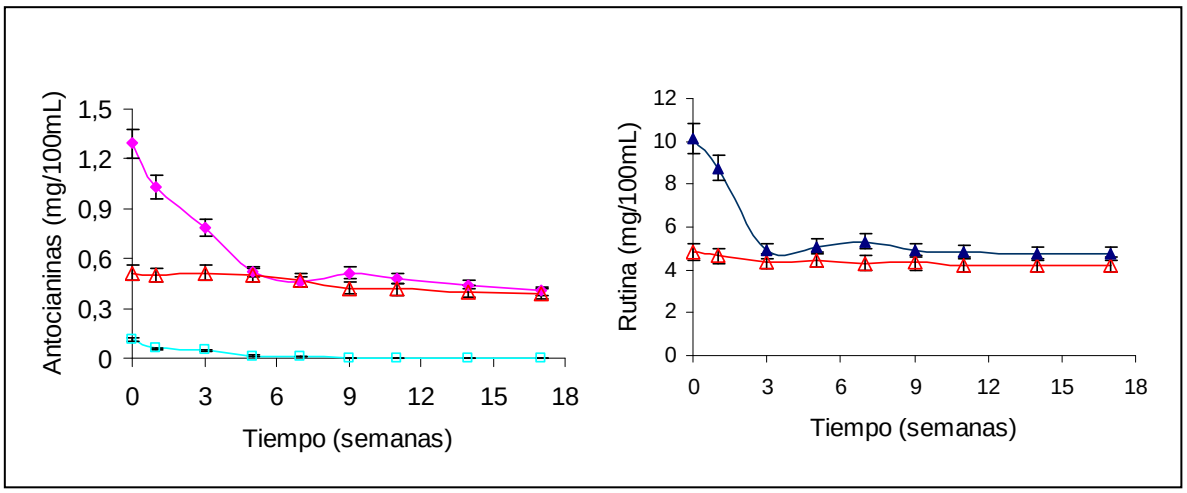


Figura 23: Evolución de la concentración de antocianinas y rutina de los zumos modelo durante la conservación. Zumos CAE (♦), CAE+AA (□), CAE+R (Δ) y R (▲). Las barras representan la desviación estándar.

4.3.3.- Estabilidad de la capacidad antioxidante.

La capacidad antioxidante inicial del zumo CAE fue de 2,4 mg VCE/100 mL (**Figura 24**). La contribución estimada de las antocianinas a la capacidad antioxidante total del zumo CAE fue 1,7 mg VCE/100 mL, calculada según la siguiente ecuación:

$$\text{VCEAC (mg VCE/100 mL)} = \Sigma[\text{CC (mg/100 mL)} \cdot \text{Ra} \cdot \text{PC (\%)} \cdot 100^{-1}]$$

donde CC: contenido de cianidina glicósido en el zumo CAE (1.3 mg/100mL) (**Figura 23**), Ra: ratio VCE/cianidina glicósido (1,4 para cianidina 3-glucósido y 1,1 para cianidina 3-rutinósido en solución acuosa de metanol (50% v/v) acidificada (Heo *et al*., 2007) y PC: proporciones de las dos cianidinas glicósido presentes en el zumo CAE (76% de cianidina 3-glucósido y 24% de cianidina 3-rutinósido).

De estos resultados se desprende que el 71% de la capacidad antioxidante del zumo CAE proviene del 9,5% de antocianinas presente en el extracto utilizado para elaborar los zumos, lo que muestra la gran contribución de las antocianinas a la capacidad antioxidante total.

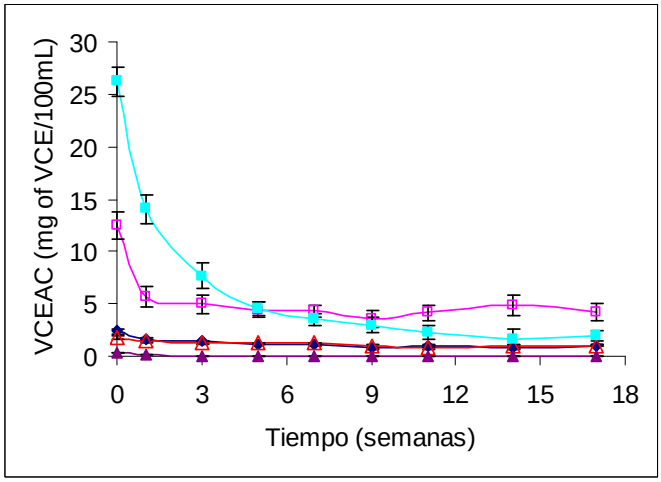


Figura 24: Evolución de la capacidad antioxidante equivalente a vitamina C (VCEAC) de los zumos modelo durante la conservación. Zumos CAE (♦), CAE+AA (□), CAE+R (Δ), R (▲) y AA (■). Las barras representan la desviación estándar.

Los resultados del zumo de rutina muestran una capacidad antioxidante casi nula, y ello se debe al bajo pH utilizado para la elaboración de los zumos modelo (pH= 3,7), ya que la máxima capacidad antioxidante de rutina se obtiene a pH 7,0 (Ignjatovic *et al.*, 2002; Gomathi *et al.*, 2003) con ratios VCE/rutina de 0,9-1,0 (Heo *et al.*, 2007; Kim *et al.*, 2002). A partir de la semana 11 de conservación, los zumos CAE+AA muestran más capacidad antioxidante que los zumos AA. Esto podría deberse al efecto protector ejercido por las antocianinas sobre el ácido ascórbico, que ya ha sido descrito en otros trabajos (García-Viguera y Bridle, 1999; Pang *et al.*, 2001). Los resultados indican la existencia de una correlación (R^2= 0,881) entre la capacidad antioxidante y la concentración de antocianinas de los zumos (**Figura 25**).

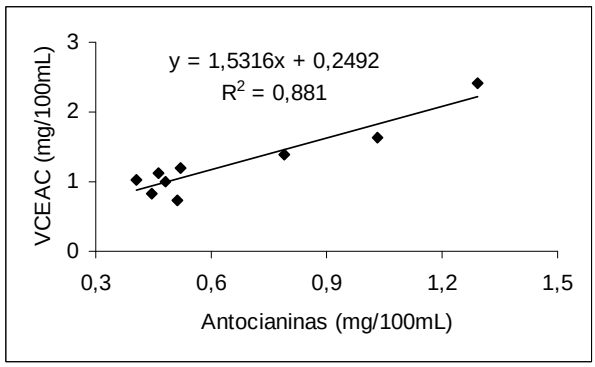


Figura 25: Correlación entre capacidad antioxidante y antocianinas en los zumos modelo elaborados durante la conservación. Las barras indican la desviación estándar.

4.4.- Efecto de la adición de zumo concentrado de ciruela sobre las propiedades físico-químicas y sensoriales de yogur.

En este ensayo se elaboraron cuatro tipos de yogur, incluyendo tanto yogures control como otros coloreados por la adición de un 9% (v/v) de zumo concentrado de ciruela (*Prunus salicina*, L.) cv. Black diamond:

1. Yogur control blanco (Control-W).
2. Yogur control coloreado (Control-C) con 2,5 (mg/L) del colorante sintético azorrubina (E 122).
3. Yogur con zumo concentrado de ciruela y un 4,85% (p/v) de leche desnatada en polvo (Y1).
4. Yogur con zumo concentrado de ciruela y un 5,33% (p/v) de leche desnatada en polvo (Y2).

Las diferentes cantidades de leche en polvo de las muestras Y1 e Y2 responden al interés en evaluar diferentes texturas.

4.4.1.- Firmeza.

El valor promedio de la firmeza de las muestras Y2 es un 13% superior a la de las muestras Control-W, y éstas a su vez un 10% superior a las muestras Y1 (**Figura 26**). Estos valores de firmeza están correlacionados con los valores de sólidos solubles totales (SST), de acuerdo con la siguiente ecuación:

$$\text{Firmeza (N)} = 1{,}5904 \times \text{SST (\%)} - 25{,}2160$$

Esta relación ya ha sido mostrada en otros estudios (Mahdian y Tehrani, 2007).

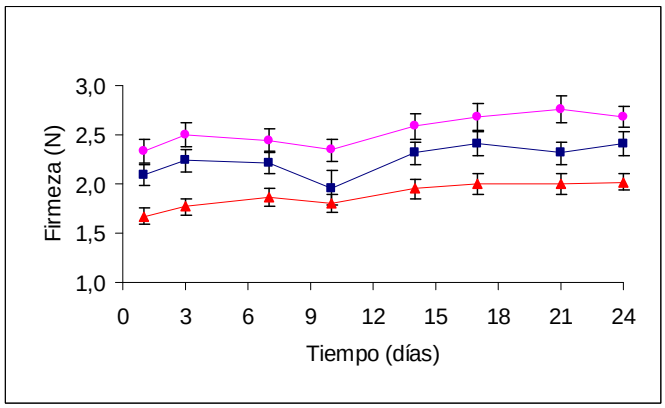


Figura 26: Evolución de la firmeza de los yogures Y1 (▲), Y2 (●) y Control-W (■) durante los 24 días de conservación a 6 ºC.

4.4.2.- Estabilidad del color.

Los resultados de color de los yogures Y1 y Control-C indican que el tono (H*) se mantuvo estable ($P \leq 0,05$) durante las primeras 10 y 7 semanas de conservación respectivamente. El color rosáceo del yogur Y1 mostró un valor H* inicial un 6% mayor que la muestra control coloreada (**Figura 27**). La intensidad de color (C*) reflejó resultados similares y estables durante las semanas iniciales, para posteriormente producirse un descenso. El periodo inicial de la conservación en el cual el color se muestra estable (entre 7 y 10 semanas) es superior al encontrado en otros estudios con yogures de fresa, donde tras la primera semana de conservación a 5 ºC ya se detectaron pérdidas significativas ($P \leq 0,05$) de color (Aryana *et al.*, 2006).

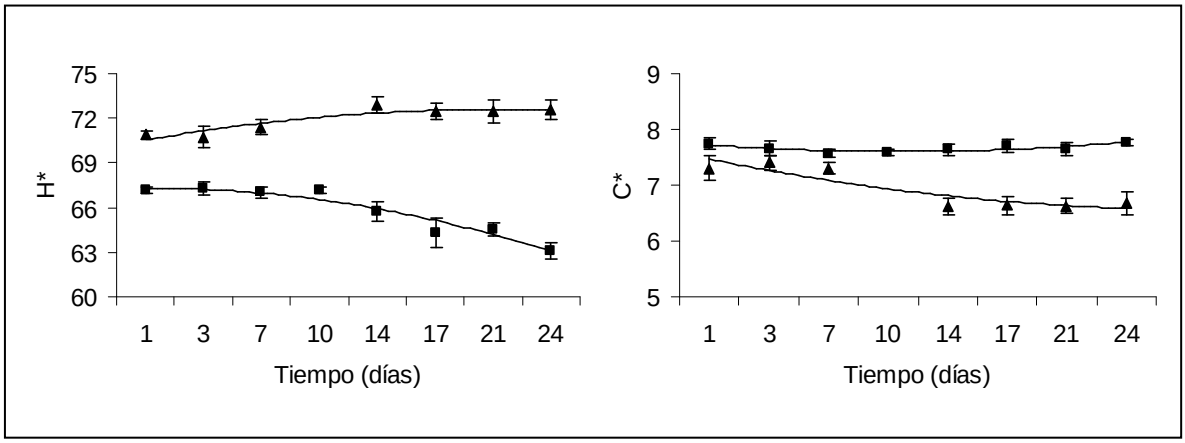


Figura 27: Evolución del tono (H*) e intensidad de color (C*) en las muestras Y1 (▲) y Control-C (■) durante los 24 días de conservación a 6 ºC.

4.4.3.- Análisis sensorial.

Los resultados de cremosidad (**Tabla 6**) muestran que los yogures Control-W e Y1 son los más cremosos ($P \leq 0,05$). Estos resultados guardan relación con los de firmeza instrumental y el contenido en sólidos totales. En cuanto al color, los yogures coloreados con E 122 (Control-C) mostraron un color más intenso que los coloreados con zumo concentrado de ciruela (Y1 e Y2). Los resultados de la prueba de ordenación de los yogures según su aceptación no reflejó diferencias significativas ($P \leq 0,05$) entre las muestras estudiadas.

	Y1	**Y2**	**Control-W**	**Control-C**
Cremosidad	$5,53^{a}$	$5,00^{b}$	$5,80^{a}$	---
Color	$4,07^{b}$	$4,00^{b}$	---	$5,87^{a}$
Aceptabilidad	144	116	---	100

Tabla 6: Evaluación sensorial de la cremosidad, intensidad de color y aceptabilidad de los yogures. Para una misma fila, los valores con letra diferente son significativamente diferentes ($P \leq 0,05$).

5.- Conclusiones

5.- CONCLUSIONES.

Primera. Los extractos de uva (*Vitis vinifera* L., cultivar Moravia), piel de ciruela (*Prunus salicina* L., cv. Santa rosa) y piel de berenjena (*Solanum melongena* L., cv. Black bell) son los más estables respecto de la concentración de antocianinas.

Segunda. El extracto de piel de ciruela (*Prunus salicina* L., cv. Santa rosa) proporciona unos niveles de color adecuados a los zumos, tanto por la tonalidad roja presentada como por su intensidad y estabilidad.

Tercera. El zumo coloreado con extracto de piel de berenjena (*Solanum melongena* L., cv. Black bell) posee una capacidad antioxidante muy superior al resto de extractos vegetales utilizados.

Cuarta. El color rojo de los zumos elaborados con extracto de piel de ciruela (*Prunus salicina* L., cv. Santa rosa) se muestra estable tanto a temperatura de refrigeración (6 ºC) como a temperatura ambiente (23 ºC) a pH inferior a 4,0.

Quinta. La capacidad antioxidante del zumo coloreado con extracto de piel ciruela (*Prunus salicina* L., cv. Santa rosa) es estable durante la conservación frigorífica (6 ºC), mientras que a temperatura ambiente (23 ºC) sufre pérdidas a partir de la cuarta semana de conservación.

Sexta. El color de las antocianinas de ciruela (*Prunus salicina* L., cv. Black gold) se estabiliza por la presencia de rutina, apreciándose este efecto a partir de la quinta semana de conservación a 20 ºC.

Séptima. El ácido ascórbico ejerce un efecto degradante sobre las antocianinas de ciruela (*Prunus salicina* L., cv. Black gold) y el color aportado por éstas.

Octava. El zumo concentrado de ciruela (*Prunus salicina* L., cv. Black diamond) aporta al yogur un color adecuado y estable durante una semana de conservación frigorífica (6 ºC).

Novena. Teniendo en cuenta todos los parámetros estudiados tanto en los zumos modelo como en el yogur (rendimiento de extracción, concentración y perfil de antocianinas, capacidad antioxidante y color), la piel de ciruela es la fuente de antocianinas con el mayor potencial para ser utilizada como colorante alimentario.

Bibliografía

BIBLIOGRAFÍA

Álvarez-Suárez J.M., Giampieri F., Tulipán S., Casoli T., Di Stefano G., González-Paramás A.M., Santos-Buelga C., Busco F., Quiles J.L., Cordero M.D., Bompadre S., Mezzetti B. y Battino M. (2014). One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular risk, oxidative stress markers and platelet activation in humans. *Journal of Nutrition Biochemistry, 25*, 289-294.

Andersen Ø.M. y Jordheim M. (2006). The Anthocyanins. En: *Flavonoids: Chemistry, Biochemistry and Applications*, (Andersen Ø.M. y Markham K.R., eds.), CRC Press, Boca Raton, pp. 471–553.

Azuma K., Ohyama A., Ippoushi K., Ichiyanagi T., Takeuchi A., Saito T. y Fukuoka H. (2008). Structures and antioxidant activity of anthocyanins in many accessions of eggplant and its related species. *Journal of Agricultural and Food Chemistry, 56,* 10154–10159.

Auroma O.I. (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American Oil Chemists' Society, 75,* 199-212.

Bagchi D., Garg A., Krohn R.L., Bagchi M., Bagchi B.J., Balmoori J. y Stohs S.J. (1998). Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. *General Pharmacology, 30*(5), 771–776.

Bąkowska A., Kucharska A.Z. y Oszmiański J. (2003). The effect of heating, UV irradiation, and storage stability of anthocyanin-polyphenol copigment complex. *Food Chemistry, 81,* 349–355.

Bąkowska-Barczak A. (2005). Acylated anthocyanins as stable, natural food colorants – a review. *Polish Journal of Food and Nutrition Sciences, 14/55*(2)*:* 107-116.

Betz M. y Kulozik U. (2011). Whey protein gels for the entrapment of bioactive anthocyanins from bilberry extract. *International Dairy Journal, 21,* 703-710.

Bobbio F.O. y Mercadante A.Z. (2008). Anthocyanins in foods: Occurrence and physicochemical properties. En: *Food colorants: Chemical and functional properties*, (Bobbio F.O. y Mercadante A.Z., eds.), CRC Press, Boca Raton, Vol. 1, pp. 241−276.

Boulton R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *American Society for Enology and Viticulture, 52,* 67−80.

Brouillard R. (1982). Chemical structure of anthocyanins. En: *Anthocyanins as food colors*, (Markakis P., ed.), Academic Press, New York, pp. 1-40.

Brouillard R. y Dangles O. (1993). Flavonoids and flower colour. En: *The flavonoids: advances in research since 1986*, (Harborne J.B., ed.), Chapman and Hall, London.

Buchert J., Koponen J.M., Suutarinen M., Mustranta A., Lille M., Törrönen R. y Poutanen K. (2005). Effect of enzyme-aided pressing on anthocyanin yield and profiles in bilberry and blackcurrant juices. *Journal of the Science of Food and Agriculture, 85*, 2548–2556.

Buchweitz M., Nagel A., Carle R. y Kammerer D.R. (2012). Characterisation of sugar beet pectin fractions providing enhanced stability of anthocyanin-based natural blue food colourants. *Food Chemistry, 132*, 1971-1979.

Burin V.M., Rossa P.N., Ferreira-Lima N.E., Hillmann M.C.R. y Boirdignon-Luiz M.T. (2011). Anthocyanins: optimisation of extraction from Cabernet Sauvignon grapes, microcapsulation and stability in soft drink. *International Journal of Food Science & Technology, 46*, 186–193.

Cacace J.E. y Mazza G. (2003). Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering, 59*(4), 379–389.

Calvo C. (1999). Uso de colorantes en el campo de la alimentación. *Alimentación, equipos y tecnología, 3(abril)*, 79-88.

Can N.O., Arli G. y Atkosar Z. (2012). Rapid determination of free anthocyanins in foodstuffs using high performance liquid chromatography. *Food Chemistry, 130*, 467–473.

Castañeda-Ovando A., Pacheco-Hernández M.d-L., Páez-Hernández M.E., Rodríguez J.A. y Galan-Vidal C.A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry, 113*, 859–871.

Carlsen C. y Stapelfeldt H. (1997). Light sensitivity of elderberry extract. Quantum yields for photodegradation in aqueous solution. *Food Chemistry, 60(3)*, 383-387.

Cavalcanti R.N., Santos D.T. y Meireles M.A.A. (2011). Non-thermal stabilization mechanisms of anthocyanins in model and food systems - An overview. *Food Research Internacional, 44*, 499-509.

Cemeroglu B., Velioglu S. y Isik S. (1994). Degradation kinetics of anthocyanin in sour cherry juice and concentrate. *Journal of Food Science, 59*, 1216-1218.

Cheoka C.Y., China N.L., Yusofa Y.A., Taliba R.A. y Law C.L. (2013). Optimization of total monomeric anthocyanin (TMA) and total phenolic content (TPC) extractions

from mangosteen (*Garcinia mangostana* Linn.) hull using ultrasonic treatments. *Industrial Crops and Products, 50*, 1-7.

Chou P.-H., Matsui S. y Matsuda T. (2007). Isolation and identification of xenobiotic aryl hydrocarbon receptor ligands in dyeing wastewater. *Environmental Science and Technology, 41*(2), 652-657.

Cuevas M.E., Antezana A. y Winterhalter P. (2008). Análisis y caracterización de antocianinas en diferentes variedades de maíz (*Zea mays*) Boliviano. En: *Memorias Red-Alfa Lagrotech*, Comunidad Europea, Cartagena (Colombia), pp. 79-95.

Cui C., Zhang S., You L., Ren J., Luo W., Chen W. y Zhao M. (2013). Antioxidant capacity of anthocyanins from *Rhodomyrtus tomentosa* (Ait.) and identification of the major anthocyanins. *Food Chemistry, 139*, 1-8.

Dangles O., Saito N. y Brouillard R. (1993). Anthocyanin intramolecular copigment effect. *Phytochemistry, 34*, 119-124.

de Pascual-Teresa S., Moreno D.A. y García-Viguera C. (2010). Flavonols and anthocyanins in cardiovascular health: a review of current evidence. *International Journal of Molecular Sciences, 11*, 1679-1703.

Delgado-Vargas F., Jiménez A.R. y Paredes-López O. (2000). Natural pigments: carotenoids, anthocyanins, and betalains – characteristics, biosynthesis, processing and stability. *Critical Reviews in Food Science and Nutrition, 40*, 173-289.

Durst R. y Wrolstad R.E. (2001). Separation and characterization of anthocyanins by HPLC. En: *Handbook of food analytical chemistry*, John Wiley & Sons, New Jersey, pp. 33-45.

Edwards W.P. (2001). Emulgentes, colorantes y aromas. En: *La ciencia de las golosinas*, (Edwards W.P., ed.), Editorial Acribia S.A., Zaragoza (España), pp. 65-88.

Eiro M.J. y Heinonen M. (2002). Anthocyanin color behavior and stability during storage: Effect of intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 50*, 7461–7466.

Es-Safi N.E., Cheynier V. y Moutounet M. (2000). Study of the reactions between (+)-catechin and furfural derivates in the presence or absence of anthocyanins and their implication in food color change. *Journal of Agricultural and Food Chemistry, 48(12)*, 5946-5954.

Es-Safi N.E., Cheynier V. y Moutounet M. (2002). Interactions between cyanidin 3-O-glucoside and furfural derivatives and their impact on food color changes. *Journal of Agricultural and Food Chemistry, 50(20)*, 5586–5595.

Felgines C., Talavera S., Gonthier M.-P., Texier O., Scalbert A., Lamaison J.-L. y Rémésy C. (2003). Strawberry anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans. *The Journal of Nutrition, 133,* 1296–1301.

Fleschhut J., Kratzer F., Rechkemmer G. y Kulling S.E. (2006). Stability and biotransformation of varius dietary antocyanins *in vitro*. *European Journal of Nutrition, 45(1),* 7-18.

Flores F.P., Singh R.K., Kerr W.L., Pegg R.B. y Kong F. (2014). Total phenolics content and antioxidant capacities of microencapsulated blueberry anthocyanins during in vitro digestion. *Food Chemistry, 153*, 272-278.

Frankel E.N., Waterhouse A.L. y Teissedre P.L. (1995). Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *Journal of Agricultural and Food Chemistry, 43,* 890–894.

Furtado P., Figueiredo P., Neves H.C. y Pina F. (1993). Photochemical and thermal degradation of anthocyanins. *Journal of Photochemistry and Photobiology A: Chemistry, 75*, 113-118.

Galvano F., La Fauci L., Vitaglione P., Fogliano V., Vanella L. y Felgines C. (2007). Bioavailability, antioxidant and biological properties of the natural free-radical scavengers cyanidin and related glycosides. *Annali Dell'Istituto Superiore di Sanita, 43(4)*, 382-393.

Gamel T.H. y Kiritsakis A. (1999). Effect of methanol extracts of rosemary and olive vegetable water on the stability of olive oil and sunflower oil. *Grasas y Aceites, 50(5)*, 345-350.

Gao L. y Mazza G. (1995). Characterization, quantitation, and distribution of anthocyanins and colorless phenolics in sweet cherries. *Journal of Agricultural and Food Chemistry, 43(2)*, 343-346.

García-Viguera C. y Bridle P. (1999). Influence of structure on colour stability of anthocyanins and flavylium salts with ascorbic acid. *Food Chemistry, 64,* 21-26.

Garde-Cerdan T., Marselles-Fontanet A.R., Arias-Gil M., Ancin-Azpilicueta C. y Martín-Belloso O. (2008). Effect of storage conditions on the volatile composition of wines obtained from must stabilised by PEF during ageing without SO_2. *Innovative Food Science & Emerging Technologies*, *9*(4), 469–476.

Garzón G.A. y Wrolstad R.E. (2001). The stability of pelargonidin-based anthocyanins at varying water activity. *Food Chemistry, 75*, 185-196.

Garzón G.A. y Wrolstad R.E. (2002). Comparison of the stability of pelargonidin-based anthocyanins in strawberry juice and concentrate. *Journal of Food Science, 67*, 1288-1299.

Ghosh D. y Konishi T. (2007). Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. Review Article. *Asia Pacific Journal of Clinical Nutrition, 16*(2), 200-208.

Giusti M.M. y Wrolstad R.E. (2003). Acylated anthocyanins from edible sources and their applications in food systems. Review. *Biochemical Engineering Journal, 14*(3), 217-225.

González-Manzano S., Santos-Buelga C., Dueñas M., Rivas-Gonzalo J.C. y Escribano-Bailón T. (2008). Colour implications of self-association processes of wine anthocyanins. *European Food Research and Technology, 226*, 483–490.

Goiffon J.-P., Mouly P.P. y Gaydou E.M. (1999). Anthocyanic pigment determination in red fruit juices, concentrated juices and syrups using liquid chromatography. *Analytica Chimica Acta, 382*, 39-50.

He F., Mu L., Yan G.-L., Liang N.-N., Pan Q.-H., Wang J., Reeves M.J. y Duan C.-Q. (2010). Biosynthesis of anthocyanins and their regulation in colored grapes: a review. *Molecules, 15*, 9057-9091.

He F., Liang N.-N., Mu L., Pan Q.-P., Wang J., Reeves M.J. y Duan C.-Q. (2012). Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expresión. Review. *Molecules, 17*, 1571-1601.

Hernández-Herrero J.A. y Frutos M.J. (2011). Degradation kinetics of pigment, colour and stability of the antioxidant capacity in juice model systems from six anthocyanin sources. *International Journal of Food Science & Technology, 46*, 2550–2557.

Hernández-Herrero J.A. y Frutos M.J. (2014a). Colour and antioxidant capacity stability in grape, strawberry and plum peel model juices at different pHs and temperatures. *Food Chemistry, 154*, 199–204.

Hernández-Herrero J.A. y Frutos M.J. (2014b). Effect of concentrated plum juice on physicochemical and sensory properties of yoghurt made at bench top scale. *International Journal of Dairy Technology, 67*(1), 123–128.

Hertog M.G.L., Hollman P.C.H., Katan M.B. y Kromhout D. (1993). Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutrition and Cancer, 20*(1), 21-29.

Ito F., Tanaka N., Katsuki A. y Fujii T. (2002). Why do flavylium salts show so various colors in solution?: Effect of concentration and water on the flavylium's color changes. *Journal of Photochemistry and Photobiology A: Chemistry, 150*(1–3), 153–157.

Jackman R.L. y Smith J.L. (1996). Anthocyanins and betalains. En: *Natural food colorants*, (Hendry G.A.F. y Houghton J.D., eds.), 2ª Ed., Chapman and Hall, London, pp. 245-309.

Jackson R.S. (2008). Wine Science: Principles and Applications. 3ª Ed., Academic Press, Burlington, USA, pp. 287-295.

Jiang Y.M. (2000). Role of anthocyanins, polyphenol oxidase and phenols in lychee pericarp browning. *Journal of the Science of Food and Agriculture, 80*, 305-310.

Jurd L. (1972). Some advances in the chemistry of anthocyanin-type plant pigments. En: *The chemistry of plant pigments*, (Chichester C.O., ed.), Academic Press, London and New York, pp. 123-142.

Kähkönen M.P., Hopia A.I. y Heinonen M. (2001). Berry phenolics and their antioxidant activity. *Journal of Agricultural and Food Chemistry, 49*(8), 4076–4082.

Kähkönen M.P. y Heinonen M. (2003). Antioxidant activity of anthocyanins and their aglycons. *Journal of Agricultural and Food Chemistry, 51,* 628-633.

Kalt W., McDonald J.E. y Donner H. (2000). Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. *Journal of Food Science, 65*(3), 390-393.

Kammerer D., Kljusuric J.C., Carle R. y Schieber A. (2005). Recovery of anthocyanins from grape pomace extracts (*Vitis vinifera* L. cv. Cabernet Mitos) using a polymeric adsorber resin. *European Food Research and Technology, 220*(3-4), 431-437.

Kapasakalidis P.G., Rastall R.A. y Gordon M.H. (2006). Extraction of polyphenols from processed black currant (*Ribes nigrum* L.) residues. *Journal of Agricultural and Food Chemistry, 54*(11), 4016–4021.

Kelebek H., Canbas A. y Selli S. (2009). Effects of different maceration times and pectolytic enzyme addition on the anthocyanin composition of *Vitis vinifera* cv. Kalecik Karasi wines. *Journal of Food Processing and Preservation, 33*(3), 296-311.

Kemp W. (1991). En: *Organic spectroscopy*, (Freeman W.H. & Co., eds.), 3ª Ed., New York.

Kong J.-M., Chia L.-S., Goh N.-K, Chia T.F., Brouillard R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 64*, 923-933.

Kopjar M., Piližota V., Tiban N.N., Šubarić D., Babić J., Ačkar Đ. y Sajdl M. (2009). Strawberry jams: Influence of different pectins on colour and textural properties. *Czech Journal of Food Science, 27*, 20–28.

Koponen J.M., Aponen A.M., Mattila P.H. y Törrönen A.R. (2007). Contents of anthocyanins and ellagitannins in selected foods consumed in Finland. *Journal of Agricultural and Food Chemistry, 55*(4), 1612–1619.

Krifi B., Chouteau F., Boudrant J. y Metche M. (2000). Degradation of anthocyanins from blood orange juices. *International Journal of Food Science and Technology, 35*(3), 275–283.

Kuhnau, J. (1976). The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Review of Nutrition and Dietetics, 24*, 117-191.

Lai K.P.K., Dolan K.D. y Ng P.K.W. (2009). Inverse method to estimate kinetic degradation parameters of grape anthocyanins in wheat flour under simultaneously changing temperature and moisture. *Journal of Food Science, 74*(5), E241–E249.

Lamikanra O. (1989). Anthocyanins of *Vitis rotundifolia* hybrid grapes. *Food Chemistry, 33*(3), 225–237.

Lapidot T., Harel S., Granit R. y Kanner J. (1998). Bioavailability of red wine anthocyanins as detected in human urine. *Journal of Agricultural and Food Chemistry, 46*(10), 4297–4302.

Lee J., Finn C.E. y Wrolstad R.E. (2004). Comparison of anthocyanin pigment and other phenolic compounds of *Vaccinium membranaceum* and *Vaccinium ovatum* native to the Pacific Northwest of North America. *Journal of Agricultural and Food Chemistry, 52*, 7039–7044.

Li Z., Pan Q.H., Cui X.Y. y Duan C.Q. (2010). Optimization on anthocyanins extraction from wine grape skins using orthogonal test design. *Food Science and Biotechnology, 19*(4), 1047-1053.

Lopes P., Richard T., Saucier C., Teissedre P.L., Monti J.P. y Glories Y. (2007). Anthocyanone A: A quinone methide derivative resulting from malvidin 3-O-glucoside degradation. *Journal of Agricultural and Food Chemistry, 55*, 2698–2704.

Lopes-da-Silva F., de Pascual-Teresa S., Rivas-Gonzalo J. y Santos-Buelga C. (2002). Identification of anthocyanin pigments in strawberry (cv Camarosa) by LC

using DAD and ESI-MS detection. *European Food Research and Technology, 214*, 248-253.

Malien-Aubert C., Dangles O. y Amiot M.J. (2001). Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. *Journal Agricultural and Food Chemistry, 49*, 170-176.

Matsufuji H., Kido H., Misawa H., Yaguchi J., Otsuki T., Chino M., Takeda M. y Yamagata K. (2007). Stability to light, heat, and hydrogen peroxide at different ph values and DPPH radical scavenging activity of acylated anthocyanins from red radish extract. *Journal Agricultural and Food Chemistry, 55,* 3692-3701.

Marín F.R., Frutos M.J., Pérez-Álvarez J.A., Martínez-Sánchez F. y Del Rio J.A. (2002). Flavonoids as nutraceuticals: structural related antioxidant properties and their role on ascorbic acid preservation. En: *Studies in natural products chemistry,* (Atta-ur-Rahman, ed.), Elsevier, London, Vol. 26, Bioactive natural products (part G), pp. 741-778.

Mazza G. y Miniati E. (1993). Introduction. En: *Anthocyanins in fruits, vegetables and grain*, CRC Press Inc., Boca Raton (Florida), pp. 1-28.

Mazza G., Cacace J.E. y Kay C.D. (2004). Methods of analysis for anthocyanins in plants and biological fluids. *Journal of AOAC International, 87*, 129-145.

McCann D., Barret A., Cooper A., Crumpler D., Dalen L., Grimshaw K., Kitchin E., Lok K., Porteous L., Prince E., Sonuga-Barke E., Warner J.O. y Stevenson J. (2007). Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: A randomised, double-blinded, placebo-controlled trial. *Lancet, 370*, 1560–1567.

Miller N.J., Diplock A.T. y Rice-Evans C.A. (1995). Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *Journal of Agricultural and Food Chemistry, 43*, 1794-1801.

Moreau C., Ambrose M.J., Turner L., Hill L., Ellis T.H.N. y Hofer J.M.I. (2012). The b gene of pea encodes a defective flavonoid 3´,5´-hydroxylase, and confers pink flower color. *Plant Physiology, 159*, 759–768.

Noda Y., Kneyuki T., Igarashi K., Mori A. y Packer L. (2000). Antioxidant activity of nasunin, an anthocyanin in eggplant peels. *Toxicology, 148,* 119–123.

Odriozola-Serrano I., Soliva-Fortuny R. y Martín-Belloso O. (2010). Changes in bioactive composition of fresh-cut strawberries stored under super atmospheric oxygen, low-

oxygen or passive atmospheres. *Journal of Food Composition and Analysis, 23*, 37−43.

Ordaz-Galindo A., Wesche-Ebeling P., Wrolstad R.E., Rodríguez-Saona L.E. y Argaiz-Jamet A. (1999). Purification and identification of Capulin (*Prunus serotina* Ehrh) anthocyanins. *Food Chemistry, 65*, 201-206.

Pang X.Q., Zhang Z.Q., Duan X.W. y Ji Z.L. (2001). The influence of oxidizing and reducing agents on the stability of anthocyanin in pericarp of lychee fruits. *Journal of South China Agricultural University, 22*(2), 15-17.

Pazmiño-Durán A.E., Giusti M.M., Wrolstad R.E. y Gloria B.A. (2001). Anthocyanins from oxalis triangularis as potencial food colorants. *Food Chemistry, 75*(2), 211-216.

Piffaut B., Kader M., Girardin M. y Metche M. (1994). Comparative degradation pathways of malvidin 3,5-diglicoside after enzymatic and thermal treatments. *Food Chemistry, 50*, 115-120.

Poei-Langston M.S. y Wrolstad R.E. (1981). Color degradation in an ascorbic acid-anthocyanin-flavonol model system. *Journal of Food Science, 46*, 1218-1236.

Prior R.L. (2004). Absorption and metabolism of anthocyanins: potential health effects in phytochemicals – mechanisms of action. En: *Phytochemicals: mechanism of action*, (Meskin M.S., Bidlack W.R., Davies A.J. Lewis D.S. y Randolph R.K., eds.), CRC Press, Boca Raton, pp. 1-19.

Quevedo R., Ronceros B., García K., López P. y Pedreschi F. (2011). Enzymatic browning in sliced and pureed avocado: A fractal kinetic study. *Journal of Food Engineering, 105*(2), 210–215.

Rechner A.R. y Kroner C. (2005). Anthocyanins and colonic metabolites of dietary polyphenols inhibit platelet function. *Thrombosis Research, 116*(4), 327-334.

Reglamento (CE) Nº 1333/2008 del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, sobre aditivos alimentarios.

Reglamento (UE) Nº 231/2012 de la Comisión, de 9 de marzo de 2012, por el que se establecen especificaciones para los aditivos alimentarios que figuran en los anexos II y III del Reglamento (CE) Nº 1333/2008 del Parlamento Europeo y del Consejo.

Rein M. (2005). Copigmentation reactions and color stability of berry anthocyanins. Doctoral dissertation, University of Helsinki, Helsinki, Fi, pp. 10–14, 87.

Rice-Evans C.A., Miller N.J. y Paganga G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933–956.

Roopchand D.E., Kuhn P., Krueger C.G., Moskal K., Lila M.A. y Raskin I. (2013). Concord grape pomace polyphenols complexed to soy protein isolate are stable and hypoglycemic in diabetic Mice. *Journal of Agricultural and Food Chemistry, 61*, 11428-11433.

Salas E., Fulcrand H., Meudec E. y Cheynier V. (2003). Reactions of anthocyanins and tannins in model solutions. *Journal of Agricultural and Food Chemistry, 51*, 7951-7961.

Sánchez-Moreno C., Cao G., Ou B. y Prior R.L. (2003). Anthocyanin and proanthocyanidin content in selected white and red wines. Oxygen radical absorbance capacity comparison with nontraditional wines obtained from highbush blueberry. *Journal of Agricultural and Food Chemistry, 51*(17), 4889–4896.

Sarma A.D., Sreelakhmi Y. y Sharma R. (1997). Antioxidant ability of anthocyanins against ascorbic acid oxidation. *Phytochemistry, 45*(4), 671-674.

Schreiber H.D., Swink A.M. y Godsey T.D. (2010). The chemical mechanism for Al^{3+} complexing with delphinidin: A model for the bluing of hydrangea sepals. *Journal of Inorganic Biochemistry, 104*, 732−739.

Stintzing F.C. y Carle R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology, 15*, 19–38.

Strack D. y Wray V. (1993). The anthocyanins. En: *The flavonoids: Advances in research since 1986*, (Harborne J.B., ed.), Chapman and Hall, London, pp. 1-22.

Sun J., Bai W., Zhang Y., Liao X. y Hu X. (2011). Identification of degradation pathways and products of cyanidin-3-sophoroside exposed to pulsed electric field. *Food Chemistry, 126*(3), 1203–1210.

Teissedre P.L. y Landrault N. (2000). Wine phenolics: contribution to dietary intake and bioavailability. *Food Research International, 33*(6), 461–467.

Timberlake C.F. y Bridle P. (1977). Anthocyanins: colour augmentation with catechin and acetaldehyde. *Journal of the Science of Food and Agriculture, 28*, 539-544.

Vázquez-Flores A.A., Alvarez-Parrilla E., López-Díaz J.A., Wall-Medrano A. y de la Rosa L.A. (2010). Taninos hidrolizables y condensados: naturaleza química, ventajas y desventajas de su consumo. *Tecnociencia Chihuahua, VI*(2), 84-93.

von Elbe M. y Shwartz R. (1996). Colorants. En: *Food Chemistry*, (Fennema O.R., ed.), Marcel Dekker, Inc., New York, pp. 681–694.

Vourela S., Kreander K., Karonen M., Nieminen R., Hamalainen M. y Galkin A. (2005). Preclinical evaluation of rapessed, raspberry and pine bark phenolics for health related effects. *Journal Agricultural and Food Chemistry, 53*(15), 5922-5931.

Wang H., Nair M.G., Strasburg G.M., Chang Y., Booren A.M., Gray J.I. y DeWitt D.L. (1999). Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *Journal of Natural Products, 62,* 294–296.

Wang L.-S. y Stoner G.D. (2008). Anthocyanins and their role in cancer prevention. Mini-review. *Cancer Letters, 269,* 281–290.

Wilska-Jeszka J. (2007). Food colorants. En: *Chemical and functional properties of food components*, (Sikorski Z.E., ed.), CRC Press, Boca Raton, pp. 245−274.

Wong, E. (1976). Biosynthesis of flavonoids. En: *Chemistry and biochemistry of plant pigments*, (Goodwin T.W., ed.), 2ª Ed., Academic Press, London, Vol 1, p. 464.

Wrolstad R.E., Durst R.W. y Lee J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science and Technology, 16,* 423-428.

Wu X., Beecher G.R., Holden J.M., Haytowitz D.B., Gebhardt S.E. y Prior R.L. (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural and Food Chemistry, 54,* 4069-4075.

Yamaguchi T., Takamura H., Matoba T. y Terao J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1,-diphenyl-2-picrylhydrazyl. *Bioscience, Biotechnology, and Biochemistry, 62,* 1201–1204.

Yoshida K., Kitahara S., Ito D. y Kondo T. (2006). Ferric ions involved in the flower color development of the Himalayan blue poppy, *Meconopsis grandis*. *Phytochemistry, 67*(10), 992–998.

Zhang W., Curtin C. y Franco C. (2002). Towards manipulation of postbiosynthetic events in secondary metabolism of plant cell cultures. *Enzyme and Microbial Technology, 30,* 688-696.

Zimman A. y Waterhouse A.L. (2002). Enzymatic synthesis of [3‘-*O*-*Methyl*-^{3}H]malvidin-3-glucoside from petunidin-3-glucoside. *Journal of Agricultural and Food Chemistry, 50*(8), 2429-2431.