UNIVERSITAT POLITÈCNICA DE VALÈNCIA

VALORIZACIÓN DEL CULTIVO DEL CAQUI

TESIS DOCTORAL

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Unas pequeñas notas…

A Dios, a través de quién entiendo toda la vida. Él es la causa y motor por la cual he podido realizar el doctorado. Me siento identificada con las palabras que dijo en su momento Alfred North Whitehead (matemático y filósofo inglés):

"El hombre fue capaz de hacerse científico porque presuponía ver a Dios en la naturaleza. Y presuponía leyes en la naturaleza porque creía en un dador de leyes".

A Joel, mi increíble acompañante con quien formo equipo en el camino de la vida.

A mis padres y hermanas, por su cariño, cuidado y ejemplo.

A mis directoras, por su apoyo, tesón y convicción de querer sacar lo mejor de mí.

A mis compañeros de laboratorio, sobre todo a Mariola y Juan, por los buenos momentos e interesantes conversaciones.

Las obras de Dios son grandes,
y quienes las aman, las estudian.
Salmo 111:2

RESUMEN

La industria de frutas y hortalizas genera millones de toneladas de residuos al año. En muchos casos, estos residuos generan problemas medioambientales por lo que resulta necesario gestionarlos adecuadamente. Sin embargo, los residuos alimentarios pueden también valorizarse por lo que en los últimos años una estrategia con gran potencial está siendo la extracción de compuestos bioactivos. El diseño de procesos integrales que permitan transformar estos subproductos orgánicos en productos que generen un beneficio económico y medioambiental, es clave para el desarrollo sostenible del sector agroindustrial.

En la Comunidad Valenciana se concentra la mayor parte de la producción de caqui *Rojo Brillante* en España. Este cultivo genera una gran cantidad de descartes que pueden llegar al 15-20%, y a esto hay que sumarle los excedentes en el mercado. Por otro lado, las hojas de caqui son una fracción del cultivo que no se está aprovechando y que resulta de gran interés por sus propiedades antioxidantes. Las hojas de caqui son caducas y su aprovechamiento permitiría aumentar el valor económico de este cultivo si se organizara adecuadamente su recolección y estabilización.

El trabajo realizado en esta tesis doctoral ha consistido en explorar alternativas de aprovechamiento de los residuos generados por el cultivo del caqui tanto en el campo (hojas) como en la industrialización de sus frutos (piel y bagazo). Por un lado, se ha querido profundizar en el conocimiento de los compuestos polifenólicos presentes en la hoja de caqui, como posible fuente de principios activos con propiedades funcionales, evaluando en qué medida estos compuestos se ven afectados por el método de secado y por las condiciones de extracción. Por otro lado, se ha estudiado la extracción y caracterización de fibra de caqui de la piel y bagazo como una de las estrategias de aprovechamiento de los subproductos del fruto. Los productos obtenidos se han caracterizado y se ha

evaluado la influencia de los métodos de secado sobre sus propiedades tecnológicas y funcionales. Por último, dado las implicaciones de los compuestos bioactivos sobre la salud se estudió la evolución de los polifenoles procedentes de los productos obtenidos (té de hoja de caqui, caqui y fibra de caqui), utilizando un sistema de simulación in vitro de la digestión gastrointestinal.

De los resultados obtenidos del estudio se concluye en primer lugar, que el secado por aire caliente a 100 ºC permite estabilizar las hojas de caqui con una adecuada preservación de sus compuestos antioxidantes. La extracción de antioxidantes se consiguió mediante infusión a 90 ºC durante 5 minutos. Se observó que el proceso de secado previo de las hojas facilita la posterior extracción de compuestos antioxidantes.

En segundo lugar, se identificaron y cuantificaron 41 compuestos fenólicos de la hoja de caqui, y la mayoría de ellos por primera vez. Entre los polifenoles identificados se encuentran: 9 ácidos benzoicos, 5 ácidos hidroxicinámicos, 11 flavanoles, 13 flavonoles, 1 flavanona, 1 flavona y 1 tirosol.

En tercer lugar, la fibra procedente del bagazo y de la piel de caqui podría utilizarse como ingrediente lo que sin duda podría contribuir a incrementar la rentabilidad del cultivo del caqui en momentos de grandes volúmenes de excedentes de producción y/o descartes. La incorporación de estas fibras como ingrediente aportaría un valor añadido al producto no solo por sus propiedades tecnológicas (hidratantes, emulsificantes, etc.) sino también por sus propiedades antioxidantes.

Por último y en cuanto al consumo de compuestos fenólicos y flavonoides procedentes de las hojas, fruto o fibras de caqui se refiere, es de destacar que si bien una infusión de hojas de caqui presenta un mayor contenido en compuestos antioxidantes frente al fruto o fibras extraídas del mismo, la bioaccesibilidad de los polifenoles totales, flavonoides y de la

fracción antioxidante total fue superior en las fibras de caqui y en el fruto que en la infusión de las hojas. Este resultado pone de manifiesto la mayor sensibilidad de las antioxidantes procedente de la infusión a las condiciones del proceso digestivo, así como el efecto protector de la matriz (fruto o fibra) frente a la degradación de estos compuestos. Cabe destacar sin embargo, que la ingesta de una infusión (1.5 g en 110 mL de agua) de hoja de caqui y de un fruto de 200 g aportarían al final de la digestión, la misma actividad antioxidante total bioaccesible, mientras que el fruto aportaría dos veces más polifenoles bioaccesibles y tres veces menos flavonoides bioaccesibles que la infusión.

RESUM

La indústria de fruites i hortalisses genera milions de tones de residus a l'any. En molts casos, aquests residus generen problemes mediambientals pel que resulta necessari gestionar-los adequadament. No obstant açò, els residus alimentaris poden també valoritzar-se pel que en els últims anys una estratègia amb gran potencial està sent l'extracció de compostos bioactius. El disseny de processos integrals que permeten transformar aquests subproductes orgànics en productes que generen un benefici econòmic i mediambiental, és clau per al desenvolupament sostenible del sector agroindustrial.

A la Comunitat Valenciana es concentra la major part de la producció de caqui *Rojo Brillante* a Espanya. Aquest cultiu genera una gran quantitat de destriaments que poden arribar al 15-20%, i a açò cal sumar-li els excedents en el mercat. D'altra banda, les fulles de caqui són una fracció del cultiu que no s'està aprofitant i que resulta de gran interès per les seues propietats antioxidants. Les fulles de caqui són caduques i el seu aprofitament permetria augmentar el valor econòmic d'aquest cultiu si s'organitzara adequadament la seua recol·lecció i estabilització.

El treball realitzat en aquesta tesi doctoral ha consistit a explorar alternatives d'aprofitament dels residus generats pel cultiu del caqui tant en el camp (fulles) com en la industrialització dels seus fruits (pell i polpa). D'una banda, s'ha volgut aprofundir en el coneixement dels compostos polifenòlics presents en la fulla de caqui, com a possible font de principis actius amb propietats funcionals, avaluant en quina mesura aquests compostos es veuen afectats pel mètode d'assecat i per les condicions d'extracció. D'altra banda, s'ha estudiat l'extracció i caracterització de fibra de caqui de la pell i polpa com una de les estratègies d'aprofitament dels subproductes del fruit. Els productes obtinguts s'han caracteritzat i s'ha avaluat la influència dels mètodes d'assecat sobre les seues

propietats tecnològiques i funcionals. Finalment, donat les implicacions dels compostos bioactius sobre la salut es va estudiar l'evolució dels polifenols procedents dels productes obtinguts (té de fulla de caqui, caqui i fibra de caqui), utilitzant un sistema de simulació in vitro de la digestió gastrointestinal.

Dels resultats obtinguts de l'estudi es conclou en primer lloc, que l'assecat per aire calent a 100 ºC permet estabilitzar les fulles de caqui amb una adequada preservació dels seus compostos antioxidants. L'extracció d'antioxidants es va aconseguir mitjançant infusió a 90 ºC durant 5 minuts. Es va observar que el procés d'assecat previ de les fulles facilita la posterior extracció de compostos antioxidants.

En segon lloc, es van identificar i quantificar 41 compostos fenòlics de la fulla de caqui, i la majoria d'ells per primera vegada. Entre els polifenols identificats es troben: 9 àcids benzoics, 5 àcids hidroxicinàmics, 11 flavanols, 13 flavonols, 1 flavanona, 1 flavona i 1 tirosol.

En tercer lloc, la fibra procedent de la polpa i de la pell de caqui podria utilitzar-se com a ingredient, el que sens dubte podria contribuir a incrementar la rendibilitat del cultiu del caqui en moments de grans volums d'excedents de producció i/o destriaments. La incorporació d'aquestes fibres com a ingredient aportaria un valor afegit al producte no solament per les seues propietats tecnològiques (hidratants, emulsificants, etc.) sinó també per les seues propietats antioxidants.

Finalment i quant al consum de compostos fenòlics i flavonoids procedents de les fulles, fruit o fibres de caqui es refereix, és de destacar que si bé una infusió de fulles de caqui presenta un major contingut en compostos antioxidants enfront del fruit o fibres extretes del mateix, la bioaccesibilitat dels polifenols totals, flavonoids i de la fracció antioxidant total va ser superior en les fibres de caqui i en el fruit que en la infusió de les fulles. Aquest resultat posa de manifest la major sensibilitat dels antioxidants procedent de la infusió a les condicions del procés

digestiu, així com l'efecte protector de la matriu (fruit o fibra) enfront de la degradació d'aquests compostos. Cal destacar no obstant açò, que la ingesta d'una infusió (1.5 g en 110 ml d'aigua) de fulla de caqui i d'un fruit de 200 g aportarien al final de la digestió, la mateixa activitat antioxidant total bioaccesible, mentre que el fruit aportaria dues vegades més polifenols bioaccesibles i tres vegades menys flavonoids bioaccesibles que la infusió.

ABSTRACT

Fruit and vegetable industries generate millions of tonnes of wastes per year. In many cases, these wastes generate environmental problems so it is necessary to manage them properly. However, food waste may also be recovered. In the last years, the extraction of bioactive compounds is becoming a strategy with great potential. The design of integrated processes which could transform these organic by-products into products that generate economic and environmental benefits is the key to sustainable development of the agribusiness sector.

In Spain, most of the production of Rojo Brillante persimmon is concentrated in Valencia region This cultivar generates a lot of cull fruit that can reach 15-20%, in addition to the market surplus. On the other hand, persimmon leaves are a fraction of the crop that is not been used yet although they have a great interest because of its antioxidant properties. Persimmon leaves are deciduous and its use would increase the economic value of this crop if the collection and their stabilization would be properly organized.

This thesis explores alternative uses of wastes generated by persimmon cultivar both those from the field (leaves) and those from the industrialization of the fruit (skin and pulp). One of the purposes was deeping in the knowledge of the polyphenolic compounds present in the persimmon leaf, as a possible source of active ingredients with functional properties, evaluating how these compounds are affected by the drying method and the extraction conditions. On the other hand, the extraction and characterization of persimmon fiber from skin and pulp as a way of using the fruits' subpoducts were studied. Products obtained have been characterized and the influence of drying methods on their technological and functional properties has been evaluated. Finally, given the implications of bioactive compounds on health, the evolution of polyphenols from the products obtained (persimmon leaf tea, persimmon fruit and

persimmon fibers) was studied by using in vitro gastrointestinal digestion.

From the results of the study, it could be firstly concluded that hot air drying at 100ºC allows stabilizing persimmon leaves with an adequate preservation of their antioxidant compounds. The extraction of antioxidants was achieved by infusion at 90 °C for 5 minutes. It was noted that the process of drying facilitates the subsequent extraction of antioxidant compounds.

Secondly, 41 phenolics compounds from persimmon leaves were identified and quantified, most of them for the first time. Among the identified polyphenols are: 9 benzoic acids, 5 hydroxycinnamic acids, 11 flavanols, 13 flavonols, 1 flavanone, 1 flavone and 1 tyrosol.

Thirdly, fiber from pulp and peel characteristics confirm that they could be used as ingredients which could certainly help to increase the profitability of the crop specifically when large amounts of surplus or cull fruit are obtained. The incorporation of these fibers as an ingredient would bring added value to the product not only for their technological properties (moisturizer, emulsifier, etc.) but also for their antioxidant properties.

Finally, regarding the consumption of phenolics and flavonoids from leaves, fruit or persimmon fibers, results showed that the bioavailability of total polyphenols, flavonoids and total antioxidant fraction was higher in fibers and persimmon fruit than in the persimmon leaves infusion; although an infusion of persimmon leaves has a higher content of antioxidant compounds compared to the fruit or the fibers extracted. This result shows that antioxidants from the infusion are more inestable under digestive conditions than the same compounds from fruit and fiber; these results are probalbly related with the protective effect of the matrix (fruit or fiber) against degradation of these compounds. However, the intake of an infusion of persimmon leaves (1.5 g in 110 mL water) and a 200 g fruit provide at the end of digestion the same bioaccessible total

antioxidant activity, while the fruit would provide twice the bioaccessible polyphenols and three times less bioaccessible flavonoids than infusion.

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I. INTRODUCCIÓN

I.1 EL CULTIVO DEL CAQUI

El caqui (*Diospyros kaki Thunb.*) es un árbol frutal originario de China, donde su cultivo se inició siglos antes de Cristo (Figura I.1). Posteriormente se introdujo en Japón y Corea en los siglos VII y XIV, respectivamente, y en Europa en el siglo XVII (Llácer & Badenes, 2002b). En España, el caqui fue introducido a finales del siglo XIX y su cultivo se desarrolló principalmente como árboles aislados, mezclados con otros frutales en los jardines, huertos familiares o en pequeñas plantaciones destinadas al consumo local (Llácer & Badenes, 2002a).

La producción mundial de caqui es de 4 millones de toneladas siendo China el productor de más del 80% de ésta. Corea y Japón son el segundo y tercer productores, respectivamente, con una producción de 0.4 y 0.2 millones de toneladas. Juntos, los tres países asiáticos concentran más del 95% de toda la producción mundial. Si bien España produce menos de 0.1 millones de toneladas (Cerdà et al., 2015), esta cifra es el reflejo de un rápido crecimiento y expansión de la producción de caqui en los últimos 15 años en toda la cuenca del Mediterráneo consecuencia, principalmente, del aumento del precio de venta y la aparición de mercados emergentes en Europa, Brasil y los países árabes (Cerdà et al., 2015).

Fig. I.1. a) Fruto de caqui, variedad *Rojo Brillante*. b) Árbol del caqui, *Dyospiros kaki*.

La Tabla I.1 presenta la composición nutricional promedio del fruto de caqui; ésta varía según el cultivar, aunque el contenido en agua suele ser muy similar y como en la mayoría de las frutas los glúcidos son fundamentalmente monosacáridos de fructosa y glucosa. Las grasas y las proteínas son escasas aunque su aporte energético es superior al de la manzana, piña o pera (55 kcal/100 g) e inferior al del plátano (122 kcal/100 g). En cuanto a su aporte en fibra, contiene pectina de tipo soluble en cantidad moderada. También contiene una gran cantidad de polifenoles, entre los que se encuentran taninos condensados y otros compuestos fenólicos, que se han utilizado tradicionalmente para el tratamiento de problemas de salud como la tos, hipertensión, disnea, parálisis, congelación, quemaduras y sangrado (Matsuo & Ito, 1978; Mowat, 1990).

Tabla I.1. Composición del fruto del caqui (por 100 g) en cuanto a macro y microconstituyentes (USDA).

INFORMACIÓN NUTRICIONAL DEL CAQUI Contenido por 100 g de sustancia comestible		
Calorías (Kcal)	70	
Agua (g)	79.59	
Proteínas (g)	0.58	
Lípidos (g)	0.19	
Hidratos de carbono (g)	Fibra (g)	4.14
	Azúcares (g)	14.44
Minerales	Potasio (mg)	161 (3%)
	Fósforo (mg)	17 (2%)
	Magnesio (mg)	9 (2%)
	Calcio (mg)	8 (1%)
	Sodio (mg)	1 (0%)
	Hierro (mg)	0.15 (1%)
	Manganeso (mg)	0.355 (18%)
	Zinc (mg)	0.11 (1%)
Vitaminas	Retinol (vit.) A	81 µg (9%)
	ß-caroteno	253 µg (2%)
	Tiamina (vit. B1)	0.03 mg (2%)
	Riboflavina (vit. B2)	0.02 mg (1%)
	Niacina (vit. B3)	0.1 mg (1%)
	Vitamina B6	0.1 mg (8%)
	Ácido fólico (vit. B9)	8 µg (2%)
	Vitamina C	7.5 mg (13%)
	Vitamina E	0.73 mg (5%)
	Vitamina K	2.6 µg (2%)

Fuente: Base de datos de Caquis, en la base de datos de nutrientes de USDA; % CDR

La disponibilidad de este cultivo en el mercado está limitada a los meses de octubre-enero debido a su corta estacionalidad. Actualmente, uno de los mayores retos que se presentan es aumentar la disponibilidad comercial de este fruto y, por tanto, mejorar la rentabilidad del cultivo. Esto se está consiguiendo mediante el empleo de diferentes tratamientos precosecha como el paclobutrazol (PBZ) y el Etefón con el fin de acelerar la maduración de los frutos (Martínez-Fuentes et al., 2013; Upreti et al., 2013) y/o retrasarla mediante aplicación del ácido giberélico (GA_3) (Zilka et al., 1997; Dagar et al., 2012). Sin embargo, no existe información sobre la influencia que estos pretratamientos tienen en la calidad del caqui (tamaño, sabor, color, textura, etc.). Adicionalmente, la correlación de los tratamientos precosecha con estos parámetros de calidad y con la concentración de compuestos bioactivos resulta de especial interés por la importancia nutricional y funcional que tienen estos últimos. Analizar y correlacionar estos parámetros permitiría verificar si realmente es posible ofrecer un producto con una calidad razonablemente homogénea durante toda la campaña de este fruto. Además, la respuesta de los compuestos bioactivos (como los antioxidantes) a estos reguladores del crecimiento tampoco se ha estudiado en profundidad.

I.1.1 Variedades de caqui

Las variedades de caqui se dividen desde el punto de vista comercial en astringentes (*Rojo Brillante, Triumph, Tomatero*, etc.) y no-astringentes (*Fuyu, Hana-Fuyu, Jiro,* etc.). La astringencia está ligada al contenido y forma de los taninos. En las variedades no-astringentes éstos están insolubilizados permitiendo su consumo sin la realización de ningún tratamiento postcosecha y sin alcanzar la madurez fisiológica. Las variedades astringentes tienen un elevado contenido en taninos solubles que va disminuyendo a medida que se alcanza la madurez. Entre las variedades más conocidas se encuentran:

(i) *Tomatero*: las principales características de esta variedad son sus cualidades organolépticas y su precocidad (aproximadamente de unos quince días en comparación al *Rojo Brillante*), pero la falta de calibre es su principal problema desde el punto de vista comercial. La principal zona productora es el Alto Palancia.

(ii) *Triunmph (Sharon)*: esta variedad presenta la ventaja de que permite su conservación en cámaras frigoríficas aproximadamente de dos a tres meses.

(iii) *Fuyu*: con este nombre algunas veces se designa, aunque incorrectamente, a un grupo de variedades de caqui importadas de Japón muy posteriormente a la primera introducción del caqui. Es un grupo de variedades muy numeroso, de las cuales algunas están relacionadas directamente con la *Fuyu* original (*Hana Fuyu, Cal Fuyu, Isahaya Fuyu*), y otras no (*Jiro, Maekawa Jiro, Gosho, Suruga,* etc.). Son variedades de diferentes características en cuanto a tamaño, forma, color, sabor, etc., aunque son prácticamente todas achatadas, y siempre comestibles inmediatamente después de la recolección. La *Fuyu* es la variedad más cultivada en todo el mundo. Dentro de las *Fuyu, Hana Fuyu* es quizás la más gruesa y *Suruga* es la más tardía.

(iv) *Rojo Brillante*: es la variedad de mayor importancia tanto productiva como comercial en España. Es una variedad astringente que tradicionalmente se ha consumido en su fase de madurez óptima y que comercialmente se conoce como caqui *Classic*. La textura blanda de los frutos maduros representa una limitación para su comercialización más allá de lo que es el mercado local por su difícil manipulación, transporte y corta vida útil. En este contexto, la posibilidad de comercializar el fruto en estadíos previos cuando presenta una textura firme ha revitalizado el cultivo de este fruto que comercialmente se conoce como caqui *Persimon*. En estos momentos el caqui en la Comunidad Valenciana está localizado principalmente en dos zonas: El Alto Palancia (Segorbe) y la

Ribera Alta del Xúquer. Existen plantaciones en otras zonas pero con una menor presencia (Hernándiz, 1999).

I.1.2 El proceso de desastringencia del caqui

La comercialización del *Rojo Brillante* ha sido posible gracias a los tratamientos postcosecha orientados a eliminar su astringencia.

Los taninos solubles responsables de la astringencia, son polimerizados bajo condiciones anaerobias por el acetaldehído producido en la respiración anaerobia pasando a su forma insoluble, de manera que ya no provocan sensación de astringencia en el paladar (Matsuo et al., 1982; Taira et al., 1997). Existen métodos de desastringencia basados en la exposición de los frutos a condiciones anaerobias (Ben Arie y Sonego, 1993), como la aplicación de atmósferas modificadas enriquecidas con etanol, CO_2 o N_2. De entre todos ellos, el método que ha demostrado ser más efectivo y el que se aplica actualmente es el de atmósferas con altas concentraciones de CO_2, ya que elimina la astringencia preservando su firmeza (Zavrtanik et al., 1999; Yamada et al., 2002; Arnal & Del Río, 2003). No obstante, la eficacia del tratamiento con CO_2 en la eliminación de la astringencia y la duración del mismo dependen de la variedad, temperatura y estado de maduración del fruto (Ben Arie & Sonego, 1993). Actualmente a nivel comercial, gracias a la actividad investigadora realizada en el Centro de Tecnología Postcosecha del Instituto Valenciano de Investigaciones Agrarias (IVIA), se ha establecido como método estándar para reducir la astringencia del caqui *Rojo Brillante* el consistente en introducir los frutos durante 24 h en cámaras estancas con condiciones constantes de 95% de CO_2 a 20 ºC y 90% de humedad relativa (Salvador et al., 2004a).

Sin embargo, las temperaturas invernales de final de campaña dificultan la eficacia del tratamiento de la eliminación de la astringencia. Esto significa que es difícil alcanzar las condiciones estándares (95% CO_2, 20 ºC, 24h) en las centrales

de manipulación, especialmente en lo que a la temperatura se refiere (Besada, 2008; Horticom, 2009). Esto se debe a la diferencia de temperatura ambiental durante la campaña de recolección del caqui, que comienza a principios de octubre y finaliza a finales o principios de enero. Por ello, el ajuste del tiempo de atemperado de los frutos para que alcancen los 20ºC es más complicado a mediados o a finales de campaña, con temperaturas más frías, que al principio. Como consecuencia, la presencia de astringencia residual en los frutos conlleva la devolución de partidas completas por parte de los mayoristas. Este motivo, conjuntamente con la problemática derivada de los excedentes de producción del fruto, hace necesaria la búsqueda de alternativas de uso y aprovechamiento del cultivo del caqui.

I.2 INDUSTRIALIZACIÓN DEL CAQUI. NUEVAS OPORTUNIDADES

El cultivo del caqui genera una gran cantidad de descartes que pueden llegar fácilmente al 15-20%, y a esto hay que sumarle los excedentes en el mercado. Estos descartes y/o excedentes podrían estar causados por: daños fisiológicos de la pulpa que reducen la calidad del fruto, caída del fruto del árbol asociada a la presencia del hongo *Mycosphaerella nawae*, la falta de eficacia de los métodos de desastringencia y el gran volumen producido en una campaña muy corta.

De manera similar a otras frutas, una de las salidas comerciales para los descartes y/o excedentes podría ser el procesamiento del caqui en productos tales como purés, néctares, zumos o mermeladas. Sin embargo, la industrialización del caqui no es siempre sencilla. La técnica más universal para estabilizar y conservar los zumos de frutas consiste en la inactivación de los enzimas endógenos y los microorganismos contaminantes mediante la aplicación de un tratamiento térmico de conservación. La intensidad del tratamiento térmico se ajusta de acuerdo con las características de la fruta (composición, pH, labilidad de los componentes

volátiles, actividad de los enzimas endógenos, etc.). Pero en el caso del caqui, si se aplica un tratamiento térmico intenso de conservación, en condiciones ácidas, las formas no astringentes de taninos se hidrolizan y dan lugar a taninos solubles, con lo que el zumo se vuelve astringente (Carbonell et al., 2012). Si el caqui se transforma en puré, éste tiende a gelificar mediante un mecanismo en el que participan las pectinas y hemicelulosas principalmente; y el proceso de gelificación se acentúa especialmente cuando la temperatura supera los 50ºC (Carbonell et al., 2012).

El desarrollo de procesos de fabricación de productos de caqui sometidos a tratamientos térmicos teniendo en cuenta las bases moleculares de la gelificación, pardeamiento y astringencia, ofreciendo un nuevo método que permite obtener derivados del caqui (puré, zumo, néctar, mermelada, entre otros) con propiedades físicas, nutritivas y sensoriales aceptables por los consumidores (Carbonell et al., 2012; nº patente: WO2012131129 A1) ha dado lugar a una patente que ha permitido resolver los problemas de reversibilidad de la astringencia en los procesos de industrialización.

Pero estos procesos industriales generan habitualmente grandes volúmenes de residuos de alta biodegradabilidad, por lo que la minimización y valorización de éstos supone una doble ventaja:

1. Se reduce la carga contaminante, contribuyendo al desarrollo sostenible del sector.

2. Se obtiene un mejor rendimiento de las materias primas mediante su uso integral.

La tendencia del sector hortofrutícola es trabajar hacia una productividad sostenible que incluya el diseño de productos innovadores, la reducción y gestión de las emisiones y la mejora de los procesos y aprovechamiento de los subproductos generados.

Durante las últimas décadas ha aumentado el interés y los estudios científicos orientados al aprovechamiento de residuos generados por las empresas agroalimentarias, ya que en muchos casos estos residuos presentan compuestos de alto valor, que una vez estabilizados, podrían ser utilizados como ingredientes por su funcionalidad tecnológica (por ejemplo como espesante en el caso de las fibras) y/o nutricional (antioxidante, prebiótico, etc).

Por tanto, una de las estrategias para el aprovechamiento de estos subproductos podría ser la extracción de fibra de la piel y bagazo de caqui para su uso como aditivo.

En la actualidad, las principales fuentes de fibra son las frutas y los cereales. De ahí que se pueden encontrar en el mercado fibras de manzana, guisantes, remolacha azucarera, soja, naranja o limón, pero no de caqui. En la industria alimentaria, la adición de fibra alimentaria conlleva una mejora de la textura, viscosidad y alargamiento de la vida útil del producto y en la actualidad se utiliza en multitud de alimentos tales como yogures, productos de panadería, etc. (Thebaudin et al., 1997). La adición de fibra no solo es interesante por las propiedades funcionales que confiere a los productos, sino también porque su consumo está asociado con la prevención y tratamiento de ciertas enfermedades como el cáncer de colon, enfermedades coronarias, el constipado, diabetes, mejora del tránsito intestinal y disminución de los niveles de colesterol en sangre (Sorensen et al., 2014; Guevara-Cruz, 2013).

Si bien es cierto, que la presencia de compuestos bioactivos en el caqui y las fibras está relacionada con los beneficios anteriormente descritos, su repercusión sobre la salud depende del estado bioquímico en el que los compuestos antioxidantes llegan al torrente sanguíneo, y por ende a los tejidos. El proceso de digestión en sí mismo conlleva una serie de cambios en los macro y micronutrientes condicionando así, su biodisponibilidad final, que se define como la cantidad de compuesto que es capaz de ser liberado por la matriz

alimentaria tras ser transformado en el proceso digestivo en una forma más soluble (bioaccesibilidad) y atravesar la barrera intestinal (bioabsorción) para ser aprovechado posteriormente por el organismo (Parada & Aguilera, 2007).

La mejor forma de determinar los beneficios derivados de la ingesta de un alimento, y por tanto su biodisponibilidad, consiste en someter al mismo al propio proceso digestivo "in vivo", evaluando los cambios que experimenta a lo largo de cada una de las etapas involucradas. Sin embargo, los ensayos in vivo son costosos y requieren de largos tiempos de estudio, en especial en muestras humanas, además de conllevar implicaciones médicas y éticas. Por ello, resulta de gran interés la sustitución de éstos por estudios "in vitro" dado que los resultados obtenidos son más reproducibles además de permitir mecanizar estudios con varios parámetros bajo control. Existen evidencias científicas que avalan positivamente la alternativa de emplear métodos enzimáticos que reproduzcan las condiciones metabólicas óptimas de digestión estomacal y posterior absorción en el intestino, frente a ensayos in vivo (Ramos, 1995; During & Harrison, 2005). Por ello, sería interesante estudiar qué cambios experimentan los polifenoles del caqui y del las fibras extraídas del caqui durante las diferentes etapas implicadas en la digestión.

I.3 APROVECHAMIENTO DE LAS HOJAS COMO FUENTE DE ANTIOXIDANTES

No solo el fruto del caqui resulta de interés por sus propiedades antioxidantes, sino que hay otra fracción del cultivo que tiene más potencial en este sentido.

En Japón, el té de hoja de caqui (conocido como *kakinoha-cha*) se consume tanto como el té verde y se usa tradicionalmente gracias a sus propiedades curativas (tratamiento en la parálisis, congelación, quemaduras y para parar el sangrado (Matsuo & Ito, 1978)). Las hojas de caqui, contienen un alto contenido en vitamina C (Mizuo, 1995; Hiromichi, 2002), flavonoides,

considerados fuente principal de compuestos bioactivos de las hojas (Liu et al., 2012), terpenoides (Chen et al., 1999), y otros compuestos como resinas, polisacáridos, clorofilas (Hospital, 1973), carotenos, kryptoxantina, celulosa, hemicelulosa, ligninas (Hu et al., 2002) y amino ácidos (Zu & Lei, 1989). Los flavonoides que se han aislado de las hojas de caqui presentan actividad antioxidante (Sun et al., 2014), hipotensiva (Kameda et al., 1987) y antialergénica (Kotani et al., 2000). Algunos estudios *in vitro* han sugerido que también pueden tener efectos beneficiosos en la diabetes (Kawakami et al., 2010; Wang et al., 2011).

Todas estas propiedades muestran que no solo la hoja de caqui puede ser usada para ser infusionada sino también sus extractos podrían ser añadidos como ingrediente funcional en otras matrices alimentarias como bebidas, galletas, bizcochos, etc. con propósitos terapéuticos y saludables. Aunque algunos compuestos fenólicos se han determinado en hojas de caqui, entre los cuales se encuentran el kaempferol y la quercitina (Chou, 1984), isoquercitina (Nakatani et al., 1989), myricitrina (Guo & Dong, 1999) y algunos glucósidos flavonoles (Chou, 1984; Chen et al., 2008); la identificación exhaustiva de su perfil polifenólico ayudaría a elucidar los efectos beneficiosos de su uso como planta medicinal.

Las hojas de caqui son caducas, por lo que su aprovechamiento permitiría aumentar el valor económico de este cultivo si se organizara adecuadamente su recolección. Una vez recogidas, las hojas necesitan ser estabilizadas, normalmente por técnicas de secado. El secado es un método muy común de conservación ampliamente usado en alimentos y la calidad del producto final depende en gran medida de las técnicas y las variables de proceso utilizadas (Doymaz, 2005). La reducción de la actividad de agua por la eliminación de la humedad no solo garantiza la estabilidad de los productos deshidratados sino que contribuye a la minimización de los costes de transporte y almacenamiento como consecuencia de

la reducción significativa del peso y volumen de estos productos (Okos et al., 1992).

La industria alimentaria hace un uso frecuente de las técnicas de secado y deshidratación con el objetivo de garantizar el almacenamiento a largo plazo, lo cuál ha sido posible gracias al conocimiento científico y al progreso de estas tecnologías como consecuencia de numerosos estudios científicos. Las características nutricionales de los productos vegetales dependen del tipo de procesamiento utilizado, y en consecuencia del proceso de deshidratación aplicado.

Por lo tanto, conocer la influencia de los diferentes tratamientos de secado en los compuestos bioactivos de las hojas de caqui es importante para optimizar el proceso y garantizar su estabilización. Sin embargo, hay muy pocas publicaciones en este sentido (Jo et al., 2003; Sakanaka et al., 2005; Lee et al., 2006; Sun et al., 2011) que arrojen información sobre la caracterización de las hojas de caqui en términos de compuestos bioactivos y el beneficio de sus antioxidantes; y no hay ninguna sobre el efecto de las diferentes técnicas de secado en la estabilización de estos compuestos.

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II. OBJETIVOS

II. OBJETIVOS

II.1. Objetivo general

El objetivo principal de este trabajo ha sido explorar alternativas de aprovechamiento de los residuos generados por el cultivo del caqui tanto en el campo (hojas) como en la industrialización de sus frutos.

II.2. Objetivos específicos

II.2.1 APROVECHAMIENTO DE LAS HOJAS COMO FUENTE DE ANTIOXIDANTES

(i) Seleccionar el método óptimo de secado en base a su influencia sobre las propiedades antioxidantes y color de extractos acuosos de hojas obtenidos a diferentes tiempos (1, 3, 5, 60 y 1440 minutos) y temperaturas (70, 80 y 90 ºC) de infusión.

(ii) Determinar y modelizar las isotermas de sorción de las hojas deshidratadas por el método óptimo.

(iii) Identificar y caracterizar los compuestos polifenólicos presentes en las hojas de caqui deshidratadas por espectrometría de masas de alta resolución (LC-ESI-LTQ-ORBITRAP-MS).

(iv) Evaluar la evolución de los compuestos antioxidantes de extractos acuosos de hojas de caqui durante el proceso gastrointestinal utilizando un método de digestión in vitro.

II.2.2 APROVECHAMIENTO DE LOS RESIDUOS DE INDUSTRIALIZACIÓN DEL CAQUI

(i) Analizar la influencia de los métodos de secado (aire caliente y liofilización) utilizados para la estabilización de fibra de caqui sobre sus propiedades físico-químicas, antioxidantes, hidratantes y emulsificantes.

(ii) Determinar la evolución de los compuestos antioxidantes del fruto de caqui y de la fibra de caqui extraída del bagazo y piel del fruto, y estabilizada por secado por aire caliente y liofilización, durante el proceso gastrointestinal utilizando un método de digestión in vitro.

II.2.3 TRATAMIENTOS EN CAMPO COMO ESTRATEGIA PARA AMPLIAR LA RECOLECCIÓN DEL FRUTO DEL CAQUI

Adicionalmente, se estudió el efecto de los tratamientos precosecha (PBZ y Etefón para acelerar la maduración y el GA_3 para retrasarla) en el tamaño, composición (sólidos solubles y contenido en humedad), color, textura y propiedades antioxidantes (contenido total de fenoles y capacidad antioxidante) con el objetivo de extender el periodo comercial del caqui *Rojo Brillante*.

III. RESULTADOS

III.1. INFLUENCE OF DRYING METHOD AND EXTRACTION VARIABLES ON THE ANTIOXIDANT PROPERTIES OF PERSIMMON LEAVES

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En Japón, el té de hoja de caqui es, conjuntamente con el té verde, una de las infusiones más consumidas tradicionalmente por sus propiedades curativas (Matsuo & Ito, 1978). Las hojas de caqui son caducas, y una vez recogidas necesitan ser estabilizadas normalmente por secado. Además, se sabe que tanto el método como las variables de secado como la temperatura y el tiempo, afectan sustancialmente a las propiedades funcionales del producto final. Aunque se han publicado estudios sobre la caracterización de las propiedades del té de hoja de caqui (Sun et al., 2011; Sakanaka et al., 2005; Jo et al., 2003), no se han encontrado referencias en las que se evalúe el efecto de las variables de secado sobre la calidad final de las hojas deshidratadas, y en especial a lo que se refiere a la preservación de sus propiedades antioxidantes.

Por lo tanto, el objetivo de este trabajo fue comparar algunas propiedades antioxidantes (contenido de fenoles totales, actividad antioxidante y flavonoides) de extractos acuosos de hojas deshidratadas por distintos métodos (secado a la sombra, secado por aire caliente a 100 y 180ºC, y por liofilización) y obtenidos bajo distintas condiciones de extracción (70, 80 y 90 ºC durante 1, 3, 5, 60 y 1440 minutos). Adicionalmente, se llevó a cabo un estudio para determinar si el tamaño de hoja puede ser una variable que influye en las propiedades antioxidantes de los extractos.

Los métodos espectrofotométricos utilizados en la caracterización de las propiedades antioxidantes de los extractos acuosos fueron: el método Folin-Ciocalteu para la determinación del contenido de fenoles totales (Sakanaka et

al., 2005), el método DPPH (Shahidi et al., 2006) para la determinación de la actividad antioxidante y el método descrito por Dewanto et al., (2002) para la determinación del contenido en flavonoides. Adicionalmente, se estudiaron las propiedades ópticas del espacio de color CIEL*a*b* de los extractos con el fin de evaluar la posible correlación de esta medida con las propiedades antioxidantes.

Los extractos de hojas secadas por aire caliente a 100ºC presentaron la mayor concentración de fenoles, seguidos por los extractos de hojas liofilizadas, los extractos de hojas secadas por aire caliente a 180ºC y, finalmente, los de hojas secadas a la sombra. La misma tendencia se observa para los flavonoides y la capacidad antioxidante. Sin embargo, si los comparamos con las hojas frescas, éstas presentaron mayores valores de flavonoides que las hojas deshidratadas y menores en el contenido de fenoles y actividad antioxidante. Este resultado podría estar relacionado con un incremento de la extractabilidad de algunos compuestos como consecuencia de cambios en la matriz durante el proceso de secado (Davey et al., 2002). La estructura de las hojas secas está más abierta e interconectada que la de las hojas frescas y por tanto el disolvente podría penetrar más fácilmente y la extracción de estos compuestos sería más eficiente.

En cuanto a la influencia del tiempo y la temperatura de infusión en la extracción de compuestos antioxidantes, los resultados mostraron que la cinética de extracción de los fenoles y flavonoides fue muy rápida en ambos casos, teniendo lugar en los primeros minutos y permaneciendo constante hasta los 60 y 1440 minutos. Estos resultados ponen de manifiesto que los valores obtenidos a 90 ºC y 60 minutos corresponden a valores de equilibrio donde la extracción es máxima. La actividad antioxidante de los extractos depende del tipo y cantidad de compuestos extraídos durante la infusión, y por eso podría estar correlacionada con el contenido de fenoles totales de los extractos. En este sentido, se encontró una

buena correlación entre la concentración de flavonoides y el contenido de fenoles totales con la actividad antioxidante; siendo la mejor correlación la de flavonoides con la actividad antioxidante.

Por otro lado, el color de los extractos se vio afectado significativamente tanto por el método de secado como por el tiempo de infusión. Así, las infusiones procedentes de hojas secadas por aire caliente se caracterizan por un color más oscuro y marrón-amarillento mientras que las infusiones de hojas liofilizadas presentaron un color más verde-amarillento y una mayor luminosidad.

Dada la heterogeneidad del tamaño de hoja de caqui, las muestras recolectadas se clasificaron en dos grupos: hojas pequeñas (eje axial: 9 ± 1 (cm); eje ecuatorial: 7,0 ± 0,9 (cm)) y hojas grandes (eje axial: 17 ± 2 (cm); eje ecuatorial: 10 ± 1 (cm)); para evaluar la influencia de este parámetro sobre las propiedades antioxidantes. Los resultados obtenidos permiten afirmar que las hojas de menor tamaño presentaron un 7% más de capacidad antioxidante y fenoles totales, y un 9% más de flavonoides que las de mayor tamaño.

Los resultados de este estudio arrojan información de gran utilidad para su implementación práctica, ya que el secado por aire caliente permite estabilizar las hojas de caqui con una adecuada preservación de sus compuestos antioxidantes. Esta tecnología, ampliamente extendida en el sector agroalimentario no requiere una gran inversión para su adaptación a esta aplicación concreta. Sin embargo, la rentabilidad del desarrollo está fuertemente ligada a los costes de recolección de las hojas y el valor del mercado que pueda alcanzar el producto. En este sentido, sería recomendable hacer un estudio económico previo a su implementación.

III.1. INFLUENCE OF DRYING METHOD AND EXTRACTION VARIABLES ON THE ANTIOXIDANT PROPERTIES OF PERSIMMON LEAVES

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ABSTRACT

The presence of antioxidant compounds and therefore the antioxidant capacity of persimmon leaves and their extracts have been reported by many authors. Furthermore, it is known that both the method of drying and the temperature at which this process takes place substantially affect the properties of the final product. However, there are no studies in the literature that examine how drying variables can affect the quality of persimmon leaves, especially as refers to preservation of their antioxidant properties. Therefore, the aim of this paper was to compare some antioxidant properties of aqueous extracts of persimmon leaves obtained under different drying methods (shade drying, hot air drying at 100 and 180 °C, and freeze drying) and infusion conditions (70, 80 and 90 °C for 1, 3, 5, 60 and 1440 min). The results in terms of total phenol content, flavonoids and antioxidant capacity indicated that air drying at 100 °C would be the optimal process for the stabilization of persimmon leaves, and their subsequent use in brewed beverages. Likewise, the best conditions of aqueous extraction in order to maximize the extractability of antioxidant compounds corresponded to 90 °C for 60 min. A short experiment performed in this study confirmed that small persimmon leaves (axial axis: 9±1 (cm); equatorial axis: 7.0±0.9 (cm)) had around 9% more flavonoids, and 7% more total phenolic content and antioxidant capacity than the large ones (axial axis: 17±2 (cm); equatorial axis: 10±1 (cm)).

Keywords: persimmon leaves; drying method; antioxidant properties; leaf size

1. INTRODUCTION

The presence of natural antioxidants in food is important, not only because they are responsible for the organoleptic characteristics of the products but also because they may play an important role in helping to prevent diseases such as cancer, cardiovascular disease, Alzheimer′s disease and macular degeneration (Peter, Wootton-Beard, & Ryan, 2011). Most antioxidants (vitamin C, vitamin E, carotenoids, glucosinolates and polyphenols among others) are found in fruit and vegetables (Wang, Melnyka, Tsao, & Marcone, 2011). Polyphenols, compounds which are bio-synthesized by plants, are responsible for most of the antioxidant activity of fruits and vegetables and some natural teas and beverages. From a chemical point of view, polyphenols have at least one or more hydroxyl group (OH-) attached to an aromatic ring in their structure. While all polyphenols present antioxidant properties, it has been established that some of these compounds also show anti-carcinogenic (Birt, Hendrich, & Wang, 2001), anti-inflammatory, antiviral, anti-allergic (Larson, 1988) and anti-bacterial properties (Piccaglia, Marotti, Giovanelli, Deans, & Eaglesham, 1993). They also exhibit estrogenic activity, modulation of the activity of numerous enzymes, including digestive enzymes, prevention of coronary diseases, as well as age-related degenerative brain disorders (Parr & Bolwell, 2000) and have beneficial effects on homeostasis, constipation, hypertension, apoplexy and atherosclerosis (Kotani et al., 2000, Matsumoto et al., 2002, Tanaka et al., 2003 and Sakanaka et al., 2005).

Beverages and herbal teas (infusions) are drunk habitually for their flavor but also, because they deliver high concentrations of functional bioactive compounds with antioxidant properties (Shahidi, 2000). In Japan, kakinoha-cha (Japanese persimmon leaf tea) is infused with hot (rather than boiling) water, drunk like green tea and used traditionally due to its healing properties (treatment of paralysis, frostbite, burns and to stop bleeding)

(Matsuo and Ito, 1978). Their principal compounds are flavonoid oligomers, tannins, phenols, organic acids, chlorophyll, vitamin C and caffeine (Matsuo and Ito, 1978 and Jo et al., 2003). In addition, persimmon trees are deciduous; therefore, making use of their leaves could be a good way to increase the economic value of this crop. Once collected, the leaves need to be stabilized, usually by drying, before their use. Dry persimmon leaves could be used in hot water infusions or as a new source of antioxidants. These antioxidants could be incorporated, once extracted, into other food matrices such as beverages, biscuits, etc. Knowing the influence of different treatments on the bioactive compounds of these leaves is important to optimize their extraction. However, there are only a limited number of publications (Sun et al., 2011, Sakanaka et al., 2005, Jo et al., 2003 and Lee et al., 2006) that yield information about the characterization of persimmon leaves in terms of bioactive compounds and antioxidant benefits, and there are none on the effect of different drying techniques on these compounds.

Drying is a very common preservation method used in foodstuffs and the quality of the final products is strongly dependent on the technique and the process variables used (Doymaz, 2005). The reduction of water activity by moisture removal also implies significant reduction of weight and volume, minimizing packaging, transportation and storage costs (Okos, Narsimhan, Singh, & Witnauer, 1992).

The food industry has evolved and often carries out freeze and air-drying processes, under controlled conditions, to achieve the objective of long-term storage. However, the health-promoting capacity and nutritional characteristics of plant products depend on the type of processing employed. Some studies report that freeze-drying increases the extraction of bioactive compounds of different products in comparison to air drying (Pinela et al., 2012, Kwok et al., 2004 and Dorta et al., 2012). This is because freeze-drying is based on the dehydration by sublimation of a frozen product (Ratti, 2001). Nevertheless, freeze-drying has

always been recognized as the most expensive process for manufacturing a dehydrated product and its application depends on the uses of the final product. Moreover, temperature and time are crucial variables to consider in this kind of process.

The objective of this study was to analyze the influence of the drying techniques (shade-drying, hot air drying and freeze-drying) on the antioxidant compounds and colour of persimmon leaf infusions obtained with different times (1, 3, 5, 60 and 1440 min) and temperatures (70, 80 and 90 °C) of infusion. A short study about the influence of leaf size on antioxidant properties was also performed.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Persimmon leaves (*Diospyros. kaki, Rojo Brillante var.*) were harvested in mid-October 2012 from 10 trees in an orchard in Valencia (Spain). The cultivar used was "*Rojo Brillante*". Folin-Ciocalteu reagent, sodium nitrite, 2,2-diphenyl-1-picrylhydrazyl, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, gallic acid, L-ascorbic acid and (+)- Catechin were obtained from Sigma-Aldrich Co. Sodium carbonate, 2,6-dichlorophenolindophenol Sodium Salt 2-hydate (DIP), meta-Phosphoric Acid stabilized with $NaPO_3$, sodium hydroxide solution were from Panreac. Aluminum chloride hexahydrate was from Fluka and methanol from Scharlab.

2.2. Drying treatments of raw material

In order to evaluate the effect of drying techniques on the antioxidant properties of persimmon leaves, the raw material was blanched in hot water (100ºC) for 1 min and after, it was subjected to three different drying methods: (i) shade-drying in which leaves were dried on trays at 20ºC for 30 days; (ii) hot air drying at 100ºC and 180ºC for 30 min in a convective drier; (iii) freeze-drying at vacuum pressure of 10^{-1} mbar for 24 hours.

Dried persimmon leaves were grounded to a fine powder (particle size ≤ 1 mm) in a SEVERIN mill previous to aqueous extraction.

2.3. Aqueous extraction from persimmon leaves

Aqueous extraction of persimmon leaves was performed with a product to water ratio of 1:10 (w/w) at 70, 80 and 90˚C for 1, 3, 5, 60 and 1440 min. The extracts were filtered with Whatman filter paper (particle retention: 20-25μm) and cooled to 25ºC before analysis.

2.4. Influence of leaf size on antioxidants properties

In order to study the influence of leaf size on antioxidant properties, an additional short study was performed in two different leaf sizes. For this purpose, leaves were classified into two groups: small leaves (axial axis: 9 ± 1 (cm); equatorial axis: 7.0 ± 0.9 (cm)) and large leaves (axial axis: 17 ± 2 (cm); equatorial axis: 10 ± 1 (cm)) and they were subjected to a blanching at 100ºC for 1 min followed by a hot air drying at 100ºC until constant weight. The antioxidant properties of persimmon leaves with regard to leaf size were analyzed after aqueous extraction at 90ºC for 5 min with a product to water ratio of 1:10 (w/w). The extracts were filtered with Whatman filter paper (particle retention: 20-25 μm) and cooled to 25ºC before analysis.

2.5. Analytical determinations

All analytical determinations were performed in triplicate for each extract.

2.5.1. Moisture

Moisture was estimated with the AOAC (2000) official method (number 934.01).

2.5.2. Determination of total phenolic content

Samples were analyzed spectrophotometrically, with a modified Folin-Ciocalteu method (Sakanaka *et. al,* 2005), in order to determine the total phenolic content. 0.5 ml of distilled water and 0.125 ml of a known dilution of the extract were added to a cuvette followed by the addition of 0.125 ml of Folin-Ciocalteu reagent. The mixture was shaken and 1.25 ml of a 7% sodium carbonate solution and 1 ml of distilled water were added after 6 min. The color was left to develop for 90 min and the absorbance was measured at 760 nm using a spectrophotometer (JASCO V-630). The measurement was compared to a standard curve of gallic acid solutions and expressed as mg of gallic acid equivalents per gram of dry matter (mg GA/g d. m.). A blank was prepared in the same way but without any sample.

2.5.3. Determination of total flavonoid content

Total flavonoid content was determined using the modified colorimetric method described by Dewanto, Wu, Adom, and Liu (2002). 0.25 ml of the extract was mixed with 1 ml of distilled water in a cuvette, followed by the addition of 0.075 ml of a 5% sodium nitrite solution. After 6 min, 0.15 ml of a 10% aluminum chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 ml of 1M sodium hydroxide solution was added. Finally, 2 ml of distilled water was added and the absorbance was measured immediately at 510 nm using a UV/vis spectrophotometer (JASCO V-630). The measurement was compared to a standard curve of (+)-catechin solutions and expressed as mg of (+)-catechin equivalents per gram. A blank was prepared in the same way but without sample.

2.5.4. DPPH radical-scavenging activity

The antioxidant activity (AA) of the extract was measured on the basis of the scavenging activities of the stable 2,2-diphenyl-1-

picrylhydrazyl free radical as described by Shahidi, Liyana-Pathirana, and Wall (2006) with some modifications. According to this method, the purple colour intensity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution decays in the presence of an antioxidant, and this absorbance change is measured spectrophotometrically at 515 nm.

A 0.1 ml of the sample diluted in methanol (5ml extract/ 25ml methanol) was added to 3.9 ml of a methanolic solution of DPPH (80:20; methanol:water) (0.025mg/ml). The solution was shaken and after 30 min the absorbance of the sample was measured at 515 nm using methanol as a blank. The antioxidant activity (%) of the samples was calculated according to Eq. (1):

$$\mathrm{AA}\ (\%) = \frac{\mathrm{A_{t=0}} - \mathrm{A_{t=30}}}{\mathrm{A_{t=0}}} \mathrm{X100} \qquad (1)$$

where $A_{t=0}$ is the initial absorbance of the DPPH (without sample) and $A_{t=30}$ is the absorbance of the sample after 30 min. The measurement was compared to a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solutions and expressed as mg of Trolox per gram.

2.5.5. Optical properties

The CIELAB color space was studied, the following color coordinates were determined by transmittance: lightness (L*), redness (a*, ± red-green) and yellowness (b*, ± yellow-blue). Color determinations were made, at 20 ± 2ºC by means of a Minolta, mod. CM- 3600d, spectrophotometer with illuminant D65 and 10˚ observer.

2.6. Statistical analysis

Statistical analysis of variance (ANOVA) was performed by Statgraphics Centurion to estimate the effect of process variables (type of drying method, temperature and time of

infusion and leaf size) on the obtained results. Evaluations were based on a 95% of significance level.

3. RESULTS AND DISCUSSION

3.1. Influence of drying method on the degradation and extractability of antioxidant compounds

The stabilization of the leaves by drying processes involves changes in the matrix that can affect not only the concentration of antioxidant compounds in the dry product but also the extractability thereof. For this reason, the concentration of total phenols and flavonoids, and antioxidant capacity was assessed in aqueous extracts of fresh and dried leaves obtained by different drying processes. For a better comparison of the results, the values have been stated in terms of grams of dry matter (Table 1) and they correspond to 1440 minutes of extraction. No statistical differences were found between antioxidant contents and antioxidant activity after 60 and 1440 minutes of extraction, this confirming that the equilibrium of extraction was achieved (data not shown).

The results of the study showed that the degradation of flavonoids varied with the different drying treatments applied to the samples. It is noted that regardless of the type of drying, extracts of dried leaves always showed lower concentrations of flavonoids than those from fresh leaves. The loss of flavonoids during drying might be due to the process conditions, in particular the temperatures and the duration used (Schieber, Stintzing, & Carle, 2001). The loss of flavonoids was found to be lower with Air Drying at 100ºC and Freeze Drying, than Air Drying at 180ºC and Shade Drying. The degradation of phytochemicals upon thermal treatments has been reported by many authors (Zhang & Hamauzu, 2004; Puupponen-Pimiä, Nuutila, Aami, & Oksman-Caldentey, 2003; Volden et al., 2008). Davey et al. (2002) found that thermal processing can affect the phytochemicals by thermal breakdown, which affects the integrity of cell structure, thereby resulting in the migration of

components, leading to breakdown by various chemical reactions involving enzymes, light and oxygen.

In the case of total phenols, a higher concentration was found in dry leaf extracts (Table 1). This result could be related to an increase in the extractability of such compounds as a consequence of the matrix changes during the drying process. The structure of dry leaves is more open and interconnected than in fresh leaves, meaning that the solvent can penetrate more easily, providing a greater surface for mass transfer, and resulting in a more efficient extraction of these compounds. As a result of this increase in the extractability of the phenolic compounds, higher values of antioxidant activity were obtained for dry leaves. However, the extracts obtained at 90°C from fresh leaves had the highest values, probably due to the higher concentration of flavonoids in these samples.

Table 1. Total phenols, flavonoids and antioxidant activity of persimmon leaf extracts from fresh and dried leaves with different infusion temperatures. Significant differences as determined by ANOVA ($p < 0.05$) are indicated by different letters

Drying method	T (ºC)	Phenols (mg GA/g d. m.)	Flavonoids (mg catechin/ g d. m.)	Antioxidant activity (mg trolox/ g d. m.)
Fresh leaves	70	59(2)h	52(3)f	105(6)h
	80	67(2)d	57.7(1.4)g	125(2)d
	90	78(2)i	62(3)h	190(9)i
AD-100	70	85(3)a	25(2)a	148.8(0.3)a
	80	99(2)b	34.60(1.07)b	164(5)b
	90	106.0234(1.1018)c	34.4(1.4)b	170(2)c
AD-180	70	67.4(0.3)d	21(2)ac	124.100(1.002)d
	80	85.28(1.14)a	26(4)ad	138.6(0.7)e
	90	88(3)e	27.6(1.2)d	146(5)af
FD	70	81(3)f	26(2)a	125(3)d
	80	83.48(1.04)a	28.1(1.2)ad	141(3)ef
	90	92(2)g	32(3)e	153(4)ag
SD	70	61(2)h	21(2)c	108(4)h
	80	72(2)j	23(2)ac	112(5)h
	90	76.6(1.3)e	27.4(0.6)ad	116(3)k

3.2. Influence of infusion time and temperature on the extraction of antioxidant compounds

Fig. 1 shows the total phenolic concentration of the aqueous extracts of persimmon leaves dried under different conditions, considering time of infusion and water temperature. The extraction kinetic of phenols was very rapid in all cases, taking place during the first few minutes of the infusion and remaining constant between 60 min (Fig. 1) and 1440 min (Table 1). Regarding to the effect of temperature, the higher the temperature of infusion the higher the phenolic concentration of the extracts. Also, the effect of temperature is observed on both, the kinetics and the concentrations achieved at 1440 min of infusion (Table 1). These results are consistent with the fact that the drying method and processing conditions greatly affect not only the degradation of bioactive compounds but also their stability and extractability. This is because method, temperature and drying time are process variables that significantly affect the structure of the resultant matrix, regardless of whether the final humidity or water activity is the same. Water removal during the drying of plant material is accompanied by significant deformation, which degrades the vegetal matrix and the functionality of its cell walls and membranes. The varying disintegration of this matrix results in a greater or lesser exposure of antioxidant compounds to oxidation reactions, hence the importance of establishing the most suitable drying conditions in each case.

The results obtained (Fig. 1) show the great influence of the drying method on the extraction of phenols during infusion. The extracts from leaves dried with air at 100°C (AD-100) gave the highest total phenolic concentration, followed by extracts from lyophilized leaves (FD), and then the extracts from air dried leaves at 180°C (AD-180) and from shade dried leaves (SD).

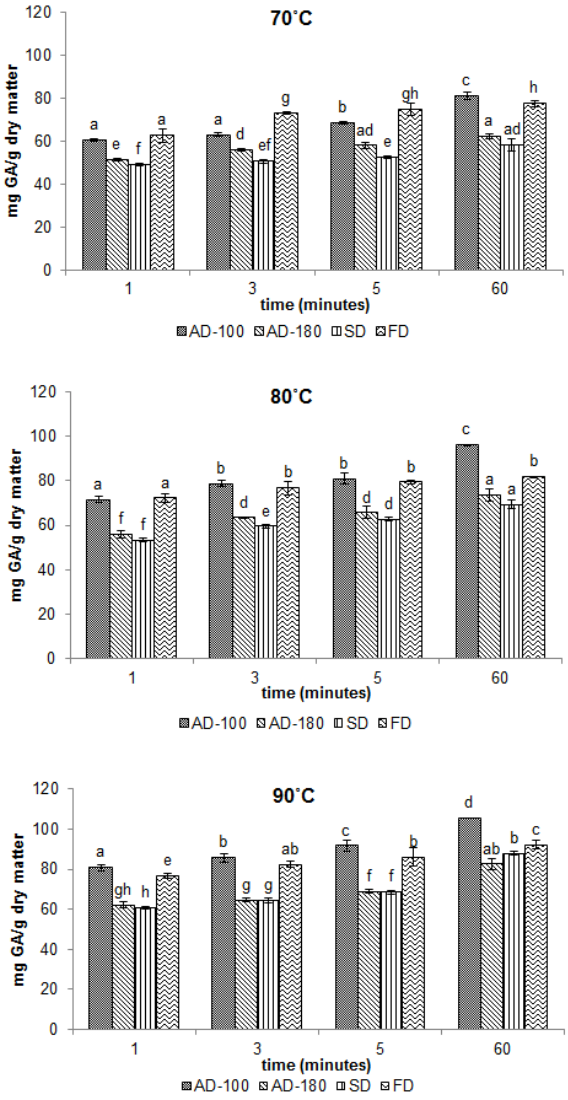

Fig. 1 – Effect of drying method, infusion time and infusion temperature on the total phenolic content (mg gallic acid/g dry matter) of the extracts. Significant differences as determined by ANOVA ($p<0.05$) are indicated by different letters.

Lyophilization is often considered to be the most adequate drying technique for preserving temperature sensitive compounds. However, it is an expensive method which is not suitable for large productions in continuous processes. In the case of persimmon leaves, it would not be a competitive process for preserving phenols since similar or better results are obtained with air drying at 100ºC (AD-100). Likewise, shade drying, which is widely used in countries producing tea and herbal teas, is cheap but has some drawbacks such as the drying time, the space required and the manpower required, etc. In view of the results obtained in this study, it could be said that the long drying time needed and the low processing temperatures involved in shade drying imply a greater exposure of the material to enzymatic and oxidation reactions than the other methods studied.

The same tendency is observed when the influence of the different processing variables (drying method, time and temperature of infusion) on the concentration of flavonoids and the antioxidant capacity of the extracts is analyzed (Fig. 2 and 3).

The antioxidant properties of food matrices are due to the presence of a complex mixture of compounds of varying polarity, such as vitamin C, vitamin E, carotenoids, and polyphenols (Cantin, Moreno & Gogorcena, 2009). When these compounds are extracted in aqueous media, the antioxidant capacity of the extracts depends on the type and quantity of the compounds extracted during infusion, and therefore it could be correlated with values of total phenols and flavonoids contents obtained in those extracts. The results obtained in this work show a good correlation between the concentration of flavonoids and total phenols and antioxidant activity, although higher values of r^2 were obtained for the correlation between flavonoids and antioxidant capacity (Table 2).

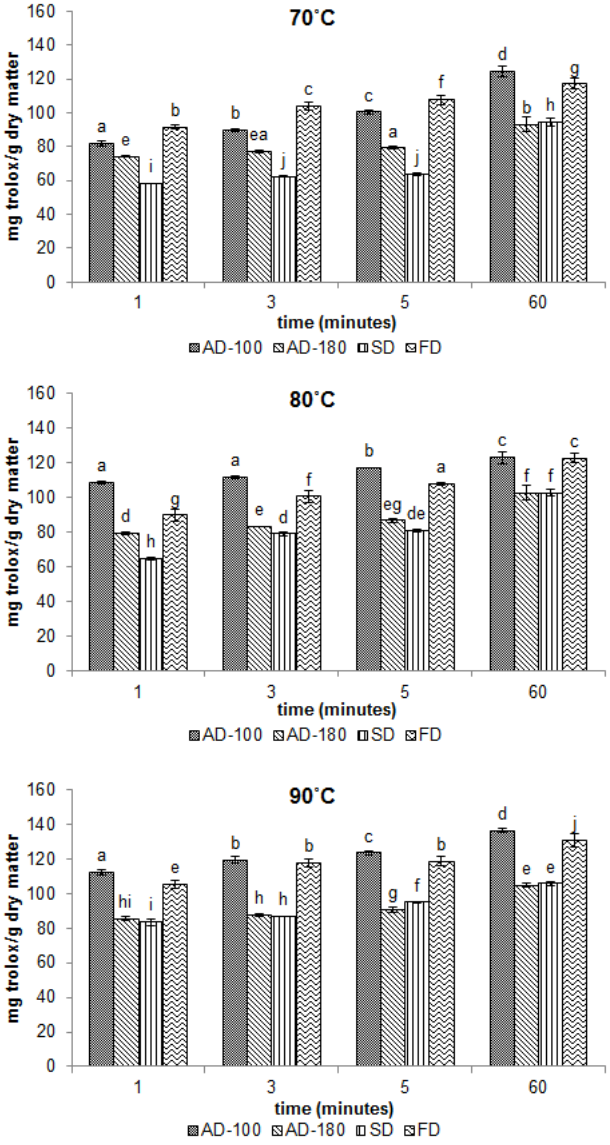

Fig. 2 – Effect of driying method, infusion time and temperature on the antioxidant capacity (mg trolox/ g dry matter) of the extracts. Significant differences as determined by ANOVA ($p<0.05$) are indicated by different letters.

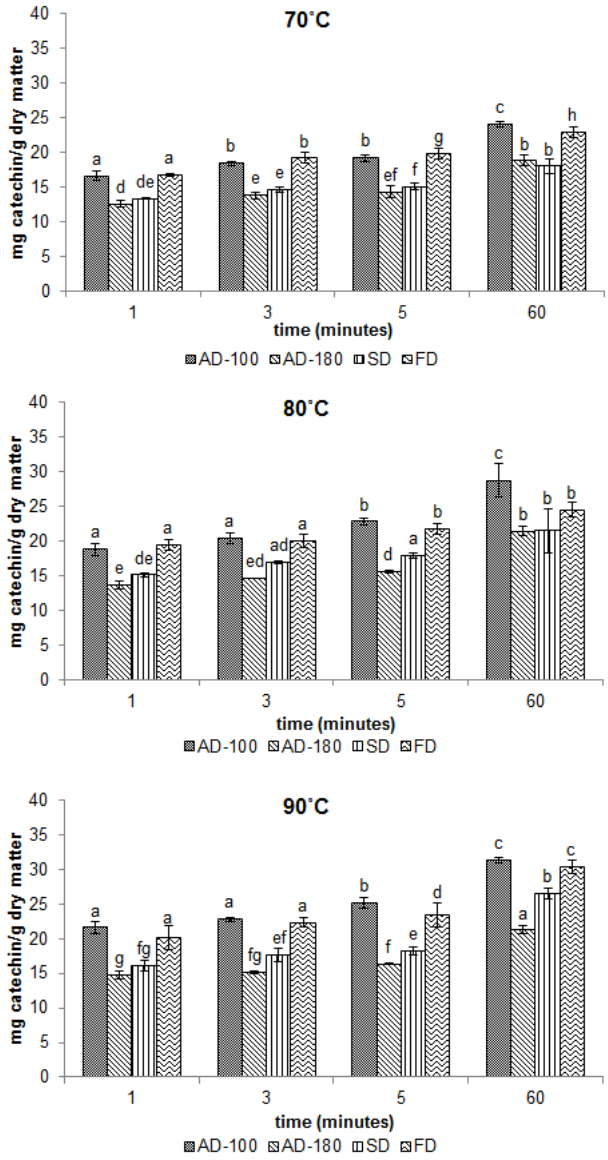

Fig. 3 – Effect of driying method, infusion time and temperature on the flavonoids (mg catechin/ g dry matter) of the extracts. Significant differences as determined by ANOVA ($p<0.05$) are indicated by different letters.

Table 2. Correlation coefficients (r^2) between total phenols vs. antioxidant capacity and flavonoids vs. antioxidant capacity, calculated from the results presented in Fig. 1, 2 and 3.

Drying method	T infusion (ºC)	r^2	
		Antioxidant activity vs. flavonoids	Antioxidant activity vs. phenols
AD-100	70	0.9490	0.9546
	80	0.8636	0.6471
	90	0.8761	0.7186
AD-180	70	0.8983	0.8506
	80	0.9372	0.9323
	90	0.9649	0.8038
FD	70	0.9603	0.9261
	80	0.9781	0.8925
	90	0.8585	0.8608
SD	70	0.9724	0.9756
	80	0.9929	0.9718
	90	0.9188	0.9241

3.3. Influence of drying method, infusion time and temperature on the colour of persimmon leaf extracts

According to the multivariant analysis of variance (ANOVA), both drying method and time of infusion, as well as their interaction, had a significance statistical influence on CIEL*a*b* colour parameters; while no influence of temperature of infusion was found on them (data not shown). Fig. 4 showed the values of L*, a* and b* of the persimmon leaf extracts in the colorimetric planes L*a* y b*a*as a function of drying method and time infusion at a set temperature of infusion of 90ºC. A loss of lightness (L*) and an increase of b* and a* values was found as time of infusion progressed, being especially notable after 1440 minutes. The change of a* coordinate from negative to positive values indicates a turn of colour from green-yellowish to brown-yellowish ones. Regarding to the effect of drying method, no statistical significance effect of drying method were

found among the colour of the samples at 1, 3 and 5 min of infusion; whereas infusions from freeze dried leaves at 60 and 1440 min presented higher lightness and lower a* and b* values than infusions obtained from shade dried leaves, and above all than infusions from air dried ones. Therefore, infusions obtained from air dried persimmon leaves are characterized by a more dark and brown-yellowish colour, while higher luminosity and green-yellowish colour is in infusions made with freeze dried leaves.

3.4. Influence of leaf size on the total phenolic content, flavonoids and antioxidant capacity

Given the heterogeneity of the size of persimmon leaves, the total phenolic content, flavonoids and antioxidant capacity were analyzed in two leaf sizes to determine if significant differences can be attributed to leaf size (Table 3). The smaller leaves presented around 9% more flavonoids, and 7% more total phenolic content and antioxidant capacity than the large leaves; in all cases the differences were statistically significant. The average values were lower than those obtained by Sakanaka et al. (2005) in all cases, while the values of antioxidant activity were higher than those obtained by Sun et al. (2011). These differences can be attributed to differences in variety, harvesting time and the extraction conditions used in each case. However, since in the present study the extraction of compounds was performed under the same conditions, the values obtained permitted evaluation of the differences between the two leaf sizes. These results may be useful to establish the criteria for collecting the persimmon leaves, in order to obtain a product for infusion with better antioxidant properties.

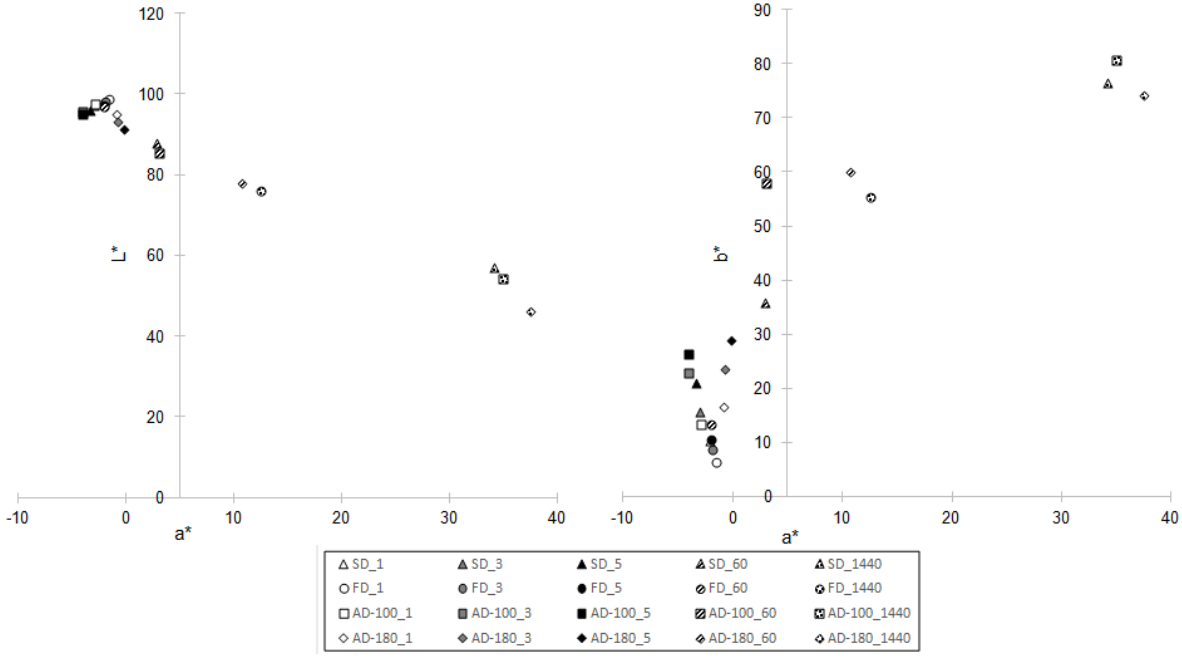

Fig. 4 – Influence of drying method and infusion time on L*, a* and b* coordinates parameters (mean values) of persimmon leaf extracts obtained at a set infusion temperature of 90 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3. Total phenols and flavonoids contents, as well as antioxidant activity, of aqueous extracts of persimmon leaves with two different sizes. Significant differences as determined by ANOVA ($p < 0.05$) are indicated by different letters.

Leave Size		Total Phenols	Flavonoids	Antioxidant activity
Axial Axis (cm)	Equatorial Axis (cm)	mg GA/g dry matter	mg catechin/g dry matter	mg trolox/g dry matter
9 ± 1	7.0 ± 0.9	83 ± 4 (a)	30,8 ± 0,5 (a)	118 ± 3 (a)
17 ± 2	10 ± 1	77 ± 4 (b)	27,9 ± 0,7 (b)	109,61 ± 1,08 (b)

4. CONCLUSIONS

Proper drying of persimmon leaves (*D. kaki, Rojo Brillante var.*) can be considered as an alternative use of the crop, due to the antioxidant properties of aqueous extracts. Hot air drying at 100°C yields better antioxidant levels compared to air drying at 180°C, drying in the shade or lyophilization. Also, the size of leaf seems to have a significant influence on the content of flavonoids and total phenols, so this aspect should be studied more in deep in order to define and to establish the optimal harvesting process.

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III.2. MOISTURE SORPTION ISOTHERMS AND ISOSTERIC HEAT OF SORPTION OF DRY PERSIMMON LEAVES

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Las propiedades y la calidad de las hojas de caqui que se preservan a través de su secado, dependen en gran medida, de su estabilidad física, química y microbiológica. Esta estabilidad está determinada por la relación entre el contenido de humedad en equilibrio (EMC) de las hojas y su correspondiente actividad de agua a una temperatura dada, lo que se conoce como isoterma de sorción (Myhara et al., 1998; Temple & van Boxtel, 1999). Entre los factores que determinan la forma y características de las isotermas de sorción de agua se encuentran la composición, el estado físico de sus componentes y la temperatura (Leung, 1986). A pesar de que el té de hoja de caqui es una de las infusiones más comercializadas y consumidas en China, no hay ningún estudio sobre las características de sorción de agua de sus hojas deshidratadas. Las isotermas de sorción son muy útiles para predecir la estabilidad durante el almacenamiento y elegir un material apropiado para su envasado (Al-Muhtaseb et al., 2004). Una vez determinadas las isotermas de sorción a diferentes temperaturas, es posible evaluar el calor de sorción, un parámetro de gran utilidad en los procesos de desorción y adsorción de agua en alimentos.

El objetivo de este estudio fue determinar el efecto de la temperatura de almacenamiento en las isotermas de sorción de agua de hojas de caqui secadas por aire caliente a 100ºC. Éstas se determinaron a 20, 30 y 40 ºC usando el método estándar gravimétrico para un rango de actividad de agua desde 0.06 hasta 0.9. Los datos experimentales se ajustaron a diferentes modelos matemáticos disponibles en la literatura científica con el objetivo de seleccionar aquel que mejor describa el comportamiento de sorción de agua de las hojas

durante el almacenamiento. Adicionalmente, se estimó el calor isostérico de sorción de agua a partir de los datos en el equilibrio utilizando la ecuación empírica de Clausius-Clapeyron.

Para la determinación de las isotermas de sorción se utilizó el método estático-gravimétrico que consiste en equilibrar las muestras en cámaras de diferente humedad relativa. Para el control de la humedad relativa se utilizaron disoluciones sobresaturadas de distintas sales. A su vez, éstas se alojaron en estufas de temperatura controlada. La transferencia de agua entre el producto y la atmósfera está asegurada por difusión natural del vapor de agua; y en el equilibrio la actividad de agua del producto se corresponde con la humedad relativa de la atmósfera de la cámara.

Las isotermas de sorción obtenidas se asemejaron a curvas de forma sigmoidal, lo que corresponde con una isoterma de Tipo II (según la clasificación de Brunauer) (Brunauer et al., 1940). Este tipo de curvas es típico de productos vegetales y son similares a las obtenidas en otros tipos de hojas como tés (Arslan & Togrul, 2005), hojas de olivo (Bahloul et al., 2008), de naranjo (Mohamed et al., 2004), etc. Los datos experimentales se utilizaron para estimar los parámetros de los diferentes modelos de sorción utilizados (Henderson, Halsey, Smith, Oswin, GAB, BET y Caurie). Los modelos de Halsey, Smith, GAB y BET fueron los que mejor describieron los datos experimentales, mientras que el modelo de Henderson presentó los peores ajustes. Los valores de humedad de la monocapa obtenidos a partir del modelo de GAB para las hojas de caqui a las diferentes temperaturas estudiadas, fueron muy similares a los publicados por Arslan & Togrul (2005) y Zanoelo (2005) para hojas de mate y té verde, respectivamente.

El calor isostérico de sorción se determinó aplicando el concepto de Clausius-Clapeyron y se estableció una ecuación empírica para predecir el valor de este parámetro en un rango entre 0.5 y 14 % de humedad (en base seca). Los valores

obtenidos del calor isostérico de sorción calculado fueron similares a los publicados por Lahsasni et al., (2003) llevados a cabo en té verde y negro.

Los resultados de este trabajo constituyen una herramienta útil para seleccionar los materiales de envasado y diseñar el envase adecuados para garantizar la calidad del producto durante su almacenamiento y vida útil.

III.2. MOISTURE SORPTION ISOTHERMS AND ISOSTERIC HEAT OF SORPTION OF DRY PERSIMMON LEAVES

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ABSTRACT

Moisture sorption isotherms of persimmon leaves were determined at 20, 30 and 40 °C using the standard gravimetric static method over a range of relative humidity from 0.06 to 0.9. The experimental sorption curves were fitted by seven equations: Henderson, Halsey, Smith, Oswin, BET, GAB and Caurie. The Halsey, Smith, GAB and BET models were found to be the most suitable for describing the sorption curves. The isosteric heat of sorption of water was determined from the equilibrium data at different temperatures. It decreased as moisture content increased and was found to be a polynomial function of moisture content.

Keywords: persimmon leaves; equilibrium moisture content; isosteric heat of sorption; modeling; sorption isotherms

1. INTRODUCTION

Persimmon crops in Spain have grown exponentially over the last decade to double their cultivated area as a result of the profitability of farming and the process of conversion of citrus and other fruit trees (Llácer & Badenes, 2002). Persimmon fruit demand has increased significantly in Germany, the United Kingdom, France, Italy and Russia, as well as in new emerging markets (the United States, Canada and Brazil). In addition, persimmon trees are deciduous; therefore, making use of their leaves could be a good way to increase the economic value of this crop. Persimmon leaves, which are well-known in Japan, are infused with hot water and are used traditionally due to their

healing properties (treatment of paralysis, frostbite, burns and to stop bleeding) (Matsuo & Ito, 1978). Their principal compounds are flavonoid oligomers, tannins, phenols, organic acids, chlorophyll, vitamin C and caffeine (Jo, Son, Shin, & Byun, 2003; Matsuo & Ito, 1978). Once collected, the leaves need to be stabilized, usually by drying, before their commercialization and use. To preserve the quality of any dried product during storage, it is necessary to know its water sorption properties.

The properties and quality of persimmon leaves preserved by drying depends to a great extent on their physical, chemical and microbiological stability. This stability is determined by the relationship between the EMC of the leaves and its corresponding water activity at a given temperature (Myhara, Sablani, Al-Alawi, & Taylor, 1998; Temple & van Boxtel, 1999), which is called sorption isotherm. Among the factors that influence the shape and characteristic of water sorption isotherms are the composition, physical state of the components and temperature (Leung, 1986). The physical state (crystalline, amorphous) in which the material components are found significantly affects water retention and depends largely on the previous technological treatments the products have been subjected to. Due to the exothermic character of the phenomenon of adsorption, an increase in temperature results in a loss of product moisture in equilibrium at a given relative humidity. In general, the effect of temperature is most significant for intermediate and low values of water activity.

There is no reported research on the moisture sorption characteristics of persimmon leaves even though they are commonly commercialized and consumed in China and Japan in infusions. Moisture sorption isotherms are important for predicting stability during storage and selecting an appropriate packaging material. Once the sorption isotherms at different temperatures have been measured, it is possible to evaluate heat sorption, which determines the interaction between the adsorbent and the adsorbate. Water activity which participates

in the degradation reactions in biosystems depends both on water content and the properties of the diffusion surface (Al-Muhtaseb, McMinn, & Magee, 2004).

Equilibrium moisture content data can be determined using two different techniques: (a) manometric or hygrometric techniques based on direct determination of the vapor pressure or the relative humidity of the interface of a solid with a known moisture content and (b) techniques based on the determination of moisture content of the sample after it has reached equilibrium in a gas environment with a known relative moisture content. This last procedure can be carried out by static or dynamic means. The static gravimetric method is the most commonly used in food engineering, and a large amount of experimental EMC data reported for many plants with medicinal and therapeutic properties was mainly found by employing the gravimetric method. Literature reported experimental EMC for bitter orange leaves (Mohamed, Kouhila, Jamali, Lahsasni, & Mahrouz, 2004), chenopodium ambrosioides (Jamali et al., 2004), citrus reticulate leaves (Jamali, Kouhila, Mohamed, Idlimam, & Lamharrar, 2005), garden mint leaves (Park, Vohnikova, & Reis Brod, 2001), green tea in powder and granules (Arslan and Togrul, 2005 and Sinija and Mishra, 2007), leaves and stems of lemon balm (Argyropoulos, Alex, Kohler, & Müller, 2012), mate leaves (Zanoelo, 2005), olive leaves and Tunisian olive leaves (Bahloul et al., 2008 and Nourhène et al., 2008) and orange peel and leaves (Citrus sinensis) (Kammoun Bejar, Boudhrioua Mihoubi, & Kechaou, 2011). The numerous studies carried out evidence the importance of the characterization of EMC for predicting the stability and shelf-life of agricultural products. There are four common specific areas of practical application of isotherms related to food processing: drying (in order to save energy and establish the optimal processing conditions), mixing (to determine the water activity of the mixture from the isotherms of each component), packaging (to determine the permeability of

the packing) and storage (to lengthen the shelf-life of foods) (Gal, 1983 and Labuza, 1984).

The objectives of this study were to determine the effect of temperature on the moisture sorption isotherms of persimmon leaves. Experimental data will be analyzed by using seven sorption isotherm equations available in the related literature in order to find the most suitable model describing the isotherms of persimmon leaves and the net isosteric heat of sorption of water will be estimated.

2. MATERIAL AND METHODS

2.1. Samples preparation

Persimmon leaves (*Diospyros kaki, Rojo Brillante var.*) were picked from trees in an orchard in Valencia (Spain), blanched at 100 °C for 5 min and then dried at 100 °C for 30 min in a convective drier. The selection of the drying conditions was based on the results obtained in a previous work (Martínez-LasHeras et al., 2014) were it was demonstrated that this drying method allowed a good preservation of the antioxidant properties of persimmon leaves.

2.2. Chemicals

Potassium Chloride, Magnesium Chloride 6-hydrate, Potassium Hydroxide, Sodium Chloride, Magnesium Nitrate 6-hydrate, Potassium Carbonate, Lithium Chloride, and Thymol were obtained from Panreac. Ammonium nitrate was obtained from VWR and dehydrated Barium chloride from Scharlab.

2.3. Determination of sorption isotherms

The sorption method used was the static gravimetric technique, which involves the use of saturated salt solutions to maintain a fixed relative humidity when equilibrium is reached. The mass transfer between the product and the ambient atmosphere is assured by natural diffusion of the water vapor and the water

activity of the product equals the relative humidity of the atmosphere at equilibrium conditions. Nine saturated salt solutions (KOH, LiCl, MgCl2, K2CO3, Mg(NO3)2, NH4NO3, NaCl, KCl and BaCl2) corresponding to a wide range of water activities ranging from 0.0610 to 0.9096 were prepared and introduced in nine glass desiccators (Hall, 1957). A small quantity of Thymol was added to the containers that had saturated salts with water activities higher than 0.6, in order to prevent microbial growth.

About 1 g of the dried leaves was placed in each glass desiccator containing the saturated salt solution. All nine desiccators were placed in an incubator and the gain or loss in weight of the samples in each desiccator was monitored every week. The EMC was acknowledged when three consecutive weight measurements showed a difference of less than 0.001 g. This took about 50–60 days, depending on the temperature inside the incubator. The procedure was carried out in triplicate for each sample and the average values of EMC were measured. This procedure was repeated at three different temperatures to obtain the sorption isotherm at 20, 30 and 40 °C.

2.4. Analytical determinations

Analytical determinations were carried out in accordance with the method proposed by the Association of Official Analytical Chemists (AOAC, 1984). Moisture content was determined using the gravimetric method, oven drying at 105 °C up to constant weight (24 h). Total protein was determined using the Kjeldahl method and protein was calculated using the general factor (6.25) (El-Shurafa, Ahmed, & Abou-Naji, 1982). Fat content was determined by the Soxhlet method using petroleum ether (40–60 °C boiling range) as a solvent. Ash content was measured using a muffle at 550 °C up to constant weight (4 h).

The sample weight was measured with a gravimetric balance (Mettler-Toledo) with a precision of 0.0001 g. Moisture content,

protein, fat and ash content were expressed in dry basis (g/100 g d.b.). All analytical determinations were performed in triplicate. Values of different parameters were expressed as the mean±standard deviation.

2.5. Analysis of sorption data

A large number of models have been proposed in the related literature for sorption isotherms. In the present study, the description of the relationship between EMC, aw and the temperature of persimmon leaves were verified according to seven models (Basunia & Abe, 2001; Henderson, 1952: Lahsasni, Kouhila, Mahrouz & Fliyou, 2003; Mohamed et al., 2004) as shown in Table 2.

The criterion used to evaluate the quality of the fit was the mean relative deviation modulus:

$$MRE = \frac{100}{N}\sum_{i=1}^{N}\frac{M_{i,exp}-M_{i,pre}}{M_{i,exp}} \quad (1)$$

where Mi,exp is the ith experimental value of EMC; Mi,pre is the ith value predicted by the model; and N is the number of data points. It is generally considered that MRE values below 10% indicate an adequate fit for practical purposes (Aguerre, Suarez, & Viollaz, 1989).

The differences between the measured and predicted EMC values for various water activities were defined as residuals, and these residuals were plotted against predicted values of EMC. A model was considered acceptable if the residual values felt in a horizontal band centered around zero, and displays no systematic tendencies towards a clear pattern (Bag, Srivastav, & Mishra, 2009).

2.6. Determination of net isosteric heat of sorption

The Clausius–Clapeyron equation derived for the vapor–liquid equilibrium (2), applied to temperature–pressure vapor data

relating to the product, allows the enthalpy change associated with the sorption process (net isosteric heat of sorption) (3) to be calculated for different levels of humidity. The net isosteric heat of sorption represents the total energy available to do work, while entropy at any temperature is equivalent to lost work and gives a measure of the energy that is not available to perform work. Thus, the energy that is available to do work is the difference between these two quantities (Aviara & Ajibola, 2002).

Differential heat of sorption (ΔH) measures the energy changes that occur during the sorption process and is indicator of the level of attractive or repulsive forces of the system. Gibb's free energy (ΔG), associated with the spontaneity of the process, may be calculated for the adsorption process (4). Entropy changes (ΔS) may be associated with spatial reorganization occurred at the water–solute interface, which can be calculated by Eq. (5)

$$\ln(p) = \frac{\Delta H}{R\frac{1}{T}+K} \quad (2)$$

$$\Delta H = \Delta H_V + Q_S \quad (3)$$

$$\Delta G = R \cdot T \cdot \ln\left(\frac{P}{P^0}\right) = R \cdot T \cdot lna_w \quad (4)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (5)$$

where: p is the water vapour pressure in equilibrium with the material at a given temperature (atm); ΔHv is the heat of water evaporation (-43.96 kJ/mol); Qs is the net isosteric heat of sorption (kJ/mol), aw is the water activity (dimensionless), T is the absolute temperature (K), R is the universal gas constant (kJ/mol K), p^o is the pure water vapour pressure (atm) and K is a constant.

A, B and C	model coefficients	Mi,pre	ith predicted moisture content (% d.b.)
aw	water activity (dimensionless)	MRE	mean relative error (%)
d. b.	dry basis	N	number of data points
d_f	number of degrees of freedom	Qst	net isosteric heat of sorption (kJ/mol)
EMC	equilibrium moisture content	R	universal gas constant (8.314 J/mol K)
K	equilibrium moisture content (% d.b.)	r	coefficient correlation
M	ith experimental moisture content (% d.b.)	T	temperature (ºC, K)
$M_{i,exp}$			

3. RESULTS AND DISCUSSION

Dried persimmon leaves presented a moisture content of about 4.34 ± 0.16% (dry basis), and protein, ash and fat contents of about 3.3 ± 0.2%, 4.7 ± 0.1% and 11.26 ± 0.23%, respectively.

3.1. Sorption isotherms

Fig. 1 shows the experimental “working sorption isotherm” of dry persimmon leaves which can be very useful to predict the moisture adsorption or desorption of this product under different storage conditions (Labuza, 1984).

The sorption isotherm obtained presents a sigmoidal shaped curve, thus reflecting a Type II isotherm (according to Brunauer’s classification) (Brunauer et al., 1940). The behaviour observed was typical of plant products and similar results have been obtained with other leaves such as tea leaves (Arslan & Togrul, 2006; Sinija & Mishra, 2007), olive leaves (Bahloul et al., 2008), orange leaves (Mohamed et al., 2004) and lemon balm leaves (Argyropoulos et al., 2012).

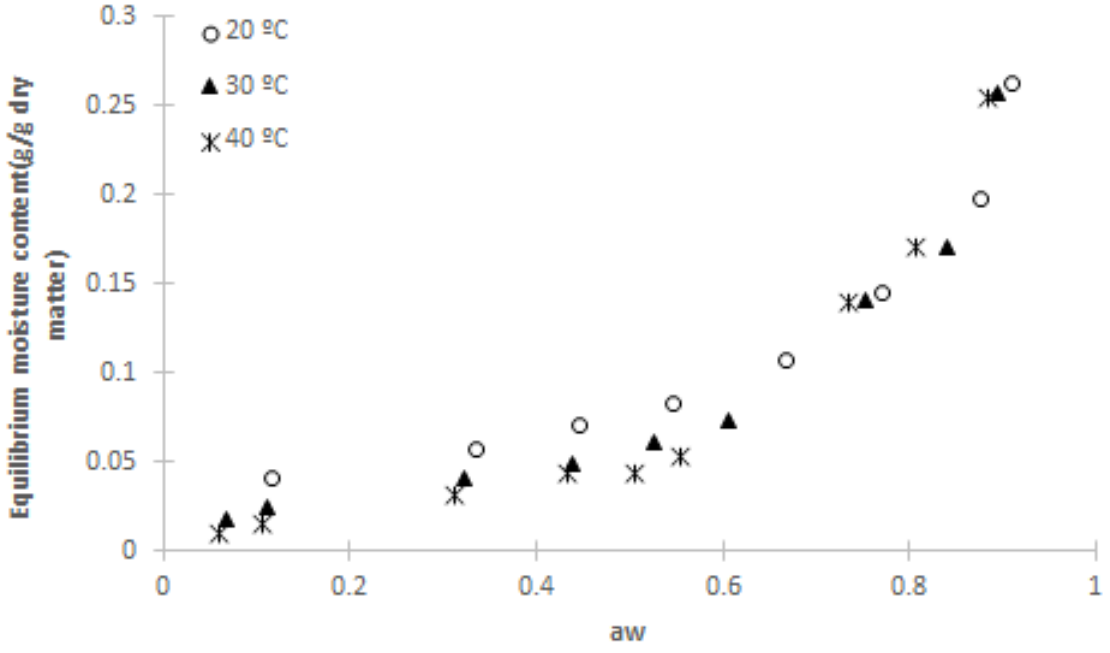

Fig. 1 - Equilibrium moisture content (EMC) vs. water activity (a_w) at 20, 30 and 40 ºC for persimmon leaves

The EMC decreases as temperature increases, at constant water activity (Fig. 1); however, this influence was not noteworthy as it occurs with many biological materials. The higher excitation state of water molecules at a higher temperature thus decreasing the attractive forces between them may explain this result (Mohamed et al., 2004). Moreover, at constant temperature, the EMC increases as water activity increases. This is consistent with similar results that have been reported in the related literature for other products (Argyropoulos et al., 2012; Arslan and Togrul, 2006; Bahloul et al., 2008; Basunia and Abe, 2011; Nourhène et al., 2008; Mohamed et al., 2004).

Table 1 shows the different model equations used to fit the experimental data as well as a brief summary of the meaning of the model parameters.

The experimental results obtained from the sorption isotherm of dry persimmon leaves were used to estimate the parameters of the different sorption models (Table 1) and the results are shown as mean values, regression coefficient (r^2) and mean relative error (MRE) in Table 2. According to r^2 and MRE values, the Halsey, Smith, GAB and BET models could be considered the best to describe experimental data; the Henderson model yielded the worst results (low values of r^2 and MRE greater than 10%); and the Oswin and Caurie models would be in the middle. Fig. 2 shows the comparison between experimental and calculated (GAB model) data of sorption isotherms of persimmon leaves obtained at 20, 30 and 40 ºC.

Table 1. Sorption isotherms models for equilibrium moisture

Model name	Formula	Parameters and Reference
Henderson	$EMC = 0.01\left(\frac{-\log(1-a_w)}{10^f}\right)^{1/n}$	n and f, product characteristic parameters (Thompson et al., 1968)
Halsey	$EMC = \left(\frac{A}{\ln(1/a_w)}\right)^{1/B}$	A and B are model constants and characteristic for each food (Iglesias and Chirife, 1976a; Smith, 1947; Oswin, 1946)
Smith	$EMC = A - B \cdot \ln(1-a_w)$	
Oswin	$EMC = A \cdot \left[\frac{a_w}{1-a_w}\right]^B$	
GAB	$EMC = \frac{W_o \cdot C \cdot K \cdot a_w}{(1-K\cdot a_w)\cdot(1+(C-1)\cdot K\cdot a_w)}$	W_o is the product moisture corresponding to the situation where the primary adsorption sites are saturated by water molecules. C is the Guggenheim constant, characteristic of the product and related with the heat of adsorption of the monolayer. K is a correction factor related to the heat of sorption of the multilayer (Van den Berg and Bruin, 1981)
BET	$EMC = \frac{W_o \cdot C \cdot a_w}{(1-a_w)\cdot(1+(C-1)\cdot a_w)}$	W_o is the product moisture corresponding to a monolayer water adsorbed C, characteristic of the material constant related to the heat released in the sorption process (Brunauer et al., 1938; Iglesias and Chirife, 1976b)
Caurie	$EMC = \exp\left(a_w \cdot \ln(r) - \frac{1}{4.5 \cdot W_s}\right)$	r, is a constant characteristic of the material W_S, moisture content security that provides maximum stability for the dehydrated food during storage (Castillo et al., 2003)

The GAB model appears to be one of the most suitable for describing the relationship between water content, water activity and temperature throughout the intervals considered. On the other hand, a model whose parameters have a physical meaning is highly interesting because it provides a link with physical phenomena and allows comparisons with other materials (Mulet, García-Pascual, Sanjuán, & García-Reverter, 2002). Thus, the GAB model was found to be the best model to describe sorption isotherm of dry persimmon leaves. The values of monolayer moisture for dry persimmon leaves from the GAB model at different temperatures were similar to those obtained for mate and tea leaves (Zanoelo, 2005; Arslan & Togrul, 2005).

3.2. Isosteric heat of sorption and sorption entropy

The differential heats of sorption, ΔH, were calculated from the slope of the plot between the values of ln (p) and 1/T for moisture content ranging from 0.5 % to 14% as shown in Fig. 3; ΔG and ΔS were calculated according to equations 4 and 5 and the variation of ΔH, ΔG and ΔS with moisture content is shown in Fig. 4. The moisture level for which ΔH and ΔS are at their maximum approximately fits with the BET monolayer value (Brunauer et al., 1938), which represents the product moisture corresponding to a situation where the primary adsorption sites are saturated by water molecules. When the most accessible points are saturated, the water vapour is adsorbed at primary points of the less accessible areas, thus the associated heat of sorption is the greatest just before the completion of the monolayer (Fig. 4).

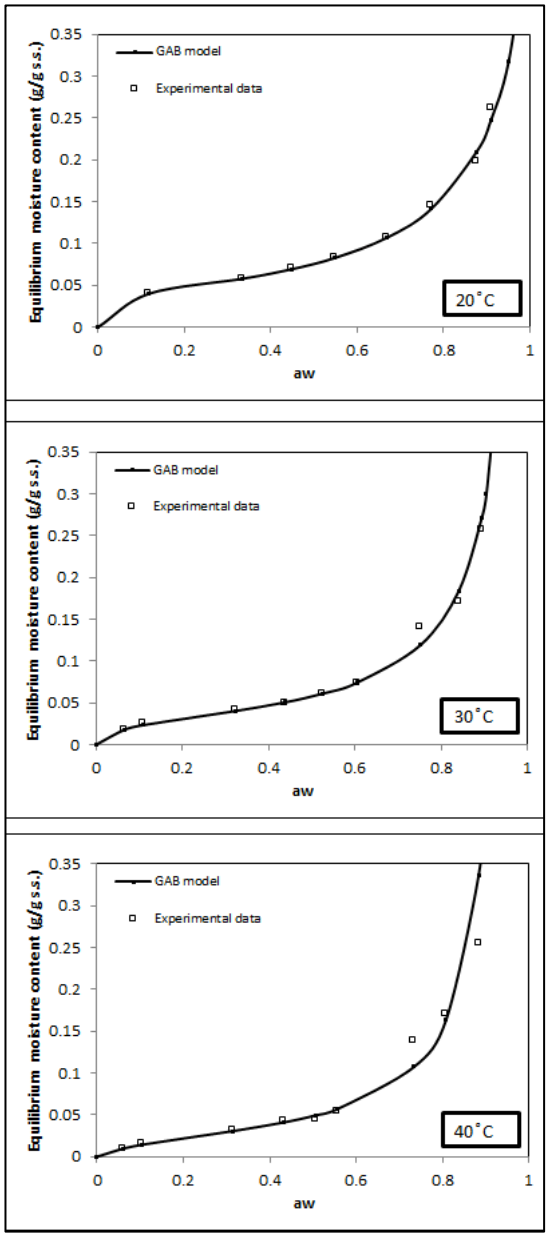

Fig. 2 - Adsorption isotherms of persimmon leaves fitted with seven models

Table 2. Estimated parameters of the different models fitted to the sorption data for persimmon leaves.

Models	Parameters	Temperature (ºC)		
		20	30	40
Henderson	f	-1.9109	-1.6545	-1.3799
	n	1.454	1.2866	1.0511
	r^2	0.9137	0.9208	0.9351
	MRE %	14.9	22.85	26.30
Halsey	A	0.0097	0.0218	0.0389
	B	1.6664	1.2201	0.9504
	r^2	0.9976	0.9931	0.9861
	MRE %	2.48	5.86	11.02
Smith	A	-0.0088	-0.0404	-0.0645
	B	0.1066	0.1263	0.1475
	r^2	0.9736	0.9731	0.993
	MRE %	5.36	7.28	5.01
Oswin	A	0.0833	0.0672	0.0569
	B	0.4416	0.5481	0.691
	r^2	0.9766	0.9736	0.9775
	MRE %	7.53	12.95	14.27
GAB	W_o	0.04225	0.03088	0.0259
	C	44.4418	17.2626	7.9654
	K	0.9127	0.9927	1.0457
	r^2	0.9906	0.969	0.9156
	MRE %	2.16	4.82	10.13
BET	W_o	0.03783	0.0299	0.0289
	C	66.5410	19.6189	6.9575
	r^2	0.9999	0.9983	0.9993
	MRE %	2.83	2.94	4.30
Caurie	r	9.6465	18.7801	41.2025
	W_s	0.0614	0.0529	0.0465
	r^2	0.9632	0.9761	0.9803
	MRE %	9.07	10.74	12.70

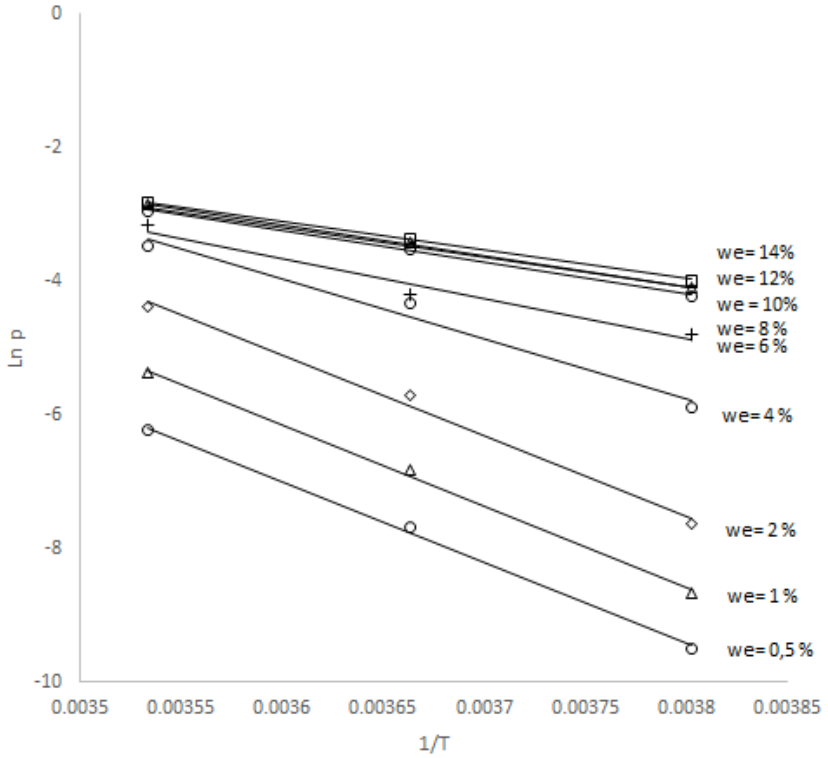

Fig. 3 - ln(p) vs. 1/T graphs for calculating the heat of sorption of persimmon leaves

The net isosteric heats of sorption were estimated from Eq. (3) and their correlation with the moisture content can be expressed mathematically as a function of moisture content (Mohamed et al., 2004):

$$Qst = 0.7397M^2 - 18.162M + 90.451 \quad (r = 0.9962) \qquad (6)$$

This mathematical relationship can be a useful tool for estimating the heat of sorption of dry persimmon leaves for other moisture contents. Results obtained are alike to those obtained for similar food materials such as withered leaves, black and green tea (Lahsasni et al., 2003).

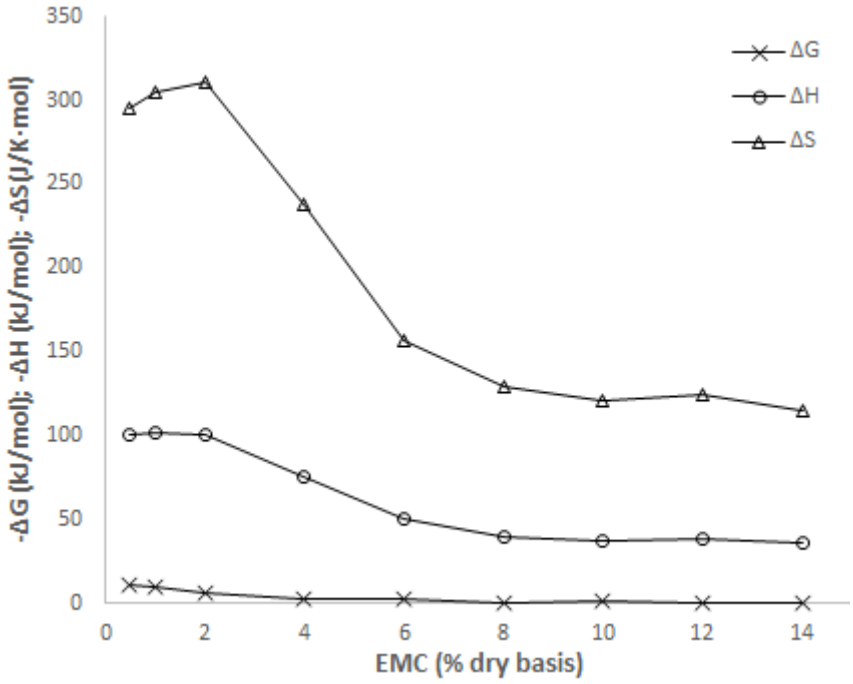

Fig. 4 - Variation of ΔH differential, ΔG and ΔS for the water vapour adsorption depending on the moisture

4. CONCLUSIONS

The sorption isotherm for dry persimmon leaves presented a sigmoid shape and belong to type II according to BET classification and a slight influence of temperature was observed on water sorption behavior of this product. Since GAB and BET models provided the best fit to experimental data, they can be used to describe its drying and storage behaviour. The net isosteric heat of sorption, that can be used to estimate the energy requirements for dehydration processes, was evaluated by applying the Clausius-Clapeyron concept, and an empirical equation was established to describe its variation between 0.5% and 14% (d.b.).

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III.3. A COMPREHENSIVE CHARACTERIZATION OF PERSIMMON LEAVES POLYPHENOLS BY HIGH RESOLUTION MASS SPECTROMETRY (LC–ESI-LTQ-ORBITRAP-MS)

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Las hojas de caqui conocidas como *Shi Ye* en China, se han usado a lo largo de la historia, en la medicina tradicional china para el tratamiento del ictus, angina de pecho, hemorragia interna, hipertensión, aterosclerosis y algunas enfermedades infecciosas (Kotani et al., 2000; Matsumoto et al., 2002; Tanaka et al., 2003; Sakanaka et al., 2005). Las hojas de caqui contienen vitamina C (Mizuo, 1995), flavonoides (Liu et al., 2012), terpenoides (Chen et al., 1999), resinas, polisacáridos, clorofilas (Hospital, 1973), carotenos, kryptoxantina, celulosa, hemicelulosa, lignina (Hu et al., 2002) y amino ácidos (Zu & Lei, 1989). Aunque en estudios anteriores (Chou, 1984; Nakatani et al., 1989; Guo & Dong, 1999; Chen et al., 2002), algunos compuestos fenólicos han sido determinados en las hojas de caqui (como el kaempferol, quercetin, isoquercetin, myricitrin y algunos flavonoles glucósidos), hasta la fecha no se había realizado una identificación exhaustiva de su perfil polifenólico mediante espectrometría de masas de alta resolución.

Por ello, el objetivo de este trabajo fue identificar y cuantificar una amplia gama de polifenoles que se encuentran en las hojas de caqui.

La espectrometría de masas LTQ-Orbitrap se ha usado en otros estudios para identificar polifenoles en diferentes matrices alimentarias como son el tomate (Vallverdú-Queralt et al., 2011), cerveza (Quifer-Rada et al., 2015), vino (Vallverdú-Queralt et al., 2015), hierbas culinarias (Vallverdú-Queralt et al., 2014), etc., y ha demostrado ser una herramienta fiable para

conocer la estructura de polifenoles desconocidos en muestras complejas.

Se han identificado 41 compuestos polifenólicos por LC-ESI-LTQ-Orbitrap, de los cuales 25 se identificaron mediante la comparación de los tiempos de retención, patrones de fragmentación y masas exactas con patrones puros. La identificación de los 17 compuestos restantes sin patrones disponibles, se basa en las mediciones de la masa exacta del ión [M-H]- y el patrón de fragmentación y éstos se compararon con los que se encuentran en la literatura científica (ya que los patrones de fragmentación de muchos de estos compuestos han sido previamente identificados en otros trabajos). Entre los polifenoles identificados se encuentran: 9 ácidos benzoicos (ácido gálico, ácido gálico 4-*O*-glucósido, ácido 3,5-dihidroxibenzoico, ácido 2,5-dihidroxibenzoico, ácido 4-hidroxibenzoico, ácido 3-hidroxibenzoico, ácido 2-hidroxibenzoico, ácido vanílico y el ácido 3,5-dimetoxibenzoico), 5 ácidos hidroxicinámicos (ácido clorogénico, ácido cafeico, ácido *p*-cumárico, ácido sinápico, ácido cumárico-*O*-hexósido), 11 flavanoles (gallocatequina, catequina-*O*-hexósido I, catequina-*O*-hexósido II, catequina-*O*-hexósido III, procianidina B1, epigallocatequina, dímero I de procianidina, dímero II de procianidina, catequina, epicatequina-3-*O*-galato y prodelfinidina dímero B3), 13 flavonoles (miricetin-*O*-hexósido I, miricetin-*O*-hexósido II, isoquercetin, quercetin-*O*-hexósido, quercetin-*O*-pentósido I, quercetin-*O*-pentósido II, kaempferol-3-glucósido, kaemfperol-*O*-hexósido I, miricetin, kaemfperol-*O*-ramnósido, quercetina, kaempferol e isorhamnetina), 1 flavanona (naringenina), 1 flavona (apigenina) y 1 tirosol (hidroxitirosol).

Estos resultados muestran que las hojas de caqui presentan un complejo perfil polifenólico que podría ayudar a explicar los efectos beneficiosos de su uso como planta medicinal.

III.3. POLYPHENOLIC PROFILE OF PERSIMMON LEAVES BY HIGH RESOLUTION MASS SPECTROMETRY (LC–ESI-LTQ-Orbitrap-MS)

Martínez- Las Heras, R., Quifer-Rada, P., Andrés, A., Lamuela-Raventós, R.

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ABSTRACT

Persimmon leaves have played an important role in Chinese medicine. Persimmon extracts and formulations have been shown to possess a wide range of pharmacological activities, including antioxidant, hypolipidaemic and antidiabetic, and they have been used to treat cardiovascular disease, improve homeostasis, as antibacterial and anti-inflammatory agents, and as a beauty treatment. In this work, liquid chromatography coupled to hybrid linear ion trap quadrupole Orbitrap mass spectrometry was used to accurately identify persimmon leaf polyphenols. Forty one phenolic compounds, including simple phenolic acids, hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavonols, flavanones, flavone-chalcones, tyrosols and their conjugated derivatives, were identified and quantified using high mass accuracy data and confirmed by MS2 experiments. To the best of our knowledge, this is the most extensive study of persimmon leaf polyphenols performed so far, since 33 phenolic compounds are reported for the first time.

Keywords: persimmon leaves; polyphenols; identification; Orbitrap-MS; liquid chromatography; flavonoids; cinnamic acid; hydroxybenzoic acids; hydroxytyrosol

1. INTRODUCTION

Persimmon (Diospyros kaki L.) leaves, known as Shi Ye in Chinese, have a long history in Chinese traditional medicine for the treatment of ischaemia stroke, angina, internal haemorrhage, hypertension, atherosclerosis and some infectious diseases. (Kotani et al., 2000 & Matsumoto et al., 2002; Tanaka et al., 2003; Sakanaka et al., 2005). Additionally, persimmon leaves are increasingly popular as constituents of health and cosmetic products in Asian countries, such as Japan, Korea and China. The leaves have been used as health-promoting beverages for centuries and are one of the most popular infusions in China and Japan.

Persimmon leaves contain a high amount of vitamin C (Mizuo, 1995; Hiromichi, 2002), flavonoids, which are the main constituents and are considered to be the active compounds (Liu et al., 2012), terpenoids, which also show certain pharmacological activities (Chen et al., 1999), and other compounds such as resins, polysaccharides, chlorophylls (Hospital, 1973), carotenes, kryptoxanthin, cellulose, hemicelluloses, lignins (Hu et al., 2002), amino acids and trace elements (Zu & Lei, 1989). Flavonoids isolated from persimmon leaves present antioxidant activity (Sun et al., 2014), hypotensive (Kameda et al., 1987) and anti-allergic effects (Kotani et al., 2000). The major polyphenols in persimmon leaves are proanthocyanidins, which have anti-hypertensive and vasorelaxant effects (Kawakami et al., 2011). Some *in vitro* studies have suggested they may also have beneficial effects on diabetes (Kawakami et al., 2010; Wang et al., 2011). Therefore persimmon leaf may be used as a functional drink or as functional ingredient to add healthy and therapeutic properties in certain foods. However, although some phenolic compounds in persimmon leaves have been reported, including kaempferol and quercetin (Chou, 1984), isoquercetin (Nakatani et al. 1989), myricitrin (Guo & Dong, 1999) and many flavonol glucosides (Chou, 1984; Chen et al., 2002), a

comprehensive phenolic profiling by high resolution mass spectrometry is lacking.

LTQ-Orbitrap mass spectrometry has been used in previous studies to identify polyphenols in different food matrices such as tomato (Vallverdú-Queralt et al., 2011), walnut (Vallverdú-Queratl, 2014) beer (Quifer-Rada et al., 2015), wine (Vallverdú-Queralt et al., 2015) and culinary herbs (Vallverdú-Queralt et al., 2014) and it has proven to be a reliable tool for structural elucidation of unknown polyphenols in complex samples. The aim of this work was to identify the full range of polyphenols found in persimmon leaves that have not yet been described.

2. MATERIALS AND METHODS

2.1. Standards, reagents and materials

All solvents were HPLC grade and all chemicals were analytical reagent grade. Vanillic acid, catechin, 3-hydroxybenzoic acid, apigenin, kaempferol-3-*O*-glucoside, myricetin, isorharmnetin and isoquercetin were purchased from Fluka (St. Louis, MO, USA). Gallic acid, caffeic acid, 3,5-dimethoxybenzoic acid, 2,5-dihydroxybenzoic acid, *p*-coumaric acid, sinapic acid, naringenin, quercetin, chlorogenic acid and 3,5-dhydroxybenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxytyrosol, kaempferol, procyanidin B1 and naringenin chalcone were purchased from Extrasynthese (Lyon, France). Ethanol, methanol and HPLC-grade formic acid were obtained from Scharlau (Barcelona, Spain) and ultrapure water (Milli-Q) from Millipore (Billerica, MA, USA). Samples were stored in the shade and protected from light until analysis.

2.2. Extraction and analysis of polyphenols

2.2.1. Samples

Persimmon leaves (Diospyros kaki, Rojo Brillante var.) were picked from trees in an orchard in Valencia (Spain), blanched at 100 ºC for 1 min and dried at 100 ºC for 30 min in a convective

drier. This drying method was chosen because in a previous study it allowed a good preservation of the antioxidant properties of persimmon leaves (Martínez-Las Heras, Heredia, Castelló & Andrés, 2014).

2.2.2. Extraction of polyphenols

Samples were treated in a darkened room with a red safety light to avoid oxidation of the analytes. The extraction of polyphenols was done following the procedure of Rodríguez-Pérez et al., 2013 with some modifications. First, 0.2 g of persimmon leaves was extracted using 8 mL of methanol/water (80:20, v/v) in an ultrasound bath (Sonorex, Bandelin) for 15 min at room temperature. Then, the samples were centrifuged for 15 min at 2700 g at 4 ºC to remove solids. After centrifugation, the pellets were again extracted with fresh solvent under the same conditions. The supernatants were combined and evaporated under nitrogen flow, and the residue was reconstituted with water 0.2% formic acid up to 3 mL and filtered through a 0.22 µm PTFE filter into an amber vial for HPLC analysis. Samples were stored at -20 ºC until analysis.

2.2.3. LC-ESI-LTQ-Orbitrap-MS

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a resolution setting of 30,000 at m/z 400. Operation parameters were as follows: source voltage, 4 kV; sheath gas, 20 (arbitrary units); auxiliary gas, 10 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 275 °C. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target 5·105 for MS mode and 5·104 for MS^n mode). Persimmon leaf samples were analysed in full scan mode at a resolving power of 30,000 at m/z 400 and data-dependent MS/MS events acquired at a resolving power of 15,000. The most intense ions detected during full scan MS triggered data-

dependent scanning. Data-dependent scanning was carried out without the use of a parent ion list. Ions that were not intense enough for a data-dependent scan were analysed in MS^2 mode with the Orbitrap resolution also set at 15,000 at m/z 400. An isolation width of 100 amu was used and precursors were fragmented by a collision-induced dissociation C-trap (CID) with normalised collision energy of 35 V and an activation time of 10 ms. The mass range in FTMS mode was from m/z 100 to 1000. The data analysis was achieved using XCalibur software v2.0.7 (Thermo Fisher Scientific, Hemel Hempstead, UK).

Liquid chromatography analysis was performed using an Accela chromatograph (Thermo Scientific) equipped with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler. Chromatographic separation was accomplished with an Atlantis T3 column 2.1x 100 mm, 3µm (Waters, Milford, MA, USA). Gradient elution of analytes was carried out with water/0.1% formic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 350 µL/min, and the injection volume was 10 µL. A non-linear gradient was applied: 0 min, 2% B; 0–2 min, 2% B; 2–5 min, 8% B; 5–14 min, 20% B; 14–18 min, 30% B; 18–22 min, 100% B; 22–24 min, 100% B; 24-25, 2%B and the column was equilibrated for 5 min to initial conditions.

Samples were quantified using pure standards when available. Some analytes, such as glycosylated forms or dimers, were quantified using the aglycon form or the monomer.

3. RESULTS AND DISCUSSION

3.1. General

Persimmon leaves are interesting for their bioactive compounds that may exert beneficial effects on human health (Xie et al., 2015). Table 1 shows a list of the 41 phenolic compounds identified by LC-ESI-LTQ-Orbitrap-MS along with their retention times, accurate mass measurements, molecular formula, error in ppm of the experimental mass compared to the theoretical

mass of each polyphenol, the product ions used for identification and the concentration they are found in persimmon leaf in mg/kg. Phenolic compounds were identified by generating the molecular formula using accurate mass with some restrictions (C=30, H=100), O=15), and matching with the isotopic pattern. This molecular formula was then identified using the polyphenol database (http://phenol-explorer.eu/).

24 phenolic compounds were further confirmed by comparing the retention times, exact mass and fragmentation patterns with pure standards. Identification of the remaining 17 compounds without available standards was based on accurate mass measurements of the $[M-H]^-$ ion and the fragmentation pattern, which was compared with the literature, since the MS^2 spectra of many of these compounds have previously been reported in other studies (Quifer-Rada et al., 2015; Dou et al., 2007; Callemien et al., 2008; Jiang et al., 2015).

In total, 41 polyphenols were identified and quantified, including 9 benzoic acids, 5 hydroxycinnamic acids, 11 flavanols, 13 flavonols, 1 flavanones, 1 flavone and 1 tyrosol (Table 1).

Figure 1 shows an FTMS chromatogram of a persimmon leaf sample.

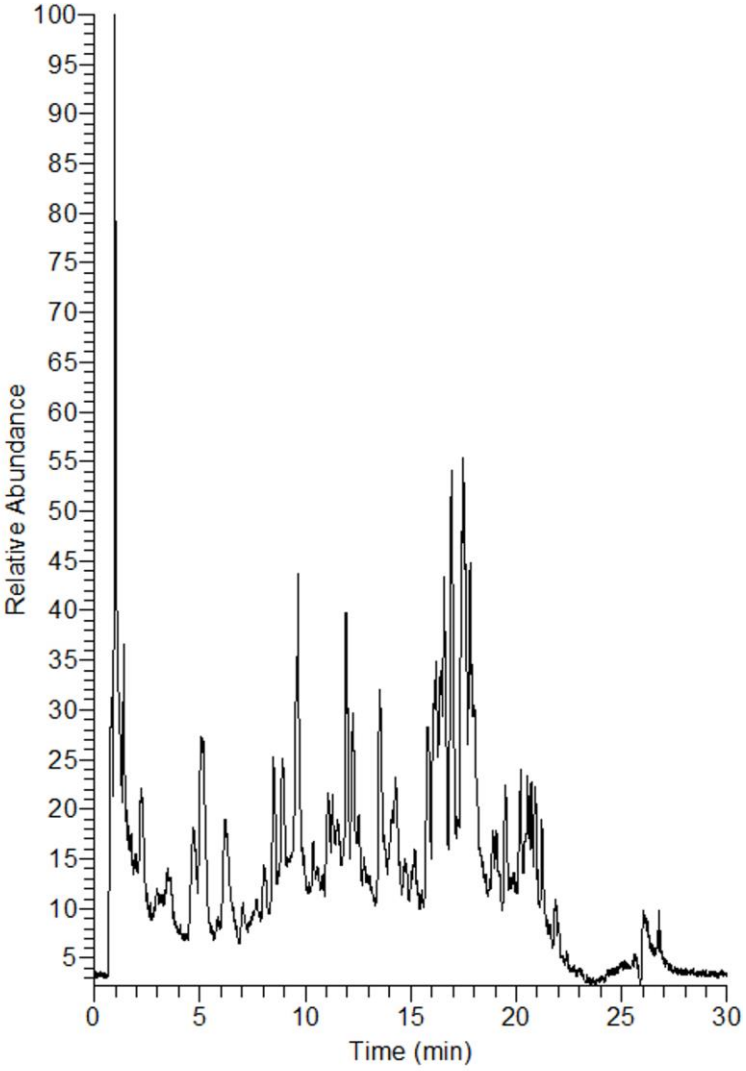

Fig. 1 – Persimmon leaf TMS chromatogram.

Table 1. Phenolic compounds tentatively identified in persimmon leaves

Compound	Rt (min)	Accurate mass [M-H]-	Fragments m/z (% intensities)	Error (ppm)	Molecular formula	Concentration[#] (mg/kg)
Benzoic acids						
Gallic acid *O*-hexoside	1.76	331.0663	169.0139 (100), 271.0452 (70), 211.0243 (20)	2.17	$C_{13}H_{16}O_{10}$	10.3(0.05)
Gallic acid*	2.31	169.0139	125.0242 (100)	1.01	$C_7H_6O_5$	32.5(1.82)
3,5-dihydroxybenzoic acid*	3.73	153.0191	108.9892 (100)	3.73	$C_7H_6O_4$	8.2(0.69)
2-Hydroxybenzoic acid*	7.29	137.0240	93.0343 (70)	2.82	$C_7H_6O_3$	4.6(0.12)
2,5-dihydroxybenzoic acid*	7.82	153.0191	109.0289 (100), 123.0445 (20)	1.71	$C_7H_6O_4$	0.1(0.03)
4-Hydroxybenzoic acid*	7.89	137.0240	93.0343 (60)	1.87	$C_7H_6O_3$	1.5(0.00)
3-Hydroxybenzoic acid*	9.63	137.0240	93.0343 (70)	4.74	$C_7H_6O_3$	4.7(0.05)
Vanillic acid*	18.27	167.0347	68.9954 (100), 123.0446 (70), 152.0110 (5)	1.6	$C_8H_8O_4$	0.04(0.003)
3,5-dimethoxybenzoic acid*	19.19	181.0502	137.0602 (100), 166.0265 (10)	0.36	$C_9H_{10}O_4$	2.8(0.19)
Hydroxycinnamic acids						
Chlorogenic acid*	10.07	353.0872	191.0552 (100)	1.77	$C_{16}H_{18}O_9$	0.6(0.02)
Coumaric acid-*O*-hexoside	10.14	325.0924	145.0292 (100), 163.0397 (80), 187.0396 (40), 265.0711 (20), 119.0500 (20), 235.0608 (10), 205.0501 (10)	0.3	$C_{15}H_{17}O_8$	0.9(0.01)
Caffeic acid*	10.42	179.0345	134.9875 (100)	0.2	$C_9H_8O_4$	0.8(0.05)
p-coumaric*	13.15	163.0397	119.0498 (100)	2.13	$C_9H_8O_3$	0.9(0.01)
Sinapic acid*	15.15	223.0607	208.0368 (100), 179.0705 (45), 164.0471 (20)	2.09	$C_{11}H_{12}O_5$	2.1(0.08)

*Polyphenols confirmed by standards; #Mean (SD)

Compound	Rt (min)	Accurate mass $[M-H]^-$	Fragments m/z (% intensities)	Error (ppm)	Molecular formula	Concentration[#] (mg/kg)
Flavanols						
Gallocatechin	5.1	305.0661	179.0346 (100), 221.0452 (75), 261.0764 (40), 125.0242 (40), 165.0190 (35), 137.0242 (25)	1.98	$C_{15}H_{14}O_7$	442.2(7.34)
Catechin-*O*-hexoside I	6.96	451.1244	289.0711 (100)	0.49	$C_{21}H_{24}O_{11}$	19.0(0.88)
Catechin-*O*-hexoside II	8.3	451.1243	289.0705 (100), 245.0809 (10)	0.58	$C_{21}H_{24}O_{11}$	3.2(0.04)
Procyanidin B1*	8.93	577.1351	425.0875 (100), 451.1030 (90), 289.0713 (60), 407.0767 (60), 299.0556 (30), 287.0557 (10)	0.12	$C_{30}H_{26}O_{12}$	203.7(1.62)
Epigallocatechin	9.08	305.0664	179.0346 (100), 221.0452 (75), 261.0764 (40), 125.0242 (40), 165.0190 (35), 137.0242 (25)	0.87	$C_{15}H_{14}O_7$	8.6(0.12)
Procyanidin dimer I	9.18	577.1352	425.0872 (100), 451.1027 (95), 407.0765 (50), 289.0711 (55), 559.1241 (30), 299.0554 (20), 467.0977 (10)	0.001	$C_{30}H_{26}O_{12}$	54.6(1.21)
Catechin-*O*-hexoside III	9.2	451.1243	289.0711 (100), 245.0814 (20)	0.56	$C_{21}H_{24}O_{11}$	4.1(0.05)
Catechin*	9.63	289.0712	245.0816 (100), 205.0503 (40), 179.0347 (20)	1.88	$C_{15}H_{14}O_6$	435.2(7.60)
Procyanidin dimer II	13.59	577.1349	425.0872 (100), 451.1027 (95), 407.0765 (70), 289.0711 (50), 559.1241 (30), 299.0554 (25), 467.0977 (10)	0.34	$C_{30}H_{26}O_{12}$	22.6(0.93)
Epicatechin-3-*O*-gallate*	15.36	441.0822	289.0705 (100), 169.0136 (30), 331.0447 (20), 271.0601 (15), 193.0135 (15)	1.42	$C_{22}H_{18}O_{10}$	2.6(0.10)

[*]Polyphenols confirmed by standards; [#]Mean (SD)

Compound	Rt (min)	Accurate mass $[M-H]^-$	Fragments m/z (% intensities)	Error (ppm)	Molecular formula	Concentration[#] (mg/kg)
Prodelphinidin dimer B3	20.18	609.1242	301.0350 (100), 463.0877 (50), 445.0773 (10)	1.44	$C_{30}H_{26}O_{14}$	24.4(0.32)
Flavanones						
Naringenin*	20.76	271.0604	151.0034 (100), 177.0189 (20), 107.0136 (5), 119.0500 (5)	2.75	$C_{15}H_{12}O_5$	1.2(0.8)
Flavones						
Apigenin*	20.82	269.0449	225.0546 (100), 149.0238 (50), 201.0548 (40), 251.1276 (25), 227.0346 (15)	2.32	$C_{15}H_{10}O_5$	1.0(0.73)
Flavonols						
Myricetin-*O*-hexoside I	14.07	479.0826	316.0221 (100), 317.0298 (90)	0.65	$C_{21}H_{20}O_{13}$	304.8(13.56)
Myricetin-*O*-hexoside II	14.29	479.0828	316.0221 (100), 317.0298 (90)	0.65	$C_{21}H_{20}O_{13}$	563.4(15.40)
Isoquercetin*	15.82	463.0877	301.0350 (100), 300.0274 (50)	1.46	$C_{21}H_{20}O_{12}$	247.4(2.65)
Quercetin-*O*-hexoside	16.11	463.0897	301.0351 (100)	1.7	$C_{21}H_{20}O_{12}$	348.8(10.96)
Quercetin-*O*-pentoside I	16.64	433.0770	301.0348 (100)	1.39	$C_{20}H_{18}O_{11}$	31.9(0.72)
Quercetin-*O*-pentoside II	16.85	433.0770	301.0348 (100)	1.39	$C_{20}H_{18}O_{11}$	52.0(1.11)
Kaempferol-3-*O*-glucoside*	16.97	447.0923	284.0324 (100), 285.0399 (80), 327.0505 (25), 255.0295 (10)	2.15	$C_{21}H_{20}O_{11}$	165.3(0.48)
Kaempferol-*O*-hexoside I	17.41	447.0930	285.0399 (100), 327.0506 (20)	0.68	$C_{21}H_{20}O_{11}$	176.8(5.80)
Myricetin*	18.09	317.0296	178.9978 (100), 151.0030 (50), 192.0056 (20)	2.3	$C_{15}H_{10}O_8$	44.7(6.51)
Kaempferol -*O*-rhamnoside	18.78	430.0905	285.0400 (100)	0.15	$C_{21}H_{19}O_{10}$	3.2(0.18)
Quercetin*	20.22	301.0347	178.9981 (80), 151.0033 (60), 273.0397 (10), 257.0448 (10)	2.17	$C_{15}H_{10}O_7$	354.7(41.5)

*Polyphenols confirmed by standards; #Mean (SD)

Compound	Rt (min)	Accurate mass $[M-H]^-$	Fragments m/z (% intensities)	Error (ppm)	Molecular formula	Concentration[#] (mg/kg)
Kaempferol*	20.9	285.0400	151.0030 (20), 229.0496(20), 257.0444 (20), 213.0548 (20), 185.0600 (20), 169.0651 (20)	1.65	$C_{15}H_{10}O_6$	206.2(13.98)
Isorhamnetin*	21.03	315.0505	300.0270 (100)	1.79	$C_{16}H_{12}O_7$	42.8(6.42)
Tyrosols						
Hydroxytyrosol*	5.78	153.0555	123.0445 (100)	1.74	$C_8H_{10}O_3$	1.5(0.08)

*Polyphenols confirmed by standards; #Mean (SD)

3.2. Phenolic acids

3.2.1. Benzoic acids and derivatives

Hydroxybenzoic acids have a C6–C1 chemical structure and show a characteristic loss of CO_2 [M-H-44]− in MS^2 experiments (Quifer-Rada et al., 2015; Kammerer et al., 2004; Schütz et al., 2005). The data-dependent scan and the examination of the chromatograms in FTMS mode revealed the presence of gallic acid (m/z 169.0139, 1.01 ppm), gallic acid 4-*O*-glucoside (m/z 331.0663, 2.17 ppm), 3,5-dihydroxybenzoic acid (m/z 153.0191, 1.77 ppm), 2-hydroxybenzoic acid (m/z 137.0240, 2.82 ppm), 2,5-dihydroxybenzoic acid (m/z 153.0191, 1.71 ppm), 4-hydroxybenzoic acid (m/z 137.0240, 1.87 ppm), 3-hydroxybenzoic acid (m/z 137.0240, 4.74 ppm), vanillic acid (m/z 167.0347, 1.6 ppm) and 3,5-dimethoxybenzoic acid (m/z 181.0502, 0.36 ppm). All ions showed a loss of 44 u in the MS^2 spectra. Moreover, 2,5-dihydroxybenzoic acid showed an extra fragmentation due to the oxygen loss from the carboxylic group [M-H-16]−; vanillic acid and 3,5-dimethoxybenzoic acid presented an extra [M-H-15]− loss due to the methyl group, and gallic acid *O*-glucoside had an [M-H-163]− loss due to the glucoside group. All benzoic acids, except for gallic acid 4-*O*-glucoside, were confirmed by comparing the retention time and the MS^2 spectrum with pure standards.

Gallic acid, 2-hydroxybenzoic acid and 4-hydroxybenzoic acid have previously been reported in leaves used for infusions such as anise, fennel and camomile (Proestos et al., 2006; Khalil et al., 2007). Moreover, gallic acid, *p*-hydroxybenzoic acid and vanillic acid were also found in persimmon fruit. Results show that persimmon leaves contain twice as much gallic acid as the persimmon fruit itself. In the case of vanillic acid, persimmon fruit has slightly higher levels than persimmon leaves. Finally, both contain almost the same quantity of *p*-hydroxybenzoic acid (Lee et al., 2012; Jiménez-Sánchez et al., 2015). To the best of our knowledge, this is the first time that benzoic acids have been identified in persimmon leaves.

3.2.2. Hydroxycinnamic acids and derivatives

Hydroxycinnamic acids have a C6-C3 structure with a double bond in the side chain in *cis* or *trans* configuration. A few hydroxycinnamic acids were identified after examination of the chromatograms: chlorogenic acid (m/z 353.0872, 1.77 ppm), coumaric-*O*-hexoside (m/z 325.0924, 0.3 ppm), caffeic acid (m/z 179.0345, 0.2 ppm), *p*-coumaric acid (m/z 163.0397, 2.13 ppm) and sinapic acid (m/z 223.0607, 2.09 ppm). The typical loss of CO_2 [M-H-44] was observed for all hydroxycinnamic acids except for the conjugated derivatives of chlorogenic acid and coumaric-*O*-hexoside, which showed a loss of the quinic acid m/z 191.0552 with a 0.3 ppm of error and the sugar moiety [M-H-162]-, respectively. Moreover, chlorogenic, *p*-coumaric and sinapic acids were confirmed by comparing the retention time and the MS^2 spectra with pure standards.

To the best of our knowledge, hydroxycinnamic acids have not been reported previously in persimmon leaves, however they have been previously found in persimmon fruit. Among them, we can find chlorogenic acid, caffeic acid, *p*-coumaric acid and ferulic acid, almost all in similar concentration as in persimmon leaves (Lee et al., 2012). Some hydroxycinnamic acids such as caffeic, ferulic, *o*-coumaric, *p*-coumaric and 5-caffeoylquinic acids have been found in other leaves used for infusions, such as Greek aromatic plants, and green and black tea (Proestos et al., 2006; Khalil et al., 2007; Stewart et al., 2005).

3.3. Flavonoids

Flavonoids are a large family of compounds with a common chemical structure: a diphenylpropane skeleton bearing two benzene rings (A and B) connected by a pyran ring attached to the A ring (Williams, Spencer, & Rice-Evans, 2004).

3.3.1. Flavanols and derivatives

Epigallocatechin (m/z 305.0664, 0.87 ppm), gallocatechin (m/z 305.0661, 1.98 ppm), catechin-*O*-hexoside I (451.1244, 0.49

ppm), catechin-*O*-hexoside II (m/z 451.1243, 0.56 ppm), catechin-*O*-hexoside III (m/z 451.1243, 0.58 ppm), procyanidin B1 (m/z 577.1351, 0.12 ppm), procyanidin dimer I (m/z 577.1349, 0.34 ppm), procyanidin dimer II (m/z 577.1352, 0.001 ppm), catechin (m/z 289.0712, 1.88 ppm), epicatechin 3-*O*-gallate (m/z 441.0822, 1.42 ppm) and prodelphinidin dimer B3 (m/z 609.1242, 1.44 ppm) were identified in persimmon leaves by analysing the chromatograms in FTMS. Product ions with the fragmentation pattern of epigallocatechin, gallocatechin and prodelphinidin dimer B3 have been described previously (Dou et al., 2007 & Callemien et al., 2008). Derivatives of catechin (catechin-O-hexoside I, II and III) showed the loss of the sugar moiety in MS^2 spectra. Procyanidin B1, catechin and epicatechin 3-*O*-gallate were confirmed with pure standards. Figure 2 shows the MS^2 spectra of procyanidin B1.

Many of these flavanols have been reported previously in other plant leaves used to prepare infusions such as green and black tea (Dou et al., 2007; Wang et al., 2008), *Byrsonima* species (Rinaldo et al., 2010), and *Styphnolobium* japonicum (Kite et al., 2007). Epigallocatechin, catechin and epicatechin gallate were also found in persimmon fruit which coincide with the flavanols found in leaves. The amount of catechin in persimmon leaves is 20 times higher than in persimmon fruit. Nevertheless, the quantity of epigallocatechin and epicatechin gallate is lower in persimmon leaves (Jiménez-Sánchez et al., 2015).

3.3.2. Flavonols and derivatives

Among the flavonols identified, quercetin, kaempferol, and myricetin are present in high concentrations in a variety of plant-based foods and beverages (Jiang et al., 2015).

Myricetin-*O*-hexoside I (m/z 479.0826, 0.65 ppm), myricetin-*O*-hexoside II (m/z 479.0828, 0.65 ppm), isoquercetin (m/z 463.0877, 1.46 ppm), quercetin-*O*-hexoside (m/z 463.0897, 1.7 ppm), quercetin-*O*-pentoside I (m/z 433.077, 1.39 ppm), quercetin-*O*-pentoside II (m/z 433.077, 1.39 ppm), kaempferol-

3-*O*-glucoside (m/z 447.0923, 2.15 ppm), kaempferol-*O*-hexoside II (m/z 447.093, 0.68 ppm), myricetin (m/z 317.0296, 2.3 ppm), kaempferol-*O*-rhamnoside (m/z 430.0905, 0.15 ppm), quercetin (m/z 301.0347, 2.17 ppm), kaempferol (m/z 285.040, 1.65 ppm) and isorhamnetin (m/z 315.0505, 1.79 ppm) were identified by analysing the chromatograms in FTMS.

Isoquercetin, kaempferol-3-*O*-glucoside, myricetin, quercetin, kaempferol and isorhamnetin were further confirmed by comparing the chromatograms with pure standards. Myricetin-*O*-hexosides, quercetin-*O*-hexoside, and kaempferol-*O*-hexoside showed the typical loss of 162 u due to the loss of the sugar moiety.

Quercetin-*O*-pentoside showed the loss of the pentoside moiety (132 u), and kaempferol-*O*-rhamnoside presented a loss of 146 u due to the loss of the rhamnoside group.

Flavonols were the main flavonoids in persimmon leaves, as reported previously. Kaempferol, quercetin, isoquercetin and myricitrin have been described (Chou, 1984, Nakatani et al., 1989, Guo and Dong, 1999). Conjugated flavonols such as quercetin-3-*O*-β-D-glucopyranoside, quercetin-3-*O*-β-D-galactopyranoside, quercetin-3-*O*-β-L-arabinopyranoside, kaempferol-3-O-α-L-rhamnopyranoside, kaempfetol-3-O-β-D-galactopyranoside, kaempferol-3-β-D-xylopyranoside and kaempefrol-3-*O*–L-arabinopyranoside have also been reported (Cai & Yang, 2001).

In this study, two quercetin-*O*-hexosides were found, one being isoquercetin and the other isomer possibly corresponding to the quercetin-3-*O*-β-D-galactopyranoside reported by Cai and Yang (2001). Two quercetin-*O*-pentosides were also identified, one of them tentatively as quercetin-3-*O*-β-L-arabinopyranoside, which was previously described by Cai and Yang (2001). Two kaempferol-*O*-hexosides were identified: kaempferol-3-O-glucoside and another hexoside isomer that may be kaempferol-3-*O*-β-D-galactopyranoside, reported by Cai and

Yang (2001). Kaempferol-*O*-rhamnoside was also found, in agreement with the Cai and Yang study (2001), where kaempferol-3-O-α-L-rhamnopyranoside is described in persimmon leaf samples.

However, to our knowledge, kaempferol-3-*O*-glucoside, isorhamnetin and myricetin-*O*-hexoside are reported for the first time in persimmon leaves. These compounds have been reported in other herbs such as *Drosera peltata* (Braunberger et al., 2013) and *Carduus acanthoides* (a traditional Tibetan herbal medicine) (Li et al., 2014), as well as extracts of apples (Schieber et al., 2002) and citrus species (Brito et al., 2014). In persimmon fruit there were reported others flavonols: quercetin-*O*-hexoside-gallate, quercetin-3-*O*-glucoside, quercetin acetyl hexoside, kaempferol-*O*-hexoside-gallate, kaempferol-3-*O*-glucoside and kaempferol acetyl hexoside (Jiménez-Sánchez et al., 2015).

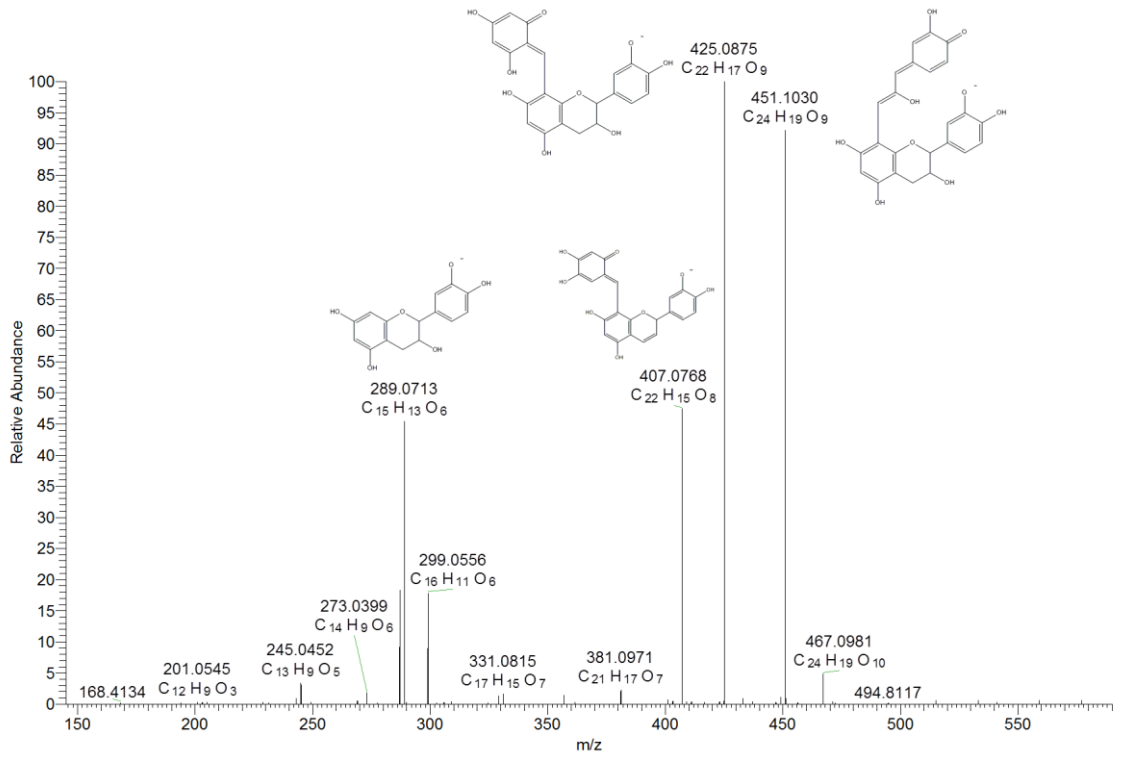

Fig. 2 – MS^2 spectra of procyanidin B1.

3.3.3. Flavanones and derivatives

Flavanones are usually glycosylated with a disaccharide at the C7 position, forming flavanonglycosides. The LTQ-Orbitrap analysis confirmed the presence of naringenin by giving the characteristic ion of the deprotonated molecule [M– H]- (m/z 271.0604, 2.75 ppm) and the ions corresponding to Retro Diels Alder fragmentation in the C-ring involving 1,3-scission (m/z 151.0034). Naringenin was further identified and confirmed by comparing its retention time with the pure standard.

3.3.4. Flavones

Flavones are a class of flavonoids based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one). The examination of the chromatograms in FTMS mode and dependent scan led to the identification of apigenin (m/z 269.0449, 2.32 ppm), which was confirmed by comparing its retention time with the pure standard.

Apigenin and apigenin derivatives such as apigenin 7-*O*-apiosyl-glucoside and apigenin 7-*O*-(6''-malonyl-apiosyl-glucoside) were also identified in the persimmon leaf samples. These compounds have also been reported in leaves of celery and oregano (Lin et al., 2007; Zheng et al., 2001).

3.4. Tyrosols

Another group of compounds found in persimmon leaves were characterised as phenylethanol-related compounds. Among them, hydroxytyrosol (m/z 153.0555, 1.74 ppm), characteristic of virgin olive oil, was identified by comparing the MS^2 spectrum with literature data (Michel et al., 2015) and with the pure commercial standard.

4. CONCLUSIONS

In conclusion, high-resolution mass spectrometry provided a powerful tool for the identification of polyphenol diversity in

persimmon leaves, even in the absence of standards. We were able to identify and quantify 41 phenolic compounds, most of them, as far as we know, for the first time. The majority of these polyphenols were hydroxybenzoic acids, hydroxycinnamic acids, flavanols and flavonols, and their respective derivatives, as well as flavanones, and hydroxytyrosol. Our results show that persimmon leaves have a complex phenolic profile that may help to explain the beneficial effects of their traditional use as a medicinal herb.

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III.4. INFLUENCE OF DRYING AND PARTICLE SIZE OF PERSIMMON FIBRE ON THEIR PHYSICOCHEMICAL, ANTIOXIDANT, HYDRATION AND EMULSIFYING PROPERTIES

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El consumo de fibra está asociado con la prevención y tratamiento de ciertas enfermedades entre las que se puede citar el estreñimiento (mejora del tránsito intestinal), cáncer de colon, enfermedades coronarias (disminución del colesterol) y diabetes (Abdul-Hamid & Luan, 2000). Se cree que no solo la fibra como tal sino también los compuestos bioactivos (antioxidantes) que pueden quedar retenidos en la misma tras su extracción y estabilización serían corresponsables de los beneficios para la salud humana asociados al consumo de la misma. En este sentido, cada vez es más común la incorporación de fibras a la formulación de alimentos con el fin de asegurar la ingesta mínima diaria recomendada, siendo esta de 35g/día (Storey & Anderson, 2014). En concreto, un estudio llevado a cabo en ratas evidenció que el consumo de caqui ayudaba a limitar la absorción de grasa debido probablemente al elevado contenido en fibra de este fruto (Gorinstein et al., 2000).

Las principales fuentes de fibra en la actualidad son las frutas y cereales. De ahí que se pueden encontrar en el mercado fibras de manzana, guisantes, remolacha azucarera, soja, naranja o limón. Además de las consecuencias para la salud humana derivadas del consumo de fibra, la adición a los alimentos también puede presentar ventajas tecnológicas tales como una mejora de la viscosidad, textura, de las características sensoriales, así como de aumento de la vida útil del producto. Actualmente muchos alimentos contienen fibra como

ingrediente en bollería, productos cárnicos cocidos, cereales, bebidas, pasta y sopas (Thebaudin et al., 1997).

En este contexto, el objetivo del presente trabajo fue analizar la influencia de la técnica de secado (aire caliente y liofilización) sobre la estabilización de fibra extraída de la piel y del bagazo de caqui, y sobre las propiedades físico-químicas, antioxidantes, hidratantes y emulsionantes de las diferentes fracciones de fibra obtenidas. Adicionalmente, dichas propiedades se compararon con las de cuatro fibras comerciales habitualmente utilizadas como ingrediente en la formulación de alimentos.

La caracterización físico-química de la fibra de caqui se llevó a cabo mediante la determinación de los contenidos de humedad, proteína y grasa, coordenadas de color del espacio CIE*L*a*b*, actividad de agua y volumen específico. Se utilizaron métodos espectrofotométricos para la determinación del contenido de fenoles totales (método Folin-Ciocalteu) (Sakanaka et al., 2005) y la capacidad antioxidante (método DPPH) (Shahidi et al., 2006). Además, se determinaron las propiedades de hidratación (capacidad de hinchamiento, capacidad de absorción de agua, capacidad de retención de agua y capacidad de retención de grasa) así como las propiedades emulsificantes (estabilidad de la emulsión y actividad emulsificante).

Los resultados evidenciaron que el método de secado y el tamaño de partícula son variables que afectan a las propiedades físico-químicas de las fibras procedentes del bagazo y de la piel. Las fibras secadas por aire caliente presentaron una menor luminosidad, un tono más anaranjado-parduzco así como menor volumen específico que las fibras liofilizadas.

El tipo de secado afecta a la capacidad de hinchamiento (SC), ya que las fibras liofilizadas presentaron valores superiores que las secadas por aire caliente y en concreto la fibra de bagazo

liofilizada presentó la mayor capacidad de hinchamiento. Se determinaron también la capacidad de absorción (WHC) y de retención de agua (WRC). En general, ambas propiedades se ven afectadas por el método de secado y en concreto, el secado por aire caliente disminuyó notablemente la WHC y la WRC de la fibra de bagazo, mientras que no influyó en la fibra de piel. En cuanto al tamaño de partícula, si que se observan diferencias sobre todo en la WRC, parámetro que aumenta conforme disminuye el tamaño de partícula.

Además de las propiedades de hidratación, las fibras poseen la capacidad de atrapar grasa, por lo que la capacidad de retención de grasa (ORC) es otro de los parámetros esenciales en la caracterización de este tipo de productos. Los resultados muestran que el método de secado afecta a esta propiedad siendo las fibras liofilizadas las que presentaron valores superiores. En cuanto a la influencia del tamaño de partícula sobre esta propiedad, si se observan diferencias en las fibras procedentes de la piel siendo la fibra de piel de caqui liofilizada y con un tamaño de partícula inferior a 125 μm la que presentó la mayor ORC. Por último, las emulsiones formadas con fibra de piel resultaron ser más estables que las que forman las fibras de bagazo y para ambas fracciones se consigue mayor estabilidad con las fibras liofilizadas.

Por otro lado, a pesar de que la piel es más rica en compuestos antioxidantes (especialmente carotenoides), la fibra de piel secada por aire caliente presentó propiedades antioxidantes similares a las de la fibra de bagazo. Adicionalmente, el método de secado únicamente influyó de forma significativa en la actividad antioxidante y en el contenido en fenoles totales de la fibra de piel, presentado mayor actividad antioxidante que la fibra de bagazo cuando se seca por liofilización, pero sus propiedades antioxidantes son menores si se seca por aire caliente. También se deduce de los resultados obtenidos, que tanto la actividad antioxidante como el contenido en fenoles totales de ambas fracciones (piel y bagazo), tienen una

dependencia estadísticamente significativa del tamaño de partícula presentando mayores propiedades antioxidantes las fracciones de menor tamaño.

Cuando comparamos las fracciones de fibra de caqui de bagazo y piel con las fibras comerciales, se observa que presentan propiedades de hidratación, emulsificación y antioxidantes, similares y en algunos casos superiores a las propiedades analizadas en distintas fibras comerciales procedentes de otras frutas.

En conclusión, la extracción de la fibra procedente de bagazo y piel de caqui debería analizarse como una opción para el aprovechamiento y valorización de los residuos de la industria o incluso para la gestión de los excedentes de producción.

III.4. INFLUENCE OF DRYING AND PARTICLE SIZE OF PERSIMMON FIBRE ON THEIR PHYSICOCHEMICAL, ANTIOXIDANT, HYDRATION AND EMULSIFYING PROPERTIES

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ABSTRACT

Given the current surplus production of persimmon, it may be an alternative source for extracting ingredients such as fibre, which are of interest for the food industry and human health. Thus, the aim of this study was to analyse the influence of hot air and freeze-drying, and the particle size of fibre extracted from persimmon peels or pulp on their physicochemical, antioxidant, hydration and emulsifying properties, as compared to commercial fibres (from peach, lemon, orange and apple). The results showed that both freeze-dried persimmon pulp followed by freeze-dried peel had better hydration properties and oil holding capacity than other fibres analysed, although the swelling capacity, was higher for lemon fibre. Freeze-dried persimmon peel fibre showed higher values of emulsion stability than commercial fibres. Finally, the antioxidant activity of the smallest sized persimmon peel fibre obtained by freeze-drying was higher than for fibres of lemon, orange and peach.

Keywords: persimmon fibre; antioxidant activity; hydration properties; freeze-drying; hot air drying

1. INTRODUCTION

Persimmon crops are traditional in the Valencian Community (Spain), although persimmons come from China. This fruit tree belongs to the botanical family Ebanaceae, genus Diospyros,

which has more than 300 species. However, only five species are of commercial importance, among which includes the genus Diospyros kaki L.f. cultivated around the world, and specifically in Spain (Hernándiz 1999).

From a commercial standpoint, Persimmon are divided into astringent (*Rojo Brillante, Triumph, Tomatero*, etc.) and non-astringent (*Fuyu, Hana-Fuyu, Jiro,* etc.) varieties. The fruits' astringency is linked to the content and solubility of tannins. In non-astringent varieties, tannins are insolubilized, allowing for consumption without carrying out any post-harvest treatment and without reaching physiological ripening. Astringent varieties have a high content of soluble tannins, which decreases as ripening is reached (Hernándiz, 1999). "*Rojo Brillante*" is the most important variety both in terms of production and commercially, and it is the only variety recognized by the Denomination of Origin Kaki Ribera del Xúquer. According to data from the Ministry of Agriculture of Spain (2013) there has been a remarkable increase in the land dedicated to persimmon, which has grown from 2,281 hectares to nearly ten thousand. In the last year, there was a 20% increase, from 7,995 to 9,580 hectares, a 20%, according to official statistics (INE, 2014). At the beginning of 2000, when consumption began to expand, production of this fruit did not exceed 20,000 tons. Today, production reaches 150,000 tons and in the next two years, it is expected to reach 500,000 tons (Alós, 2014).

Fibre intake is associated with the prevention and treatment of some diseases related to oxidative stress (Rajendran et al., 2014) and others such as: constipation (improvement of intestinal transit), colon cancer, coronary heart disease (lowering of cholesterol) and diabetes (Abdul-Hamid & Luan, 2000). It is believed that not only the fibre but also bioactive compounds (antioxidants) may be retained after extraction and stabilization of the fibre, would also be partially responsible for the health benefits associated with the consumption of fibre. In this sense it is increasingly common to incorporate fibre in

formulations in order to guarantee the minimum recommended daily intake, which is 35 g/day (Lairon, 1990; Storey & Anderson, 2014). More specifically, and bearing in mind the bioactive compounds of the persimmon fruit, a study conducted on rats showed that the consumption of persimmon helped to limit fat absorption, probably due to the high fibre content of the fruit (Gorinstein et al., 2000). Additionally, studies have recently been carried out to stabilize fat food products by using dietary fibres with antioxidant properties to improve their stability to oxidation and extend their shelf life (Zha et al., 2009)

Nowadays, the main sources of fibre are fruits and cereals. Hence fibres from apple, pea, sugar beet, soybean, orange or lemon can be found in the market, but not fibres from persimmon. In addition to the health benefits associated with human consumption of fibre, its addition to food also leads to technological advantages such as improvements in viscosity, texture, sensory characteristics, as well as an increase in shelf life. Currently, fibre is included as an ingredient in many kinds of foods such as cakes, cooked meat products, cereals, beverages, pasta and soups (Thebaudin et al., 1997).

The aim of this study was to analyse the influence of the drying method (hot air and freeze-drying) on the stabilization of the fibre extracted from the persimmon peel and pulp, and to assess the influence of the particle size of the fibre on the physico-chemical, antioxidant, moisturizing and emulsifying properties of the different fractions obtained. These properties were compared with those of four commercial fibres (orange, lemon, peach and apple) to determine the possible uses of this persimmon fibre as an ingredient in food formulation.

2. MATERIALS AND METHODS

2.1. Raw material

Persimmon (Diospyros kaki Thumb. cv. *Rojo Brillante*) fruit were harvested in Alginet (Spain) and then treated to remove

astringency in closed containers with 95% CO2 for 24 h at 20ºC and 90% of relative humidity (Arnal & Del Río, 2003).

The fresh persimmon fruits were cleaned and peeled. Pulp and peel were homogenized separately by using a stirrer (IKA T 18 basic ULTRA-TURRAX®). Each sample was put into contact with boiling ethanol (96% v/v) and stirred (600 rpm) for 15 min in a ratio 1:2 (w/v). Finally, ethanol was separated from fibre by means of a sieve and the resulting solid fraction was divided in two even parts. One of them was dried at 40 ºC till constant weight (approximately 7 h), obtaining the products denominated PULP-A (obtained from persimmon pulp) and PEEL-A (obtained from persimmon peel). The other one was frozen at -40ºC for 24 h and then it was freeze-dried (vacuum pressure of 10-1 mbar for 24 hours). In this case, the products were named PULP-F and PEEL-F when they came from persimmon pulp and peel, respectively. Then, each fraction was separated into three sub-fractions based on the particle size using three sieves with different apertures (125 µm, 250 µm and 500 µm). As a result, persimmon fibre was divided into three particle size ranges comprising between 500 to 250 µm, 250 to 125 µm and <125 µm. Samples were stored at -40 ºC until analysis.

Furthermore, four commercial fibres (lemon, orange, peach and apple) (Indulleida, particle size <125 µm) were characterized in order to compare the properties of these fibres with the persimmon fibres obtained.

Following is a description of the analytical determinations made, each of which was carried out in triplicate for all the fibres studied.

2.2. Physical and chemical characterization of fibre powders

Moisture content (g water/100g sample) was determined by drying the fibre to constant weight at 60 ºC in a vacuum oven at 10 kPa (adaptation of method 934.06 AOAC, 2000).

Protein (g protein/100g sample) was analysed using the Kjeldahl method (AOAC, Method 920.152, 1990). Factor 6.25 was used for conversion of nitrogen to crude protein.

Fat (g fat/100g sample) was calculated by weight loss after a six-cycle extraction with petroleum ether (40-60ºC boiling range) in a Soxhlet extractor.

The colour of fibres was measured using a Minolta spectrocolorimeter (Minolta CM-3600 d, Tokyo, Japan). CIEL*a*b* coordinates were obtained using D65 illuminant and 10º observer as the reference system.

Water activity (aw) was determined at 25ºC with a hygrometer (Aqualab, USA).

Specific volume, defined as the inverse of apparent density, was determined by measuring the volume occupied by a sample (5 g) using a 10 mL-graduated and calibrated cylinder. The bottom of the cylinder was gently tapped on a laboratory bench several times until there was no further decrease of the sample level (Chau et al., 2007).

2.3. Hydration properties

2.3.1. Swelling capacity (SC)

A sample (≈0.2 g) of persimmon fibre was weighed and placed in a graduated conical tube and 10 mL of water was added. It was hydrated for 18 h at 25ºC. After this time, the final volume attained by the fibre powder was measured (Raghavendra et al., 2004; Robertson et al., 2000) and SC was calculated as:

$$SC\ \left[\frac{mL}{g}\right] = \frac{\text{volume occupied by sample}}{\text{original sample weight}} \qquad \text{(Equation I)}$$

2.3.2. Water holding capacity (WHC)

A sample (≈0.2 g) of persimmon fibre was weighed and placed in a graduated conical tube and 10 mL of water was added. It

was hydrated for 18 h at 25ºC. The supernatant was removed and the decanted residue was weighed. The weight of the hydrated residue was recorded (HR). After freeze-drying, the weight of the dried residue was also recorded (DR). This assay was performed in triplicate and WHC was calculated as:

$$WHC\ [g/g] = \frac{HR-DR}{DR} \qquad \text{(Equation II)}$$

2.3.3. Water retention capacity (WRC)

A sample (≈1 g) of persimmon fibre was weighed and placed in a graduated conical tube and 10 mL of water was added. It was hydrated for 18 h at 25ºC. Centrifugation for 30 min at 2000 rpm was then performed in the same tube. The supernatant was separated and the residue was weighed. The remaining wet fibre was weighed (R + W2), as well as the freeze-dried residue (R) (Raghavendra et al., 2004; de Escalada Pla et al., 2012) and WRC was calculated as:

$$WRC\ [g\ water\ /g\ dried\ residue] = \frac{W_2}{R} \qquad \text{(Equation III)}$$

where R is the dried residue and W2 is the retained water.

2.4. Oil holding capacity (OHC)

OHC was measured according to Garau et al., (2007). Samples (≈0.2 g) were mixed with sunflower oil (≈1.5 g), left overnight at room temperature and then centrifuged (1500xg; 5 min). The supernatant was decanted and the sample was weighed. OHC was evaluated based on the increase in weight and expressed as g of oil absorbed/g dry sample.

2.5. Emulsifying properties

2.5.1. Emulsifying activity (EA)

Emulsifying activity was measured using the method of Yasumatsu et al. (1972). 7 mL of 2% aqueous dispersion of the fibre (w/v) was mixed with 7 mL of sunflower oil and stirred for 5

min at high speed (Vortex, Heidolph). An aliquot was then centrifuged at 10000 rpm for 5 min and the emulsion volume formed was then measured using the following equation:

$$\%EA = \frac{VEL}{V} \cdot 100 \qquad \text{(Equation IV)},$$

where VEL refers to the volume of the emulsified layer (mL) and V is the total volume of fluid (mL).

2.5.2. Emulsion stability (ES)

The emulsion stability was determined using the method of Yasumatsu et al., (1972) adapted as follows: 7 mL of 2% aqueous dispersion of the fibre (w/v) was mixed with 7 mL of sunflower oil and stirred for 5 min at high speed (Vortex, Heidolph). The emulsions were heated to 80 ° C for 30 minutes, cooled for 15 minutes in running water and centrifuged at 2000 rpm for 5 minutes. The stability of the emulsion was calculated using the equation:

$$\%ES = \frac{VREL}{V} \cdot 100 \qquad \text{(Equation V)},$$

where VREL refers to the volume of the remaining emulsion layer (mL) and V is the total volume of fluid (mL).

2.6. Total Phenolic Content and Antioxidant Activity

Samples were analysed spectrophotometrically, using a modified Folin-Ciocalteu method (Sakanaka et al., 2005), in order to determine the total phenolic content (TPC). The TPC were extracted with methanol (3 grams of crushed fruit/5 mL of methanol) and then kept stirring at 200 rpm for one hour (horizontal shaker COMECTA WY-100). The test tubes were centrifuged for 10 minutes at 10000 rpm (Medifriger BL-S, P-Selecta). 0.5 mL of distilled water and 0.125 mL of the supernatant of the extract were added to a cuvette followed by the addition of 0.125 mL of Folin-Ciocalteu reagent. The mixture was shaken and 1.25 mL of a 7% sodium carbonate solution

and 1 mL of distilled water were added after 6 min. The colour was left to develop for 90 min and the absorbance was measured at 760 nm using a spectrophotometer (JASCO V-630). The measurement was compared to a standard curve of gallic acid solutions and expressed as mg of gallic acid equivalents per gram (mg GA/g dry matter). A blank was prepared in the same way but without any sample.

The antioxidant activity (AA) of the persimmon fruit was measured on the basis of the scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl free radical as described by Shahidi et al. (2006) with some modifications. According to this method, the purple colour intensity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution decays in the presence of an antioxidant, and this absorbance change is measured spectrophotometrically at 515 nm.

3 grams of the crushed sample were diluted in 5 mL of methanol and were subjected to stirring for 5 minutes. Then, the test tubes with the sample-methanol mixture were centrifuged at 10000 rpm for 10 minutes (Medifriger BL-S, P-Selecta). 0.1 mL of the supernatant was added to 3.9 mL of a methanolic solution of DPPH (80:20; methanol:water) (0.025 mg/mL). The solution was shaken and after 30 min the absorbance of the sample was measured at 515 nm using methanol as a blank. The antioxidant activity (%) of the samples was calculated using the equation I:

$$AA\ (\%) = \frac{A_{t=0} - A_{t=30}}{A_{t=0}} \cdot 100 \qquad \text{(Equation VI)}$$

where At=0 is the initial absorbance of the DPPH (without sample) and At=30 is the absorbance of the sample after 30 min. The measurement was compared to a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solutions and expressed as mg of Trolox per gram.

2.7. Statistical analysis

All experiments were carried out in triplicate. Results are expressed as mean values ± standard deviation. Data were subjected to analyses of variance (ANOVA), and multiple comparisons between means were determined using the LSD test ($P \leq 0.05$) by means of the Statgraphics Plus 5.1 software application (Manugistics, Inc., Rockville, MD, USA).

3. RESULTS AND DISCUSSION

3.1. Influence of drying method and particle size on the optical and physicochemical properties of pulp and peel fibre from persimmon

The organoleptic characteristics of a food system may be affected after the incorporation of different ingredients such as fibre. In fact, their addition typically leads to an undesirable change in the colour and texture of the products (de Escalada Pla et al., 2012), which represents a challenge for the food industry. In this sense, analysing the colorimetric properties of new ingredients, such as persimmon fibre, is essential.

In Figure 1, the chromatic planes L*-a* and b*-a*coordinates of the different persimmon fibres studied (freeze-dried and hot air dried fibres, classified by particle size) along with the results for the commercial fibres are presented. The hot air dried fibres showed lower lightness (L*) than those stabilized by freeze-drying, except for the hot air dried pulp fibre with a particle size lower than 125 μm (PULP-A <125). Freeze-dried fibres also showed similar L* values to commercial analysed fibres. Statistical analysis (ANOVA) showed the existence of significant differences in this parameter depending on the drying method and particle size in all cases except for the pulp fibre with a particle size between 500-250 and 250-125μm. Regarding to the a* and b* coordinates, the hot air drying resulted in more brownish-orange fibres than freeze-dried fibres, which presented tones which were more orange with a tendency

towards yellow shades. This is a consequence of an increase in the a* coordinate as a result of Maillard reactions occurring during the hot air drying (Perez-Jimenez et al., 2014). However, the hot air dried fibres resulted in tonalities which were more orange with higher chroma than freeze-drying. This was more significant in the case of persimmon pulp fibre. As for the particle size, and regardless of the origin of the fibre or technique applied for stabilization, the results showed a gradual increase in the a* coordinate as the particle size increased. This effect was very clear in the case of hot air dried pulp and freeze-dried peel fibres. In general, the b* parameter was instead unaffected by particle size.

Table 1 shows the physicochemical characteristics of pulp and peel fibres from persimmon dried by freeze-drying (F) or hot air drying (A) and the values of these properties obtained for the commercial fibres. The difference in the specific volume suggests differences in the capillary structure: the more porous the system, the higher water content it can absorb, assuming the chemical composition is constant.

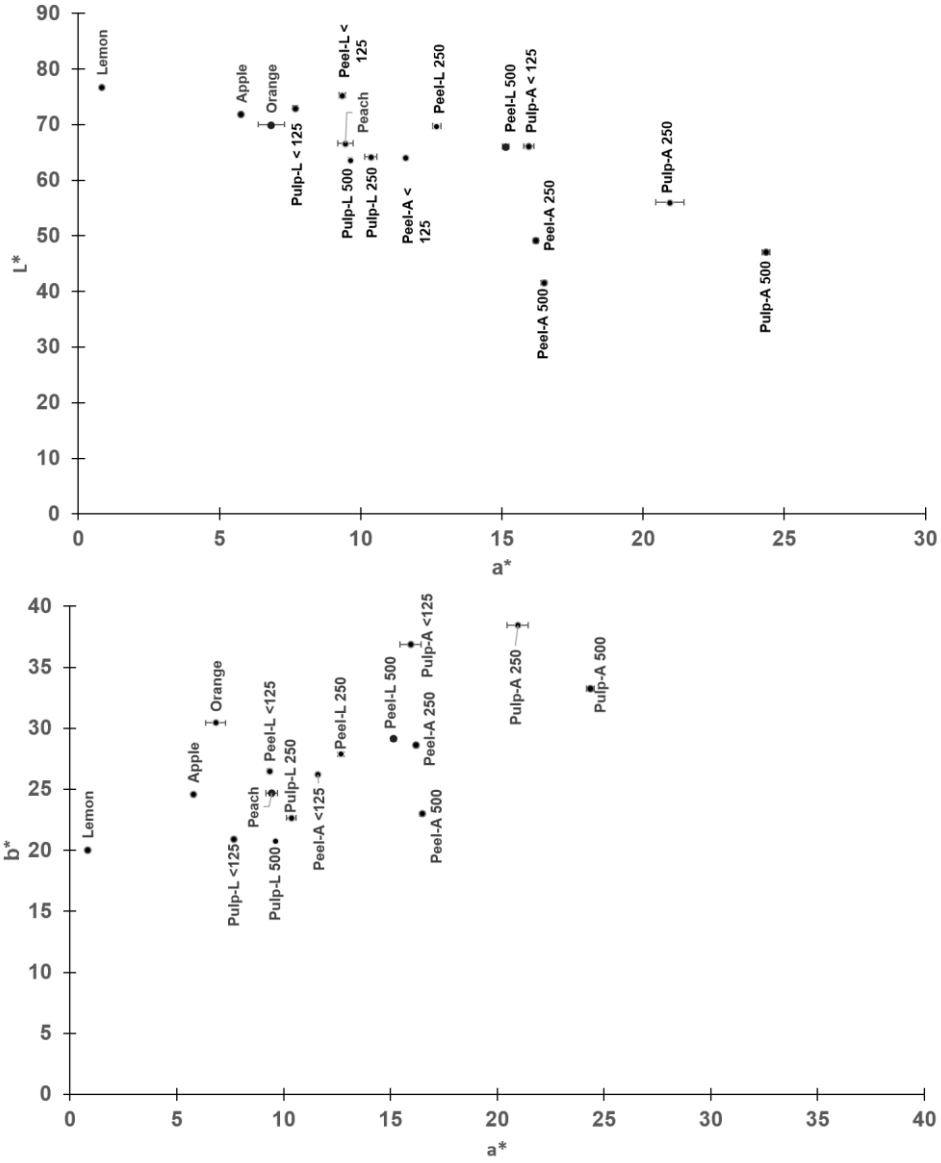

Fig. 1 – L*-a* and b*-a* colour planes of commercial fibres (lemon, orange, peach and apple) and persimmon fibres (from pulp or peel) obtained by different techniques (hot air drying: A or freeze-drying: F) and particle size (500: 500-250 μm; 250: 250-125 μm and <125 μm). Three replicates were carried out.

Table 1. Physicochemical characteristics of persimmon pulp and skin fibres obtained by freeze-drying (F) or hot air drying (A) and different commercial fibres (lemon, orange, peach and apple) on the chemical composition is constant.

	Lemon fibre	Orange fibre	Peach fibre	Apple fibre	Persimmon pulp fibre		Persimmon peel fibre	
					F	A	F	A
Specific volume*	1.84 ± 0.04$^{(a)}$	1.53 ± 0.02$^{(a)}$	1.52 ± 0.01$^{(a)}$	1.598 ± 0.008$^{(a)}$	2.8 ± 0.4$^{(b)}$	1.60 ± 0.01$^{(a)}$	2.9 ± 0.4$^{(b)}$	1.6 ± 0.2$^{(a)}$
Apparent density**	0.54 ± 0.01$^{(b)}$	0.652 ± 0.008$^{(c)}$	0.656 ± 0.004$^{(c)}$	0.626 ± 0.003$^{(c)}$	0.37 ± 0.05$^{(a)}$	0.624 ± 0.007$^{(c)}$	0.35 ± 0.04$^{(a)}$	0.64 ± 0.07$^{(c)}$
Water activity	0.46 ± 0.01$^{(e)}$	0.35 ± 0.01$^{(b)}$	0.29 ± 0.02$^{(a)}$	0.37 ± 0.008$^{(b)}$	0.47 ± 0.02$^{(e)}$	0.42 ± 0.03$^{(cd)}$	0.44 ± 0.04$^{(de)}$	0.41 ± 0.01$^{(c)}$
Moisture***	3.5 ± 0.1$^{(b)}$	2.6 ± 0.1$^{(b)}$	1.0 ± 0.1$^{(a)}$	3.70 ± 0.07$^{(b)}$	7.8 ± 0.3$^{(d)}$	6 ± 1 $^{(c)}$	8 ± 1 $^{(d)}$	6.9 ± 0.5 $^{(c)}$
Protein***	≥7%	≥7%	≥4%	≥4%	≥4%	≥4%	≥4%	≥4%
Fat***	<2%	<2.5%	<3%	<3%	<2%	<2%	<2%	<2%

***(cm^3/g); **(g/cm^3); *** (g/100g)**

Table 2. Hydration properties, antioxidant activity and total phenols content of commercial fibres

	Lemon fibre	Orange fibre	Peach fibre	Apple fibre
Swelling capacity (SC)	12.1 ± 0.8$^{(d)}$	7.6 ± 0.2$^{(a)}$	10.5 ± 0.3$^{(c)}$	8.9 ± 0.7$^{(b)}$
Water holding capacity (WHC)	14.4 ± 0.4$^{(b)}$	9.9 ± 0.2$^{(a)}$	14 ± 1$^{(b)}$	15.4 ± 0.7$^{(c)}$
Water Retention Capacity (WRC)	12.0 ± 0.4$^{(bc)}$	9.3 ± 0.3$^{(a)}$	11.0 ± 0.5$^{(ab)}$	12.9 ± 0.8$^{(c)}$
Oil holding capacity (OHC)	2.9 ± 0.3$^{(b)}$	2.67 ± 0.08$^{(b)}$	2.7 ± 0.2$^{(b)}$	2.486 ± 0.009$^{(ab)}$
Emulsifying activity (AE)	11.9 ± 0.4$^{(b)}$	8 ± 2$^{(a)}$	11 ± 2$^{(b)}$	8 ± 1$^{(a)}$
Emulsifying stability (EE)	36 ± 3$^{(b)}$	41 ± 3$^{(c)}$	38 ± 1$^{(bc)}$	42 ± 2$^{(c)}$
Antioxidant activity (mg Trolox/g dry matter)	2.3 ± 0.3$^{(a)}$	4.1 ± 0.3$^{(c)}$	3.8 ± 0.3$^{(c)}$	7.8 ± 0.2$^{(e)}$
Total phenols content (mg GA/g dry matter)	3.7 ± 0.7$^{(c)}$	15.7 ± 0.7$^{(f)}$	4.4 ± 0.4$^{(d)}$	9.0 ± 0.2$^{(e)}$

According to Vetter and Kunzek (2003), the drying method affects the porosity of the product, hence persimmon fibres dried with hot air showed a specific volume similar to those of commercial fibres analysed commonly obtained using this method. However, freeze-dried fibres presented significantly higher values, which is probably related to the sublimation water process, which leads to a different capillary structure, which is more open and porous (Basanta et al., 2012). Most fibre moisture content is usually between 11% and 2% (Femenia et al., 1999) and although the moisture content of persimmon fibre is found to be in this interval, the main difference observed between commercial and extracted fibres relates precisely to moisture content, which is significantly higher in persimmon fibres and especially in freeze-dried ones. This result evidences a higher hygroscopicity of persimmon fibres and this could also justify the higher specific volume recorded in this type of fibre. However, these higher levels do not result in significantly higher values of water activity, which indicates that the interactions of persimmon fibre with water are stronger, ensuring the product stability against lipid oxidation reactions, hydrolytic reactions, non-enzymatic browning and microbial spoilage (Labuza et al, 1972; Adams&Moss, 1997). Protein levels in persimmon fibres were similar to those in apple and peach fibres and lower than in other commercial fibres, and fat content was lower than in all commercial fibres analysed, although both parameters are within the range of values found in literature for other types of fibre fruits (de Moraes Crizel et al., 2013).

3.2. Functional properties: hydration, oil retention and emulsification

Hydration properties are related to the quantity and characteristics of polysaccharides contained and are influenced by porosity and particle size (Femenia et al., 1997). The swelling capacity (SC) of fibre is a property that is usually evaluated in this type of products due to the implications it has not only in the food matrix (in which this ingredient is included),

but also the satiating effect that it can provide due to swelling during the digestive process in the stomach. In this regard, Figure 2 shows the swelling capacity (SC), the water holding capacity (WHC) and the water retention capacity (WRC) of persimmon fibres depending on the type of drying (freeze or hot air) and particle size. Additionally, Table 2 shows the results of these hydration properties (SC, WHC, WRC and oil holding capacity: OHC) along with the emulsifying properties (emulsifying activity: EA and emulsion stability: ES) and the antioxidant properties (antioxidant activity: AA and total phenolic content: TPC) of the four commercial fibres.

The swelling capacity (SC) of the different fractions of persimmon fibre was very similar for pulp and peel and also comparable to the commercial fibres from orange, peach and apple. The influence of the particle size on this property is not very significant, although there is a slightly greater value of SC for the smallest size, especially for freeze-dried fibre.

When fibre was stabilized by freeze-drying, the water holding capacity (WHC) and the water retention capacity (WRC) were similar for persimmon fibre from peel and pulp. However, hot air drying noticeably decreased the WHC and WRC of pulp fibre, but had no influence on peel fibres, which had the same values as freeze-dried persimmon fibres. Comparing these results to those obtained for commercial fibres, WHC of persimmon fibres were in the same range as those for commercial fibres, whereas WRC were higher for persimmon fibres, except for the pulp fibre obtained by hot air drying which showed similar values to the commercial fibres.

In addition to hydration properties, fibre has the ability to trap fat, and therefore the oil holding capacity (OHC) is another essential parameter in the characterization of dietary fibres. This parameter is affected by the type, size, shape and superficial area of the particles of fibre, but also by its chemical composition (López et al., 1996).

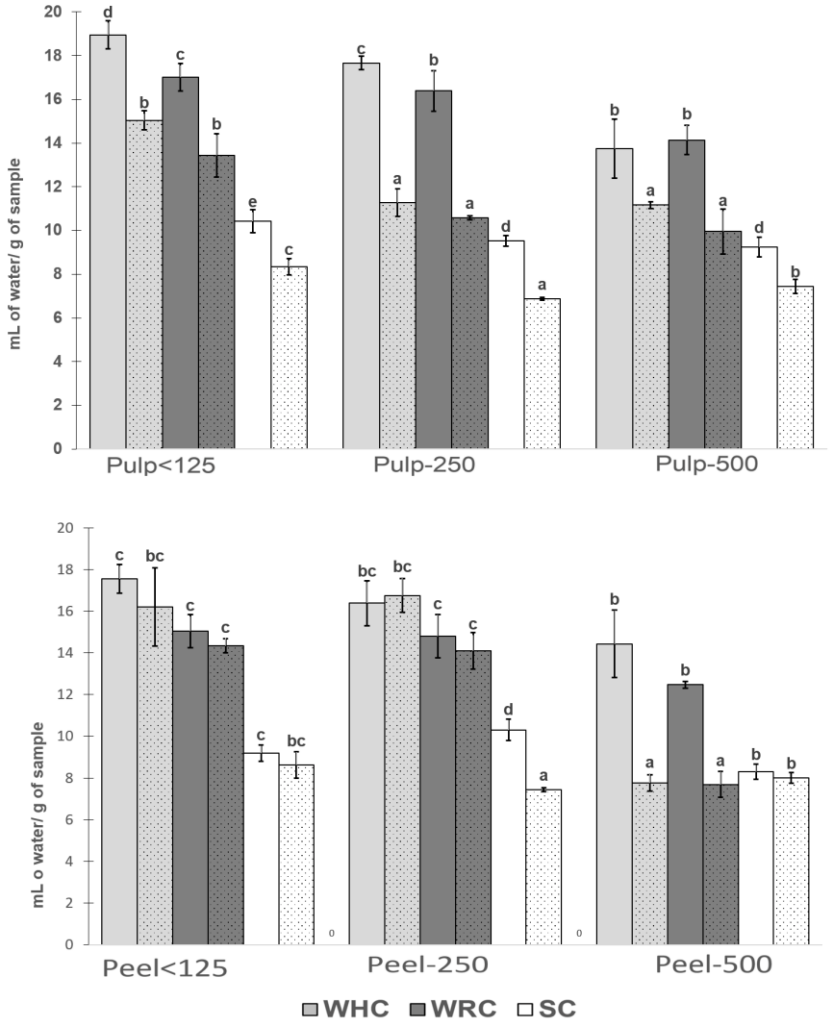

Fig. 2 – Swelling capacity (SC), water holding capacity (WHC) and water retention capacity (WRC) of persimmon fibres (from pulp or peel) obtained by different techniques (hot air drying: bars with points or freeze-drying: bars without points) and particle size (500: 500-250 μm; 250: 250-125 μm and <125 μm). Equal letters mean homogenous groups for each parameter. Three replicates were carried out.

Figure 3 shows the results of OHC of the persimmon fibres studied, depending on the type of drying and the particle size. As can be observed, the type of drying significantly affected the OHC of the fibre, being lower for hot air drying than for freeze-drying. In general, hot air dried pulp registered significantly less OHC than hot air dried peel. As for particle size, there was a noteworthy decrease in the OHC for peel in the case of increased particle size in fibres obtained by hot air drying. However, no remarkable differences were found in the other cases. Moreover, freeze-dried persimmon fibres reached values of OHC similar to all the commercial fibres. This behaviour could be related to the changes in the microstructure of the fibres, which is affected not only by the type of drying but also by the composition of the tissue. In freeze-drying, all types of tissues are less collapsed than in hot air drying, giving place to wider cavities that can retain more fat. In the case of hot air drying, there was a higher collapse in pulp than in peel.

As for emulsion properties, Figure 4 presents the percentages of emulsifying activity (EA) and the emulsion stability (ES) for persimmon fibres of 125 μm. These properties are related to the protein solubility in water, which contributes to the decrease in the interfacial tension among the hydrophobic and hydrophilic compounds, giving place to a greater disposition of molecules to act in the interphase (Hutton and Campbell, 1977; Kinsella, 1976; Singh, 2001). The emulsifying activity of persimmon fibres was comparable to commercial fibres and no significant differences were found due to the drying method in pulp, while only a slight reduction in AE was recorded when fibre was obtained by hot air drying from peels. As for emulsion stability (ES), it is noteworthy that emulsions formed by fibres from peel were more stable than those obtained from pulp and in both cases the highest stability was reached by freeze-drying.

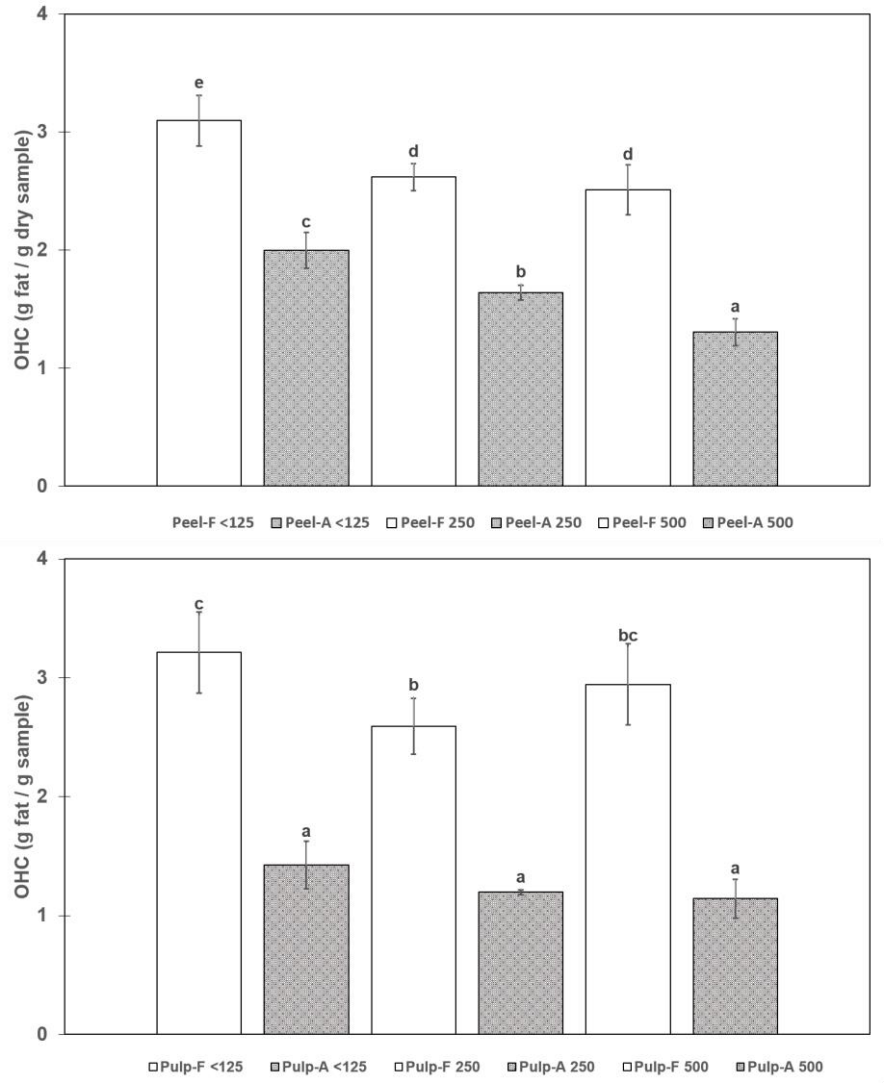

Fig. 3 – Oil holding capacity (OHC) of persimmon fibres (from pulp or peel) obtained by different techniques (hot air drying: A or freeze-drying: F) and particle size (500: 500-250 μm; 250: 250-125 μm and <125 μm). Equal letters mean homogenous groups for each parameter. Three replicates were carried out.

3.3. Antioxidant properties

Persimmon is a fruit that is a rich source of fibre, but also contains vitamin C and phenols that confer antioxidant properties (George and Redpath, 2008). Its antioxidant capacity is higher than for tomato, strawberry, apple and grape (Chen et al., 2008) and according to Achiwa et al., (1997) and Gorinstein et al., (1998) it is one of the fruits which is richest in bioactive compounds. Figure 5 shows the values of antioxidant activity (AA) and total phenolic content (TPC) of persimmon fibres obtained in this study. Antioxidant compounds in fruits usually present higher concentrations in the peel as in the case of persimmon, whose peel is rich in carotenoids, which is the reason for its characteristic colour. However, due to the instability of these compounds, the drying process used for stabilization significantly affected their final content; and consequently, peel fibre obtained by hot air drying showed antioxidant properties which were similar to those of pulp fibre. Conversely, freeze-drying led to the highest values of AA and TPC in peel, but no significant differences were found in AA in the pulp based on the drying method used. Based on these results point, the drying method has a notable influence on the stability of carotenoids, the freeze-drying being the most suitable technique for stabilizing peel fibre. Also noteworthy was that the particle size had a significant influence on the AA and the TPC in both fractions (peel and pulp), the fibres with the lowest size being with the highest values of antioxidant properties. If the results of AA and TPC of the persimmon fibres from peel with the smallest particle size are compared to the values obtained for the four commercial fibres (Table 2), it can be seen that the antioxidant activity was higher for persimmon fibre in all cases, except for in the case of apple fibre. However, the TPC of persimmon fibre was only similar to lemon and peach fibres, being much lower than for orange and apple fibres.

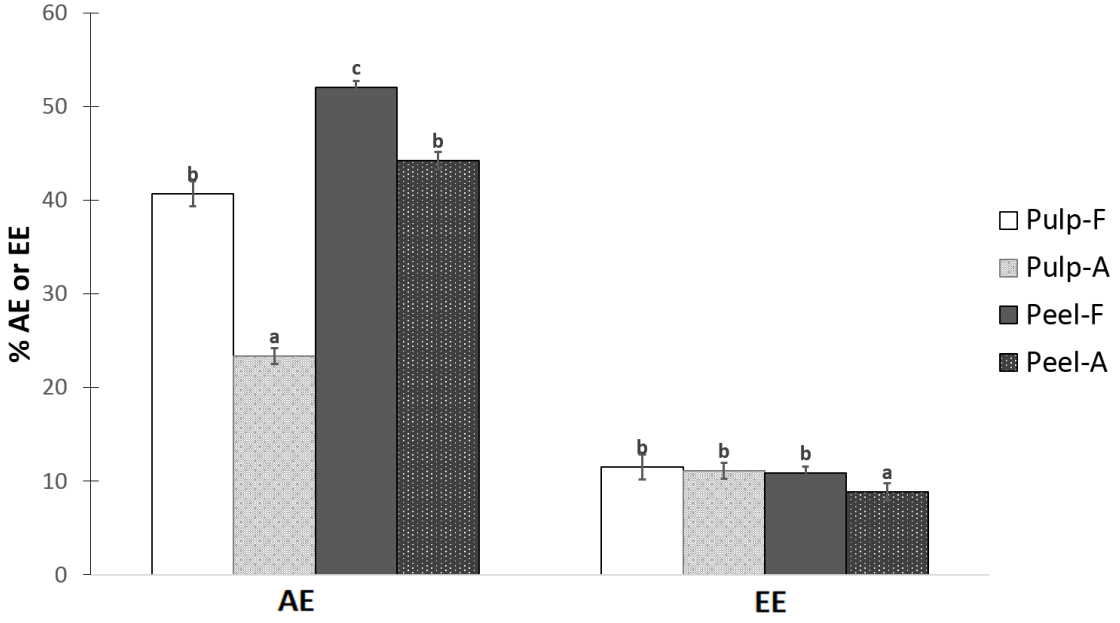

Fig. 4 – Emulsifying activity (AE) and emulsifying stability (EE) of persimmon fibres (from pulp or peel) obtained by different techniques (hot air drying: A or freeze-drying: F). Equal letters mean homogenous groups for each parameter. Three replicates were carried out.

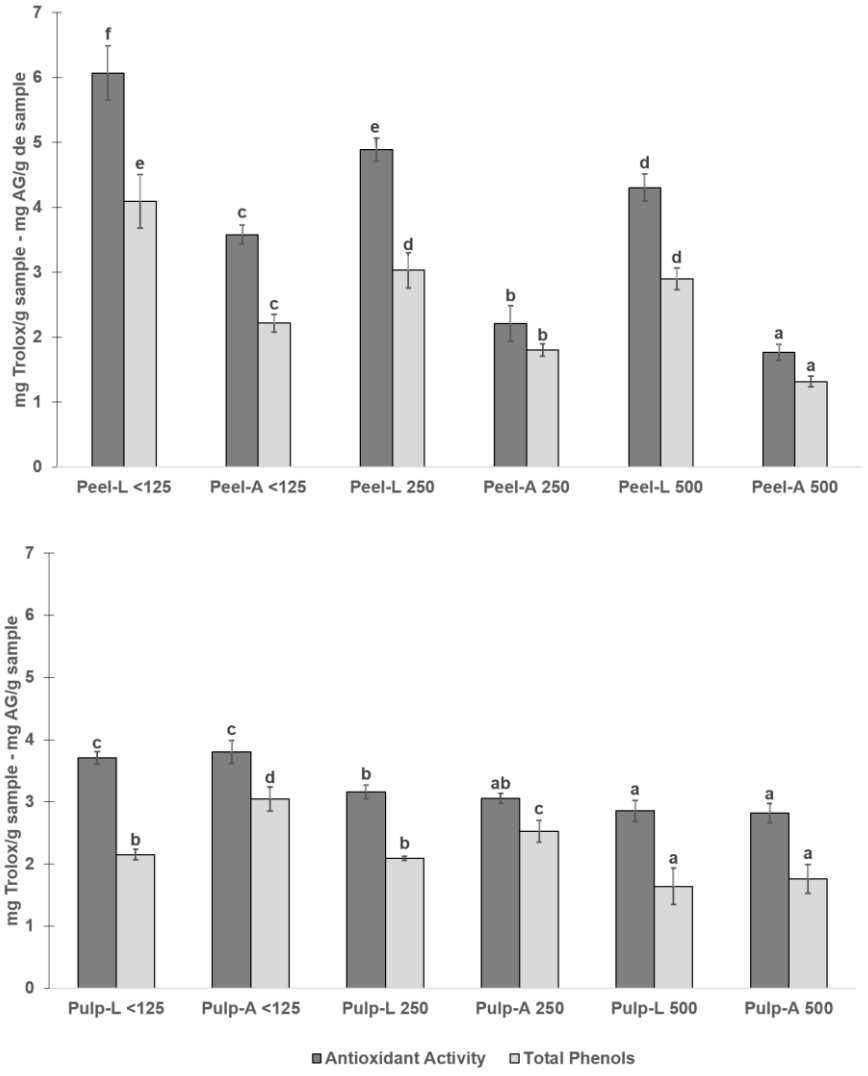

Fig. 5 – Antioxidant activity (AA) and total phenols content (TPC) of persimmon fibres (from pulp or peel) obtained by different techniques (hot air drying: A or freeze-drying: F) and particle size (500: 500-250 μm; 250: 250-125 μm and <125 μm). Equal letters mean homogenous groups for each parameter. Three replicates were carried out.

4. CONCLUSIONS

The results of fibre characterization confirm that both persimmon pulp and peel fibre present hydration, emulsification and antioxidant properties which are similar and in some cases better than the properties analysed for the commercial fibres from other fruits. Therefore, persimmon fibre is an ingredient that can help to enhance the persimmon crop when there is an excess of production. As for the process used to obtain the fibre from this fruit, freeze-drying improves fibre properties in many cases and in some cases even leads to better results than in the case of commercial fruits. However, the cost of this technique is not competitive. For this reason, the hot air drying is recommended to obtain high quality persimmon fibre.

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III.5. EVALUATION STUDIES OF PERSIMMON PLANT (DIOSPYROS KAKI) FOR PHYSIOLOGICAL BENEFITS AND BIOACCESSIBILITY OF ANTIOXIDANTS BY IN VITRO SIMULATED GASTROINTESTINAL DIGESTION

Martínez-Las Heras, R., Pinazo, A., Heredia, A., Andrés, A.

Food Chemistry 214: 478-485 (2017)

Si bien es cierto que la presencia de compuestos bioactivos en la dieta está relacionada con la prevención de numerosas enfermedades, su repercusión sobre la salud depende de su bioaccesibilidad, es decir de la fracción soluble de los mismos que supera el proceso digestivo y es susceptible de llegar al torrente sanguíneo y órganos tras atravesar la barrera intestinal (bioabsorción), y por tanto de su biodisponibilidad (Parada & Aguilera, 2007). Una de las herramientas utilizadas para reproducir las condiciones fisiológicas de la digestión gastrointestinal y poder evaluar los cambios experimentados por los macro y micronutrientes durante este proceso, son los modelos de digestión “in vitro” (During & Harrison, 2005).

Concretamente, el objetivo de este trabajo fue estudiar la evolución del contenido en polifenoles totales, flavonoides y actividad antioxidante total a lo largo de la digestión gastrointestinal de las hojas y fruto del caqui así como de fibras extraídas del bagazo y de la piel del fruto, y estabilizadas por secado por aire caliente y liofilización. Adicionalmente, se calculó la bioaccesibilidad final de polifenoles totales, flavonoides y la fracción antioxidante total para cada una de las matrices de caqui estudiadas.

La metodología experimental consistió en simular las fases oral, gástrica y duodenal de acuerdo al protocolo armonizado de INFOGEST (Minekus et al., 2014) en presencia y ausencia de enzimas digestivos y sales biliares con el fin de analizar el rol intrínseco del pH digestivo sobre los cambios experimentados por las familias de compuestos antioxidantes

analizadas. Los tiempos de digestión a los que se llevaron a cabo las determinaciones analíticas fueron: 0 (antes de la digestión), tras 2 min de fase oral, 10, 30 y 120 min de fase gástrica, y 30, 60, 90 y 120 min de duodenal. Los métodos espectrofotométricos utilizados en la caracterización de las propiedades antioxidantes de los extractos acuosos de hoja, fruto y fibras de caqui fueron: el método Folin-Ciocalteu para la determinación del contenido de fenoles totales (Sakanaka et al., 2005), el método DPPH (Shahidi et al., 2006) para la determinación de la actividad antioxidante y el método descrito por Dewanto et al., (2002) para la determinación del contenido en flavonoides. Los índices de recuperación (%) de polifenoles totales, flavonoides y actividad antioxidante total fueron calculados a cada tiempo de digestión, así como la bioaccesibilidad (%) al final de la misma.

Los resultados obtenidos ponen de manifiesto que los antioxidantes procedentes de la infusión de hoja de caqui presentaron mayor vulnerabilidad a las condiciones del proceso digestivo que aquellos procedentes del fruto o de las fibras. Este hecho podría indicar una cierta protección de la matriz alimento (fruto y fibra) frente a la degradación de estos compuestos a lo largo de la digestión. Concretamente, la fase oral fue la etapa digestiva con un mayor impacto sobre el contenido en polifenoles totales, flavonoides y actividad antioxidante total, no registrándose marcadas pérdidas adicionales durante la fase gástrica. Es de destacar la contribución de la etapa intestinal al aumento del contenido en polifenoles y flavonoides observado durante la digestión del fruto y algunas fibras de caqui. Este aumento podría ser consecuencia de la liberación de ciertos compuestos fenólicos, antes ligados o presentes en forma reducida, bajo las condiciones propias de esta etapa (Gião et al., 2012). En cuanto a la actividad antioxidante total se refiere, se produjo siempre una pérdida acumulativa de la misma durante la digestión gastrointestinal, si bien es de destacar que la pérdida fue significativamente inferior en el caso del fruto de caqui.

Los resultados revelan además un claro efecto protector de las enzimas digestivas frente a la degradación de los compuestos antioxidantes a lo largo de la digestión si se comparan los resultados obtenidos al simular la digestión en presencia y ausencia de enzimas y sales biliares.

En lo referente a la bioaccesibilidad, los resultados muestran, en general, una mayor bioaccesibilidad de los antioxidantes procedentes del consumo de las fibras de caqui, en segundo lugar del fruto, y en última instancia de la infusión de las hojas.

Finalmente, es posible concluir que que si bien la bioaccesibilidad de los compuestos antioxidantes resultó ser superior en el fruto de caqui o fibras extraídas del mismo que en el extracto acuoso de las hojas, la ingesta de una infusión (1.5 g en 110 mL de agua) de hojas de caqui y de un fruto de 200 g aportan, al final de la digestión, la misma fracción bioaccesible antioxidante, mientras que el fruto aportaría 2 veces más polifenoles pero 3 veces menos flavonoides que una infusión de hojas de caqui.

III.5. EVALUATION STUDIES OF PERSIMMON PLANT (DIOSPYROS KAKI) FOR PHYSIOLOGICAL BENEFITS AND BIOACCESSIBILITY OF ANTIOXIDANTS BY IN VITRO SIMULATED GASTROINTESTINAL DIGESTION

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ABSTRACT

This study aims to analyze the antioxidant benefits from persimmon leaf tea, fruit and fibres taking into account their changes along gastrointestinal digestion. The evolution of polyphenols, flavonoids and antioxidant capacity was studied using the recent harmonized in vitro protocol published by Minekus et al. (2014). The digestion was performed with and without digestive enzymes. Results showed aqueous leaf extract was richer in antioxidants than the fruit or the extracted fibres. Nevertheless, persimmon-leaf antioxidants were more sensitive to the digestive environment. In general, the oral conditions greatly affected the antioxidants, while gastric digestion led to slight additional losses. The intestinal step enhanced polyphenols and flavonoids solubility coming from the fruit and fibres. Additionally, the presence of digestive enzymes positively contributed to antioxidant release throughout digestion. Finally, the bioaccesibility of polyphenols, flavonoids and antioxidant activity of persimmon fruit were 1.4, 1.0 and 3.8 times higher than in aqueous leaf extract.

Keywords: persimmon; fibres; leaf-aqueous extract; antioxidant compounds; gastro-intestinal digestion; bioaccessibility.

1. INTRODUCTION

Since it is better to prevent chronic diseases than to treat them, reducing the risk of cardiovascular diseases or cancer has become one of the priorities of sanitary authorities, scientists and the food industry. The consumption of functional foods enriched in antioxidants as well as fruits and vegetables naturally rich in bioactive compounds would contribute to reducing the risk of suffering from diseases associated with oxidative stress. Although it is true that the presence of bioactive compounds in the diet is linked to the aforementioned benefit, their repercussion in human health mainly depends on biochemical state they are in when they reach the bloodstream and, consequently the tissues. The digestion process itself leads to a series of changes in the macro and micronutrients conditioning their final bioaccessibility and bioavailability (Parada & Aguilera, 2007). The bioavailability of dietary compounds in general, and phytochemicals in particular, is dependent on their digestive stability, their release from the food matrix and the efficiency of their transepithelial passage. The best way to determine the benefits arising from the intake of foods, and its bioavailability, consists of subjecting the product to in vivo gastrointestinal digestion. However, in vivo trials are expensive and require long periods of observation, especially in human samples. Besides, they involve medical and ethical implications. Due to these limitations, scientific evidences positively support the alternative to employing in vitro models to accurately reproduce the biochemical conditions of different phases involved in the gastrointestinal digestion (During & Harrison, 2005). In vitro simulated digestion presents additional advantages compared with in vivo trials such as the possibility of sampling at different times of digestion process and eluding the inter-human body variability. Therefore, the evaluation of the influence of matrix food structure on antioxidant changes along gastrointestinal digestion by means of an in vitro simulation could considered as a valid approach. In this sense, authors decided to explore the influence of structure of food matrices all coming from Persimmon crop on the changes experimented by antioxidants during

gastrointestinal digestion. Concretely, food matrices selected for this study were the persimmon fruit, the fibre, a functional ingredient extracted from the fruit, and the aqueous extract of persimmon dried leaves. Persimmon crop is native to China where it has been cultivated for centuries before Christ. It spread to Japan (c. VII) and Korea (c. XIV) in Middle Ages, and posteriorly to other continents. Persimmon cultivars can be divided into two distinct groups: astringent (Rojo brillante, Triumph, etc.) and non-astringent varieties (Fuyu, Hana-Fuyu, Jiro, etc.). Astringency is linked to the chemical structure of the tannins in the fruit, being insolubilized in non-astringency varieties and soluble in astringent ones. Soluble tannins can interact with proteins of saliva that gives rise to a bitter taste in mouth. As ripening progresses, the tannin content decrease and the risk of bitter taste in astringent varieties as well (Hernándiz, 1999). Nevertheless, some astringent varieties such as Rojo brillante are harvested unripened because of its demanding texture. Rojo brillante is the most important commercial variety in Valencian Community (Spain). According to data from Agricultural Minister of Spain, the land dedicated to persimmon crop has significantly increased in the recent past years (only in the last year it has increased an additional 20%) (Alòs, 2014), and subsequently the fruit production. This fact is partially due to the implementation of new technologies capable to remove astringency in unripened fruits that allow offering persimmons with firm texture and without astringency to consumers (Arnal & Del Río, 2005). Parallely, this rapid increase of production has led to an overproduction due to both the seasonality of the persimmon culture and fruits do not meet market standards. Due to this situation, alternatives to obtain higher economical return from the culture have being explored. In this sense, the use of dried persimmon leaves in infusions or the inclusion of aqueous leaf-extract in functional food formulations could be attractive because of the high antioxidant content in this food matrix (Martínez-Las Heras, Quifer-Rada, Andrés & Lamuela-Raventós, 2016). The extraction of functional ingredients from persimmon fruit such as fibre could

be also interesting (Landines, Martínez-Las Heras, Heredia, & Andrés, 2014). Currently, fibres are used in many foods such as yoghourts or bakery products in order to improve the texture and viscosity of the product, and because of their prebiotic potential (Thebaudin, Lefebvre, Harrington, & Bourgeois, 1997; Guevara-Cruz, 2013). Fibre consumption is associated with the prevention and treatment of some diseases such as colon cancer, coronary diseases, constipation and, diabetes as well as antioxidants (Sorensen, Hsi, Chi, Shara, Wactawski-Wende, Kahn, & Stoller, 2014). In this context, the aim of this study was to determine the changes undergone by total polyphenols, flavonoids and antioxidant capacity of persimmon aqueous leaf extract, fruit and fibre, extracted from the pulp and peel of persimmon fruit and stabilized by hot air drying or freeze-drying, during gastrointestinal digestion by means of an in vitro simulation. Besides, the bioaccessibility of these antioxidant families was determined.

2. MATERIALS AND METHODS

2.1. Raw material

The raw materials used were persimmon tree (Diospryos kaki, Rojo brillante) leaves, fruit and fibre extracted from peel or pulp of the fruit.

Persimmon leaves were collected from an orchard in Alginet (Valencia, Spain). They were washed and blanched for 1 min at 100 ºC and then dried by convection air current at 100 ºC in an oven (30 min). Once they were dehydrated, they were grounded in a grinder (Severn) till they had a particle size of less than 1 mm. The resulting powder was finally used to prepare infusions. For this purpose, 1.5 g of leaf powder was mixed with 110 mL of boiling distilled water and were filtered with a Whatman paper (particle retention: 20-25 µm) after 5 min (Martínez-Las Heras, Heredia, Castelló, & Andrés, 2014).

The persimmon fruits were also collected in Alginet (Valencia, Spain). They were kept at environmental conditions for approximately 24 h. They were then subjected to a deastringency treatment in closed chambers with 95% of CO2 at 20ºC and 90% of relative humidity for 24 h. Subsequently, the persimmon fruits were washed, peeled and the pulp cut into small cubes for digestion. Additionally, fibre was extracted from persimmon peel or pulp according to Escalada, González, Sette, Portillo, Rojas, & Gerschenson (2012). For this purpose, each fraction (peel and pulp) was separately homogenized with a high-performance dispersing instrument (T25 digital Ultraturrax IKA). Each fraction was mixed with boiling ethanol (96% v/v) in a ratio 1:2 (w/v) and the mixture was stirred at 600 rpm for 15 min. Finally, the ethanol was discarded by a sieve from the mixture and the semi-solid residue subjected to the Van Soest official method (Van Soest, Robertson, & Lewis, 1991) in order to confirm that it was fibre. Once the presence of fibre confirmed, it was dehydrated in a hot-air oven at 40ºC till it reached a constant mass (approximately 7 h), or frozen at -40ºC for 24 h and then freeze-dried (vacuum pressure of 10-1 mbar for 24 h). Therefore, four different fractions of fibre were obtained: PULP-A or PULP-F for hot-air dried or lyophilized pulp fibres; and PEEL-A or PEEL-F for hot air dried or freeze-dried/lyophilized peel fibres. The final moisture content of each fraction was: 6±1, 7.8±0.3, 6.9±0.5 and 8±1% for PULP-A, PULP-F, PEEL-A and PEEL-F, respectively.

2.2. In vitro simulated gastrointestinal digestion (GI) of persimmon food matrices

In vitro gastrointestinal digestion (oral, gastric and intestinal phases) was simulated following the harmonized INFOGEST protocol published by Minekus et al., (2014). According to this method, simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared from stock solutions fresh daily and kept at 37 ºC before their use. Stock solutions and simulated fluids were prepared with KCl,

KH2PO4, NaHCO3, NaCl, MgCl2(H2O)6 and (NH4)2CO3 at the same molarity indicated in Minekus et al. (2014). Additionally, salivary α-amylase from human saliva was added to the SSF in a final concentration of 75 U/mL; pepsine from porcine gastric mucosa to the SGF in a final concentration of 2000 U/ mL; and pancreatin from porcine pancreas and bile salts (bovine bile extract) to the SIF in a final concentration of 100U/ mL and 10 mM, respectively. All reagents and enzymes were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Once simulated fluids prepared, in vitro digestion was carried out. For oral phase simulation, the different food matrixes (persimmon leaf aqueous extract, fruit and fibres) were mixed in a ratio food: SSF of 1:1 (w/v), and properly homogenized. The mixture was then placed in 50 mL falcon tubes and incubated without agitation for 2 min at 37 ºC. Then, simulated gastric fluid (SGF) (pH 3) was added in a ratio of 1:1 (v/v) to each tube containing the oral mixture and the pH adjusted to 3 with HCl (1N). The falcon tubes were placed in a head- over-heels stirrer (Intell-Mixer RM-2, Elmi Ltd, Riga, LV-1006, Latvia) at 55 rpm for 2 h at 37 ºC in an incubator chamber (JP Selecta SA, Barcelona). Finally, simulated intestinal fluid (SIF) (pH 7), was added in a ratio of 1:1 (v/v) to each tube containing the gastric mixture, pH adjusted to 7 with NaOH (1N) and the tubes placed in the head-over-heels stirrer at 55 rpm and in the incubator chamber to conduct the intestinal phase simulation for 2 h at 37 ºC.

For analytical purposes, aliquots were taken at the end of each phase: oral (2 min), gastric (120 min) and intestinal one (120 min) as well as the following intermediate residence times: 10, 30, and 120 min for gastric and 30, 60, 90 and 120 min of intestinal digestions. Samples were placed in an ice bath for ten minutes and pH was adjusted to 9 to ensure enzyme inactivation.

The role of the pH of digestion on the antioxidants compounds changes along digestion was parallely evaluated by means of

carrying out the entire simulation without digestive enzymes and bile salts. Simulation in the absence of enzymes served as control.

All simulations were performed twice and three aliquots were extracted at each of the specified sampling times in each one of the simulations.

2.3. Determination of the antioxidant activity, total polyphenols and flavonoids

The antioxidant capacity (TAC) of samples was analyzed on the basis of the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl free radical (Brand-Williams, Cuvelier & Berset, 1995; Shahidi, Liyana-Pathirana & Wall, 2006). Three grams (3 g) of sample was diluted with 5 mL of methanol and stirred for 5 min. Then the test tubes with the sample-methanol mixture were centrifuged at 10,000 rpm for 10 min (Medifriger BL-S, P-Selecta). About 0.1 mL of supernatant was added to 3.9 mL of a methanolic DPPH solution (80:20, methanol: water) of 0.025 mg/mL. The solution was shaken and after 30 min, the absorbance of the sample was read at 515 nm in a spectrophotometer (JASCO V-630) and using methanol as blank. Quantification was performed considering a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the results were expressed as mg of Trolox equivalent per g of dry matter.

Total phenolic content (TPC) of different samples was spectrophotometrically determined by the modified Folin-Ciocalteu method (Sakanaka, Tachibana & Okada, 2005). This method is based on an extraction with methanol (3 g of sample/ 5 mL of methanol) in stirring conditions (200 rpm) for 1 h (COMECTA WY-100 horizontal stirrer). Thereafter, the mixture was centrifuged for 10 min at 10.000 rpm (Medifriger BL-S, P-Selecta). Then, 0.125 mL of supernatant were transferred to a cuvette and 0.5 mL of distilled water and 0.125 mL of Folin-Ciocalteu reactive added and left to rest for 6 minutes. After that

time, 1 mL of distilled water and 1.25 mL of a solution of sodium carbonate at 7% were added. The prepared simple was left to rest for 90 min before measuring absorbance at 760 nm using a spectrophotometer (JASCO V-630). The absorbance was compared to a standard curve of gallic acid (GA) and the content of total polyphenols was expressed as mg of GA equivalent per g of dry matter.

Total flavonoid content (TFC) was measured according to the colorimetric method described by Zhishen, Mengcheng & Jianming, 1999). Briefly, three grams (3 g) of sample were mixture with 3 mL of water and the mixture centrifuged for 10 min at 10,000 rpm (Medifriger BL-S, P-Selecta). Next, 0.25 mL of the supernatant were mixed with 1 mL of distilled water and 0.075 mL of a solution of sodium nitrite at 5%. After 6 min of rest, 0.15 mL of a solution of aluminum chloride were added and 5 min later, 0.5 mL of sodium hydroxide (1M) and 2 mL of distilled water were also added and absorbance read at 510 nm using a spectrophotometer (JASCO V-630). The total content of flavonoids was expressed as mg catechin equivalent per g of dry matter.

2.4. Assessment of bioaccessibility and recovery index of antioxidant compounds

To evaluate the effect of the food matrix on the changes undergone by the polyphenols, flavonoids and total antioxidant activity along gastrointestinal in vitro digestion, the recovery and bioaccesibility indexes (RI and BI) were calculated according to equations I and II (Ortega, Macià, Romero, Reguant & Motilva, 2011), respectively:

$$RI(\%)=100\cdot A/C \qquad \text{(Equation I)}$$

$$BI\ (\%)=100\cdot B/C \qquad \text{(Equation II)}$$

where A is either polyphenols content (mg gallic acid eq./ g dry matter) or flavonoid content (mg catechin eq./ g dry matter) or antioxidant capacity (mg trolox eq./ g dry matter) quantified in

each tested food at each digestion time, B is either polyphenols or flavonoid contents or antioxidant capacity quantified in the supernatant as previously described after the complete digestion process (Granado-Lorencio, Olmedilla-Alonso, Herrero-Barbudo, Blanco-Navarro, Pérez-Sacristán & Blázquez-García, 2008), and C is either polyphenols or flavonoid contents or antioxidant capacity quantified in the tested food before digestion and expressed in the same units.

The recovery index is related to the percentage of polyphenols, flavonoids and antioxidant capacity present in the digest at each digestion time; whereas the bioaccessibility index is defined as the percentage of polyphenols, flavonoids and antioxidant capacity that is solubilized in the chime soluble fraction after the intestinal step. Thus, this index defines the proportion of the compounds that could become available for absorption into the systematic circulation (Ortega et al., 2011).

2.5. Statistical analysis

Statgraphics plus (version 5.1) software was used to perform the two-way analysis of variance (multi-factor ANOVA) in order to assess the effect of the type of food matrix and presence of digestive enzymes on the recovery index of polyphenols, flavonoids and antioxidant capacity along gastrointestinal digestion and their final bioaccesibility. In cases when ANOVA showed significant differences, post hoc tests (Tukey HSD test) were applied.

3. RESULTS AND DISCUSSION

3.1. Antioxidant properties of food matrices previous to digestion

Table 1 shows antioxidant capacity (mg trolox eq./ g dry matter), polyphenol (mg gallic acid eq./ g dry matter) and flavonoid (mg chatechin eq./ g dry matter) contents of the different persimmon test foods (leaves, fruit and fibres) prior to digestion. As was expected, the persimmon leaves aqueous

extract presented the highest polyphenol content and antioxidant capacity. In comparison to other infusions, persimmon leaves could be considered more antioxidant than red tea but less than green and black teas (Anesini, Ferraro & Filip, 2008). Fibres, and especially those extracted from the peel and stabilized by freeze-drying (PEEL-L and PULP-L), contained twice the amount of antioxidants as persimmon fruit. In a previous study carried out by the same authors (Landines, 2014), in which antioxidant, emulsifiers and rehydration properties of different commercial and persimmon fibres were analyzed, it was found that freeze-dried peel persimmon fibre was also richer in antioxidants than commercial orange, peach or lemon fibres. Therefore, persimmon fibre could be used as a functional ingredient in high-fibre food formulations. In order to have a more realistic idea of the antioxidant benefits derived from one serving of each of these persimmon test foods, it was estimated the antioxidant contribution of each serving to health. In this sense, a aqueous extract of persimmon leaves (consisting of 1.5 g of dried leaves in 110 mL of water) would provide 183 mg trolox eq., 129 mg gallic acid eq. and 34.3 mg catechin eq., a piece of persimmon fruit (200 g) 129.6 mg trolox eq., 74.4 mg gallic acid eq. and catechin 11.2 mg eq.; and finally, the ingestion of 100 g of 5% fibre-rich product, considering that all antioxidants come from persimmon fibre would contribute to health with 31 mg Trolox eq., 20.5 mg gallic acid eq. and 29 mg catechin eq. (values calculated with data of Peel-L fibre).

Table 1. Antioxidant capacity (mg trolox eq./g dry matter), polyphenol (mg gallic acid eq. /g dry matter) and flavonoid (mg catechin eq./ g dry matter) contents of persimmon matrices before digestion: persimmon leaves, fruit and fibres extracted from pulp and peel and stabilized by hot-air drying (A) or lyophilization (L) to generate PEEL-L, PULP-L, PEEL-A and PULP-A samples. Mean and standard deviation (n=3).

	AA (mg Trolox/ g dry matter)	Phenols (mg GA/g dry matter)	Flavonoids (mg catechin/ g dry matter)
Persimmon leaf	122(3)^{a}	86(2)^{a}	22.9(0.7)^{a}
Persimmon fruit	3.24(0.12)^{c}	1.86(0.03)^{c}	0.70(0.05)^{e}
PEEL-L Fibre	6.2(0.2)^{b}	4.10(0.14)^{b}	5.8(0.5)^{b}
PULP-L Fibre	3.78(0.2)^{bc}	2.17(0.05)^{c}	2.95(0.14)^{d}
PEEL-A Fibre	3.11(0.06)^{c}	2.02(0.12)^{c}	2.7(0.3)^{d}
PULP-A Fibre	3.78(0.08)^{bc}	2.94(0.18)^{bc}	4.6(0.3)^{c}

Different letters in the same column denote significant differences (Turkey's test, $p<0.05$) among persimmon matrices in terms of antioxidant properties

3.2. Changes experimented by total phenolic content and flavonoids during simulated gastrointestinal in-vitro digestion

Figure 1 shows the recovery index (%) of total polyphenols during the simulated in vitro digestion for persimmon leaf aqueous extract, persimmon fruit and persimmon fibres in the presence and absence of digestive enzymes and bile salts; while the recovery index (%) of flavonoids for the same food matrices is depicted in figure 2. As can be observed, the simulated gastrointestinal in vitro digestion affected the polyphenol and flavonoid compounds similarly. More specifically, the oral phase, and mainly the salivary pH, was the step affecting the most both polyphenol and flavonoid contents regardless of the digested persimmon matrix. During the gastric digestion, slight additional losses of both families of compounds occurred without the influence of the residence time in the stomach. The modificaction of polyphenols and flavonoids along the digestive process has been widely studied in other vegetables and fruits. Losses of polyphenols of 70-75% were reported in some products such as pomegranade juice (Pérez-Vicente, Gil-Izquierdo & García-Viguera, 2002), apple (McDougall, Fyffe, Dobson & Stewart, 2007), cherries (Fazzari, Fukumoto, Mazza, Livrea, Tesoriere & Marco, 2008) or red cabbage (Bouayed, Deußer, Hoffmann & Bohn, 2012). By contrast, the biochemical conditions of the intestinal phase led to an increase in polyphenol content of persimmon fruit and fibres, and especially those extracted from the lyophilized peel (PEEL-L). This fact was also observed in the digestion of broccoli, wine, juices and cashews as a consequence of the biochemical changes undergone by these compounds in the small intestine (Wootton-Beard, Moran & Ryan, 2011; Chandrasekara & Shahidi, 2012). It could be with an improved of the solubility of certain phenolic compounds, before being linked or present in a reduced form (Gião et al., 2012). Additionally, the interactions of phenolic compounds with sugars or other dietary compounds released during digestion could play a protective role in their changes during the digestion

process, affecting their solubility and potential bioavailability (Ortega et al., 2011).

The same pattern was observed in flavonoids (figure 2) with a significant increase in these compounds during the intestinal digestion of persimmon leaf infusion and fruit. The increase of total phenolics and flavonoids in the intestinal phase could be explained by the additional time of digestion (plus 2 h) as well as the effect of the intestinal enzymes and bile salts, which migh be facilated the release of phenolics bound to the matrix. Record & Lane (2001) and Green, Murphy, Schulz, Watkins & Ferruzzi (2007) reported losses of around 80% of different catechins in different types of tea at the end of digestion, which is therefore higher than those recorded in the infusion of persimmon leaf. Likewise, Cilla, González-Sarrías, Tomás-Barberán, Espín & Barberá, (2009) obtained losses of between 64.5 and 70.1% in the content of flanan-3-oles in grape, orange and peach juices. In the study published by Bouayed, Hoffman & Bohn, (2011), losses of flavonoids were 56% at the end of digestion. These authors partially attributed the losses to the instability of some anthocyanins under alkaline conditions of the intestine. However, in a study also carried out on apples, catechins were degraded in an acidic stomach medium and posteriorly, their concentration increased in the presence of duodenal secretions because of a possible isomerization (Kahle et al., 2011). Finally, in other study published on the bioaccesibility of bioactives compounds of cacao, catechin and epicatechin contents augmented throughout digestion. This fact could be attributed to the protector role of certain macronutrients such as lipids and the formation of micellar structures which exert protection against the degradation of these compounds and consequently increase their final bioaccesibility (Ortega, Reguant, Romero, Macià & Motilva, 2009).

As mentioned before, the in vitro digestion was also carried out in absence of digestive enzymes and bile salts as a control. The

results show that the presence of enzymes at any step of digestion contributed positively to the preservation and/ or release of total polyphenols and flavonoids. According to these results, it could be affirmed that the final bioaccessibility of compounds, and indeed bioavailability, will be highly dependent on the correct secretion of enzymes by the human body during digestion and specifically during the duodenal step. Pancreatin (lipase, protease and amylase enzymes) hydrolises macronutrients of the matrix given as a result a degradation of the food matrix. This phenomenon could assist the antioxidants release. Therefore, it could be concluded that polyphenol and flavonoid stability would be compromised in persons suffering of enzymes secretion insufficiencies, such as exocrine pancreatic insufficiency. Exocrine pancreatic insufficiency is a condition characterized by deficiency of the exocrine pancreatic enzymes, resulting in the inability to digest food properly, or maldigestion.

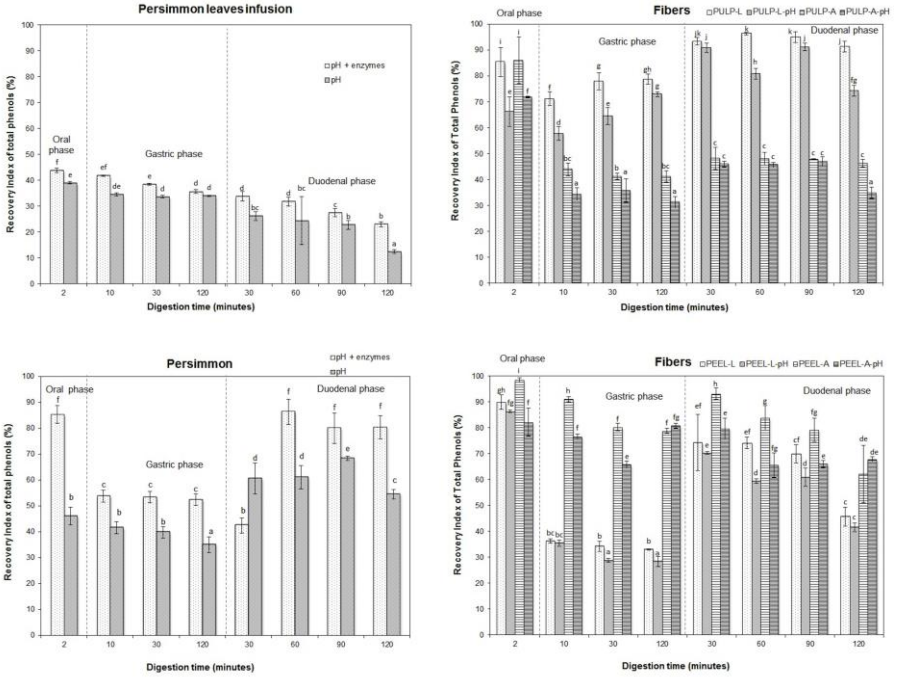


Fig. 1 – Recovery index (%) of total polyphenols along the in-vitro simulated gastrointestinal digestion of persimmon leaves infusion, persimmon fruit and fibres extracted from pulp and peel and stabilized by hot air drying (A) or lyophilization (L) to generate PEEL-L, PULP-L, PEEL-A and PULP-A matrices. Figure shows the results obtained in presence and absence of digestive enzyme and bile salts (n=3).

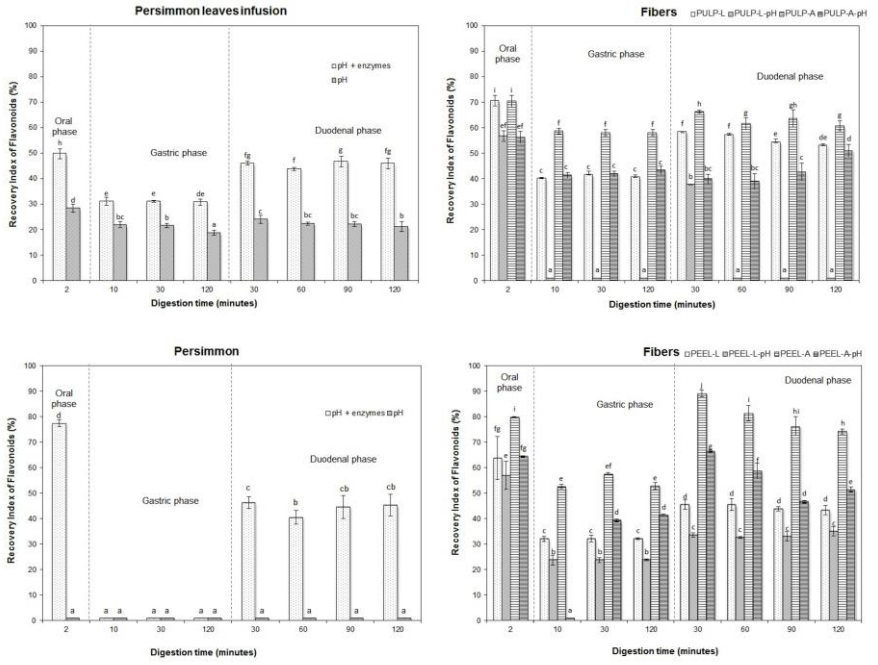
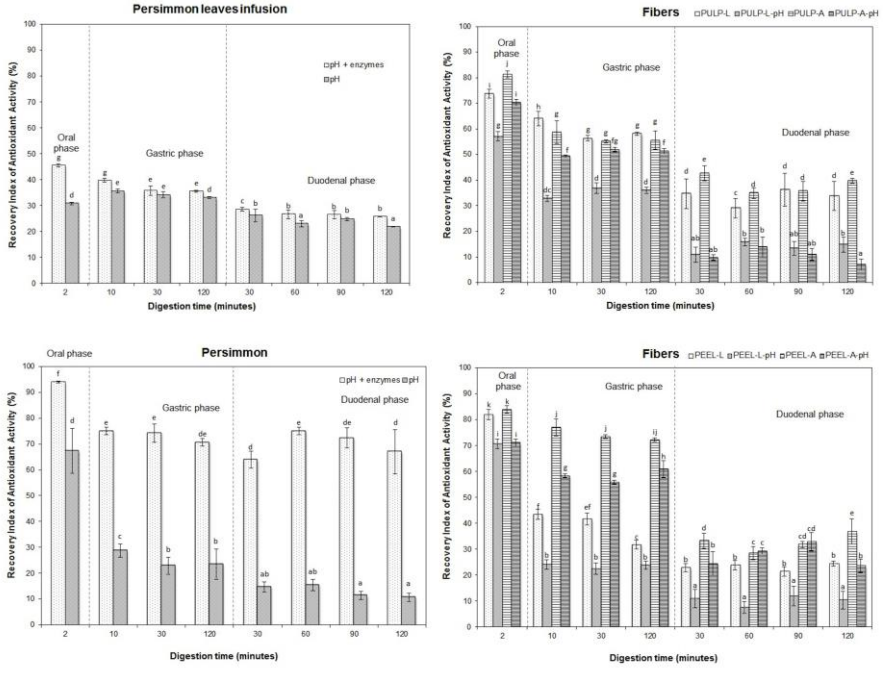


Fig. 2 – Recovery index (%) of flavonoids along the in-vitro gastrointestinal digestion of persimmon leaves infusion, persimmon fruit and fibres extracted from pulp and peel and stabilized by hot air drying (A) or lyophilization (L) to generate PEEL-L, PULP-L, PEEL-A and PULP-A matrices. Legends called as "food matrix-pH" correspond to data obtained without digestive enzymes. Figure shows the results obtained in presence and absence of digestive enzyme and bile salts (n=3).

3.3. Changes experimented by total antioxidant activity during simulated gastrointestinal in-vitro digestion

Figure 3 shows evolution of antioxidant capacity along the gastrointestinal in vitro digestion in the presence and absence of digestive enzyme and bile salts. Changes in antioxidant capacity during digestion resulted in total losses at the end of digestion of 74, 68, 63, 76, 60 and 66 % for the persimmon leaf infusion, persimmon fruit and each of the fibres: PEEL-A, PEEL-L, PULP-A, PULP-L, respectively.

The residual values of antioxidant activity at the end of digestion are similar to those obtained by other authors for example, the digestion of strawberries (Cerezo, Cuevas, Winterhalter, Garcia-Parrilla & Troncoso, 2010). However, Tavares et al., (2012) reported losses exceeding 84% of antioxidant activity in blueberries, which was higher than those obtained in this study. In contrast, some authors reported moderated losses in green and black teas (about 25%) (Record & Lane., 2001) or even an increase of the antioxidant capacity in fruit juices, bread or millet (Cilla, Perales, Lagarda, Barberá, Clemente & Farré, 2011; Wootton-Beard et al., 2011, Chandrasekara & Shahidi, 2012).



Recovery index (%) of total antioxidant capacity along the in-vitro gastrointestinal digestion of persimmon leaves infusion, persimmon fruit and fibres extracted from pulp and peel and stabilized by hot air drying (A) or lyophilization (L) to generate PEEL-L, PULP-L, PEEL-A and PULP-A matrices. Legends called as "food matrix-pH" correspond to data obtained without digestive enzymes. Figure shows the results obtained in presence and absence of digestive enzyme and bile salts (n=3).

3.4. Bioaccesibility of polyphenols, flavonoids and total antioxidant activity of tested persimmon products

The bioaccesibility index (%) of total polyphenols, flavonoids and total antioxidant capacity present in persimmon leaf infusion, fruit and fibres is shown in table 2. As can be observed, the bioaccesibility of any of the antioxidants studied in the persimmon fibres was higher than in the fruit and much higher than in the persimmon leaf infusion. Moreover, the bioaccesibility of the total antioxidant capacity was generally lower than those of polyphenols and flavonoids and never exceded 40%. Among fibres, fibre from pulp and lyophilized is the most recommended one considering its higher values of polyphenol and flavonoid bioaccesibilities (90 and 70%, respectively).

However, taking into account the total content of polyphenols, flavonoids and antioxidants capacity of a serving of persimmon leaf infusion (1.5 g in 110 mL of water), 200 g of fruit and 100 g of a food enriched with 5 % fibre previously mentioned in this section and their bioavailability (table 2), the intake of one serving of persimmon leaf infusion would provide the same bioaccesible antioxidant capacity, half the polyphenols and three times more flavonoids than one serving of a fruit. These values are far lower for the intake of 100 g of a persimmon fibre-rich food despite the fact that the bioaccesibility (%) of the compounds of this matrix (table 2) was the highest.

The analysis of variance multifactor (ANOVA multifactor) applied on changes in total antioxidant activity, polyphenols and flavonoids considering the digestion step and the presence or absence of digestive enzymes as factors, evidenced that the presence of digestive enzymes was the factor which most influenced (higher F-ratio) the evolution of the antioxidant properties along the simulated digestion of persimmon leaf infusion. In the case of the fruit, both factors showed a similar significant influence (similar values of F-ratio for both factors) on changes undergone by antioxidants during digestion; whereas

the statistical significance of either of the factors on antioxidant changes of fibres was different for each one of the four studied fibres. Finally, in order to evaluate the influence of the raw material (pulp or peel) and the drying method (hot-air or freeze drying) on the changes of fibre-antioxidants, an additional multifactor ANOVA was performed. The analysis revealed that the drying method was the factor that influenced the most the evolution of antioxidants during digestion, and especially of the total antioxidant capacity.

Table 2. Bioaccesibility index (%) of polyphenols, flavonoids and total antioxidant capacity of persimmon leaf infusion, fruit and fibres extracted from pulp and peel and stabilized by hot air drying (A) or liophilization (L) to generate PEEL-L, PULP-L, PEEL-A and PULP-A samples. Means and standard deviation of bioaccesibility index (%) (n=3) at the end of the gastrointestinal digestion carried out with or without digestive enzymes and bile salts.

	Bioaccesibility Index (%)					
Test food	**Persimmon leaves**	**Persimmon Fruit**	**PEEL-A Fibre**	**PEEL-L Fibre**	**PULP-A Fibre**	**PULP-L Fibre**
	Presence of digestive enzymes and bile salts					
Poliphenols	20.0(0.6)^{e}	76(3)^{b}	59(3)^{c}	51.43(0.13)^{d}	46(2)^{d}	91(6)^{a}
Flavonoids	47.6(0.8)^{d}	48(3)^{d}	60(3)^{b}	43.0(0.4)^{d}	53.3(0.8)^{c}	69.4(0.5)^{a}
Antioxidant capacity	24.6(1.0)^{c}	33(2)^{b}	32(1)^{b}	25(2)^{c}	36(2)^{a}	38(2)^{a}
	Abscence of digestive enzymes and bile salts					
Poliphenols	12.1(0.4)^{f}	55(2)^{c}	67.5(0.9)^{b}	42.44(0.13)^{d}	33(2)^{e}	74(6)^{a}
Flavonoids	21(2)^{c}	ND	35.9(0.6)^{b}	39(2)^{a}	21.7(0.9)^{c}	ND
Antioxidant capacity	21.4(0.6)^{b}	11(2)^{d}	28(2)^{a}	11.7(1.0)^{d}	8(4)^{d}	16(3)^{c}

Different letters in the same row denote significant differences (Turkey's test, $p<0.05$) among persimmon matrices in terms of antioxidant properties; ND: not detected

4. CONCLUSIONS

It can be concluded that the polyphenols, flavonoid and total antioxidant capacity of persimmon leaf aqueous extract were more sensitive to the biochemical conditions of the gastrointestinal environment than those coming from persimmon fruit or fibres, despite being the richest source of the antioxidant compounds. More specifically, mouth digestion was the step in which all antioxidant content decreased the most, especially during the digestion of persimmon leaf infusion and persimmon fruit, while gastric digestion led to slight additional losses. The duodenal environment had a positive effect on the antioxidants evaluated. The duodenal pH and the presence of pancreatin and bile salts increased the solubility and release of polyphenols as well as improved the final bioaccesibility of flavonoids in all studied matrices. Additionally, results revealed a positive effect of the digestive enzymes on the bioactive compounds, thus reducing their loss along digestion and increasing their release of the food matrix.

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III.6. INFLUENCE OF PREHARVEST TREATMENTS TO REDUCE THE SEASONALITY OF PERSIMMON PRODUCTION ON COLOR, TEXTURE AND ANTIOXIDANT PROPERTIES DURING STORAGE

Martínez- Las Heras, R., Amigo-Sánchez, J. C., Heredia, A., Castelló, M.L., Andrés, A.

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La disponibilidad del caqui en el mercado se concentra en los meses de octubre-diciembre, debido a su corta estacionalidad. Por eso, uno de los retos de la industria hortofrutícola es aumentar el periodo comercial para evitar la caída de los precios durante su comercialización y mejorar así su rentabilidad. Esto se puede lograr mediante el uso de diferentes tratamientos en las etapas de precosecha tales como el paclobutrazol (PBZ) y el Etefón con el fin de acelerar la maduración de las frutas y el ácido giberélico (GA_3) para retrasarlas. La influencia de estos tratamientos en la calidad del caqui (tamaño, sabor, color, textura, etc.) deberían ser estudiados con el fin de verificar si realmente es posible ofrecer un producto homogéneo durante su campaña. Además, la respuesta de estos reguladores del crecimiento vegetal en los compuestos bioactivos (tales como los antioxidantes) tampoco ha sido estudiada.

Por todo ello, el objetivo de este estudio fue analizar el efecto de los tratamientos precosecha (PBZ, Etefón y GA_3) en el tamaño del caqui, color, textura y propiedades antioxidantes durante su almacenamiento postcosecha.

Los frutos analizados procedían de la misma parcela en la que los árboles se dividieron en tres grupos: el primer grupo, etiquetado como *natural*, fueron los árboles de referencia porque no fueron tratados y los frutos se recogieron a mitad de noviembre; al segundo grupo de árboles se les aplicó un tratamiento para retrasar la maduración (tratados con GA_3) y

los frutos se recogieron a mediados de diciembre y se etiquetaron como *"delayed" (atrasada)*; el tercer grupo de árboles se trataron con PBZ y Etefón para acelerar la maduración, se recogieron sus frutos a mediados de septiembre y se etiquetaron como *"accelerated" (acelerada)*. En todos los casos, tras la recolección y pasadas 24 horas de almacenamiento a temperatura ambiente, los frutos se sometieron a un tratamiento de desastringencia (95%, CO_2, 24h, 20 ºC y 90% de humedad relativa) (Arnal & Del Río, 2003), momento a partir del cual se llevó a cabo el seguimiento de las propiedades evaluadas

Los métodos espectrofotométricos utilizados en la caracterización de las propiedades antioxidantes del caqui fueron: el método Folin-Ciocalteu (Sakanaka et al., 2005), para la determinación del contenido de fenoles totales y el método DPPH (Shahidi et al., 2006) para la determinación de la actividad antioxidante. Además, se estudiaron las propiedades ópticas de los frutos determinando los parámetros Hunter L*a*b* y el índice de color (CI) en dos zonas (ecuatorial y apical). La evolución de la textura se realizó mediante un test de compresión. El seguimiento de todas estas propiedades se llevó a cabo tras 1, 3, 5, 7, 9 y 11 días de almacenamiento de los frutos en cámaras termostatadas a 4 ºC.

El tamaño del caqui es un factor limitante a la hora de su comercialización, de ahí el interés de estudiar si los tratamientos precosecha pudieran tener alguna influencia sobre el calibre. Los resultados mostraron que los frutos madurados de forma natural presentaron un mayor tamaño que los frutos que fueron sometidos a pretratamientos en campo.

Otro de los atributos que caracteriza al caqui *Persimon* es su textura firme. Mientras que los frutos que maduraron de forma natural mantuvieron constante su textura, los frutos sometidos a un tratamiento precosecha sufrieron una reducción de su firmeza a lo largo del tiempo de almacenamiento.

Respecto al color, los frutos que maduraron sin aplicación de ningún tratamiento, presentaron valores más altos de CI durante la primera semana de almacenamiento, alcanzando valores similares a los frutos pretratados después de este tiempo. Esto puede estar relacionado con el efecto que tiene el GA_3 y el etefón sobre el desarrollo de los carotenoides. En la zona apical los cambios de color medidos no fueron significativos.

Por último, se analizó mediante el ajuste de un modelo de primer orden (Gonçalves et al., 2010; Martins et al., 2000) la cinética de degradación de compuestos fenólicos y de la actividad antioxidante. Se observó que los frutos pretratados en campo presentaron una mayor concentración de polifenoles inicialmente y, por lo tanto, una mayor actividad antioxidante que los no tratados, al menos durante los primeros días de almacenamiento. Sin embargo, la tasa de degradación de compuestos fenólicos, así como su actividad antioxidante, fue mayor en el caso de los caquis pretratados. Los frutos que maduraron de forma natural y los tratados para acelerar la maduración alcanzaron valores similares de antioxidantes al final del periodo de almacenamiento. Sin embargo, los frutos tratados para retrasar la maduración mostraron los valores más altos de polifenoles y por tanto de actividad antioxidante tras los 11 días de almacenamiento.

Los resultados de este trabajo constituyen una aportación útil sobre las repercusiones de los tratamientos precosecha destinados a ampliar el periodo de recolección sobre la calidad del fruto.

III.6. INFLUENCE OF PREHARVEST TREATMENTS TO REDUCE THE SEASONALITY OF PERSIMMON PRODUCTION ON COLOR, TEXTURE AND ANTIOXIDANT PROPERTIES DURING STORAGE

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ABSTRACT

Persimmon production has increased considerably, thanks to techniques for removing astringency whilst maintaining the strong consistency. Currently, the needs of cooperatives are focused on increasing the commercial period. Thus, the aim of this study was to analyze the effect of preharvest treatments (paclobutrazol (PBZ) and Ethephon to accelerate ripening and GA_3 to delay it) on persimmon size, composition, color index (CI), texture and antioxidant properties over 11 days of postharvest storage at 4ºC. The results showed that the size of fruits subjected to preharvest treatment was smaller than in untreated fruit. Moreover, CI of the apical zone was higher in samples of standard ripening throughout the first few days of storage. It is also noteworthy that the treated fruits at the beginning of storage reported greater antioxidant properties. Finally, the evolution of the antioxidants has been fitted with a first-order model to predict their kinetic degradation depending on the persimmon harvest period.

Keywords: persimmon; harvest ripening; texture; color index; total phenolic compounds; antioxidant activity; kinetics

1. INTRODUCTION

The persimmon species (*Diospyros kaki Thunb*.) is a fruit tree originating from China. In Spain, the species spread for

ornamental and wood uses at the end of the nineteenth century. The tree was later used to produce fruit for consumption and became a traditional crop located in Mediterranean gardens or orchards for domestic consumption. However, in the last 15 years, there has been a high increase in production of the cultivar "*Rojo Brillante*" in the region of Valencia due to application of techniques to remove the astringency from fruits whilst maintaining their firm texture (Arnal & Del Río, 2003; Ben-Aire & Sonego, 1993; Taira & Matsumoto, 1997). These techniques mainly consist of using CO_2 rich atmospheres for 24 hours at 20-24ºC to insolubilize the tannins in the pulp of persimmon, which are responsible for the astringency. Thus, there are two ways to commercialize this fruit: "Classic", fruits with natural ripening and therefore with soft texture and "Persimmon", fruits subjected to techniques to remove astringency and keep their firm texture, but with a good taste. This fact has led to a wide increase in the distribution of this product and 70% of this production is now exported to countries like Germany, France, Holland, Czech Republic, Russia and Brazil (IVIA, 2014).

However, availability of this crop in markets is restricted to October–December due to its short seasonality. One of the major challenges today is to extend the period this fruit is commercially available to avoid price drops during maximum production and hence improve profitability. This can be achieved by using different treatments in preharvest stages such as paclobutrazol (PBZ) and Etephon in order to accelerate the ripening of fruits and gibberellic acid (GA_3) to delay it. PBZ has been found to be predominantly effective in the induction of early flowering and thus finding scope for offseason production in different crops such as mango (Upreti et al., 2013), citrus (Martínez-Fuentes et al., 2013), wheat (Kondhare, Kettlewell, Farrell, Hedden, & Monaghan, 2012; 2013; 2014), among others. Ethephon treatment caused a transient increase in ethylene production and inhibited vegetative growth (Tamari, Pappa, Zered, & Borochov, 1998). Moreover, ethylene is known

to generally inhibit shoot extension whilst promoting root proliferation (Jaiswal & Sawhney, 2006; Liu, Mukherjee, & Reid, 1990; Pan, Wang, & Tian, 2002) and significantly speeds up senescence (Hodges & Forney, 2000; Philosoph-Hadas, Meir, & Aharoni, 1991). The treatment of orchards with GA_3 is one of the most widely used plant growth regulators for manipulation of fruit development and ripening in a number of crops (Dagar et al., 2012; Facteau et al., 1985; Southwick & Yeager, 1995; Zilka et al., 1997). It has recently been found to be useful in reducing flower density, which consequently reduces crop load and increases fruit size in peaches and nectarine (Stern & Ben-Arie, 2009). GA_3 applied at the end of pit hardening led to a delay in fruit ripening and in the development of internal breakdown in stored nectarines (Dagar et al., 2012; Zilka et al., 1997).

The influence of these pretreatments on the quality of persimmon (size, taste, color, texture, etc.) when fruits are in the orchard (preharvest period), at the moment of consumption and during storage (post-harvest period) should be studied in order to verify if it is really possible to offer a homogenous product during the campaign of this fruit. Besides, the response of bioactive compounds (such as antioxidants) to these plant growth regulators has not been widely studied. Persimmon fruit contains a large amount of polyphenols, including condensed tannins and other phenolic compounds, which have conventionally been used to treat health problems such as coughs, hypertension, dyspnea, paralysis, frostbite, burns and bleeding (Matsuo & Ito, 1978; Mowat, 1990). Gorinstein et al. (2000) proved that a persimmon-supplemented diet significantly lowered total cholesterol, triglycerides in plasma and LDL-cholesterol in rats fed on a high-cholesterol diet. According to many authors (Al-Maiman & Ahmad, 2002; Mirdehghan & Rahemi, 2007; Siriwoharn et al., 2004; Odriozola-Serrano et al., 2008), fruit chemical, especially phenolic and antioxidant properties, is influenced by cultivar, growing region and degree of fruit ripeness at harvest. The decrease in total phenolic content in many fruits has been attributed to the oxidation of

polyphenols by polyphenoloxidase during ripening (Amiot et al., 1995; Kulkami & Aradhya, 2005; Shwartz et al., 2009). Other possible reasons related to this phenomenon could be the regulation of polyphenols biosynthesis during fruit ripening (a dilution effect as fruit increased in size), as well as the contribution of phenolic compounds to the biosynthesis of flavylium ring of anthocyanins (Kulkami & Aradhya, 2005). Recently, Sanchís, Mateos, and Pérez-Gago (2015) have observed that the maturity stage of fresh-cut *Rojo Brillante* persimmon at harvest had an effect on both fruit firmness and the efficacy of the antioxidants to control enzymatic browning. Thus, the persimmon harvested at the beginning of the season could be processed as a fresh-cut commodity, even after 3 days of storage at 15ºC if treated with 0.01 kgL^{-1} ascorbic acid or 0.01 kgL^{-1} citric acid. However, processing fruits from late season immediately after harvest and being treated with ascorbic acid were recommended.

The aim of this study was to assess the effect of preharvest treatments (PBZ and Ethephon to accelerate ripening and GA_3 to delay ripening) on the size, soluble solids, moisture content, color, texture and antioxidant properties (total phenolic content and antioxidant activity) with a view to extending the commercial availability season of "*Rojo Brillante*" persimmon.

2. MATERIALS AND METHODS

2.1. Plant materials and preharvest treatments

Persimmon (*Diospyros kaki Thunb. cv. Rojo Brillante*) trees were grown in different orchards in Alginet (Spain). Trees were divided in three groups; the first group, labeled as "*natural*", was used as a reference group because the trees were not treated and the fruit was harvested in mid-November; the second group of trees was treated with GA_3 (CEKU-GIB, 1.6%) to delay fruit ripening and the fruit (labeled as "*delayed*") was harvested in mid-December; the third group of trees was treated with Paclobutrazol (PBZ, Cultar) and Ethephon (Fruitel® 48% Bayer)

to accelerate the ripening process and the fruit (labeled as "*accelerated"*) was harvested in mid-September.

2.2. Post-harvest treatment

After harvest, fruits were stored at room temperature for 24 h before applying deastringency treatment, which was carried out in closed chambers with 95% CO_2 for 24 h at 20ºC and 90% of relative humidity (Arnal & Del Río, 2003).

2.3 Experimental design

After this treatment to remove astringency, 40 fruits from each group were stored at 4ºC and the evolution of moisture content, color, texture and antioxidant properties were evaluated after 1, 3, 5, 7, 9 and 11 days.

2.4. Analytical Methods

Moisture content was determined using the 934.06 method (AOAC, 2000) for sugar-rich fruits.

Soluble solids content

Soluble solids content was determined using a Carl Zeiss Abbe Atago refractometer model 89,553 with thermostat set to 20 ºC using a solution of polyethyleneglycol at 5% (w/w) (PEG 6000, Panreac) to precipitate the tannins still present in the juice (Sugiura, Kataoka, & Tomana, 1983).

Diameter

Diameter was determined using a universal fruit calliper (TR 53307 with a range 25-95 mm).

Total Polyphenols and Antioxidant Activity

Samples were spectrophotometrically analyzed, employing a modified version of the Folin-Ciocalteu method (Sakanaka *et al.,* 2005), in order to determine the total phenolic content

(TPC). The TPC was extracted with methanol (3 grams of crushed fruit/5 mL of methanol) and then continuously stirred at 200 rpm for one hour (horizontal shaker COMECTA WY-100). The test tubes were centrifuged for 10 minutes at 10000 rpm (Medifriger BL-S, P-Selecta). 0.5 mL of distilled water and 0.125 mL of the supernatant of the extract were added to a cuvette followed by the addition of 0.125 mL of Folin-Ciocalteu reagent. The mixture was shaken and 1.25 mL of a 7% sodium carbonate solution and 1 mL of distilled water were added after 6 min. The color was left to develop for 90 min and the absorbance was measured at 760 nm using a spectrophotometer (JASCO V-630). The measurement was compared to a standard curve of gallic acid solutions and expressed as mg of gallic acid equivalents per 100 grams of persimmon (mg GAE/100 g of fruit). A blank was prepared in the same way but without any sample.

The antioxidant activity (AA) of the persimmon fruit was measured on the basis of the scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described by Shahidi *et al.* (2006) with some modifications. According to this method, the intensity of the purple color of DPPH solution decreases in the presence of an antioxidant, and this absorbance change is measured spectrophotometrically at 515 nm.

Three grams of the crushed sample were diluted in 5 mL of methanol and were stirred for 5 minutes. The test tubes with the sample-methanol mixture were then centrifuged at 10000 rpm for 10 minutes (Medifriger BL-S, P-Selecta). 0.1 mL of the supernatant was added to 3.9 mL of a methanol solution of DPPH (80:20; methanol:water) (0.025mg/mL). The solution was shaken and after 30 min the absorbance of the sample was measured at 515 nm using methanol as a blank. The differences in absorbance of the samples with respect to the blank in that time were compared with a standard Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) curve

and expressed as mg of Trolox equivalents per 100 grams of persimmon (TE/100 g of fruit).

2.5. Texture measurements

Flesh firmness was determined with a Texturometer TA.XTplus Texture Analyser by Stable using a 10 mm flat plunger at a test speed of 2 mm/s. The plunger penetration depth was 20 mm. Six persimmon fruits from each ripening stage and day of storage were used to analyze texture. Fruits were cut in halves longitudinally and the flat side was placed on the platform of the texture analyzer to keep the sample fixed. The parameters recorded in the compression test were the force required by the plunger to pass through the sample, expressed in newtons (N).

2.6. Color measurements

Skin color was evaluated using a Minolta Colorimeter (Model CM-3600d) spectrophotometer with D65 illuminant, 10º observer and SCE mode. L*, a*, b* Hunter parameters were measured in two zones (equatorial and apical) and results were expressed as a skin color index (CI) (Equation I), as described by Jiménez-Cuesta et al. (1981).

$$CI = \frac{1000 \cdot a}{L \cdot b} \qquad \text{(Eq I)}$$

2.7. Statistical analysis

At least three replicates were carried out for each determination. Results are expressed as mean values ± standard deviation. Data were subjected to analyses of variance (ANOVA), and multiple comparisons between means were determined using the LSD test ($P \leq 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics, Inc., Rockville, MD, USA).

3. RESULTS AND DISCUSSION

Preharvest treatments to control the ripening process can result in changes in fruit quality and storage capacity. The influence of the applied preharvest treatments on the moisture content, Brix and equatorial diameter, was analyzed just after harvest and the LSD averages given by the ANOVA analysis (number of observations = 72) are shown in Table 1.

Table 1. Average values for moisture content, ºBrix and equatorial diameter of naturally ripened fruits (natural), preharvest treated to delay ripening (delayed) and preharvest treated to accelerate ripening (accelerated).

Ripening type	Moisture Content (%)	Brix	Equatorial Diameter (mm)
Delayed	80.67 ± 0.13 (a)	15.2 ± 0.4 (a)	75.2 ± 1.7 (a)
Natural	82.12 ± 0.09 (b)	11.1 ± 0.3 (b)	82.6 ± 1.2 (b)
Accelerated	82.87± 0.07 (c)	10.9 ± 0.2 (b)	74.2 ± 0.9 (a)

Note: Equal letters in parentheses indicate homogenous groups.

Fruits harvested from the trees pretreated to delay ripening had a lower moisture content and higher soluble solids (ºBrix) at the time of harvest than those ripened naturally or those subjected to accelerated ripening. Since small fruit size is one of the limiting factors in marketing persimmon fruit, and many other species, the equatorial diameter was measured. The size of the harvested fruits (equatorial diameter) was affected by both preharvest treatments; the naturally ripened fruits were larger than those harvested from the treated trees.

The evolution of texture, color and antioxidant properties of fruits ripened under different conditions (natural, delayed and accelerated) during the post-harvest period after removing astringency was analyzed.

In terms of texture, the maximum force (F_{max}) was the parameter selected to discuss the results (Fig. 1). The F_{max} recorded in the reference samples ("*natural"*) remained nearly constant throughout the testing period while a decrease is

observed in the fruits treated to accelerate or delay ripening. The drop in the maximum force required for samples that underwent delayed ripening is observed after the 7-day control period in spite of the initially higher values, reaching similar values to those recorded in the accelerated maturation samples.

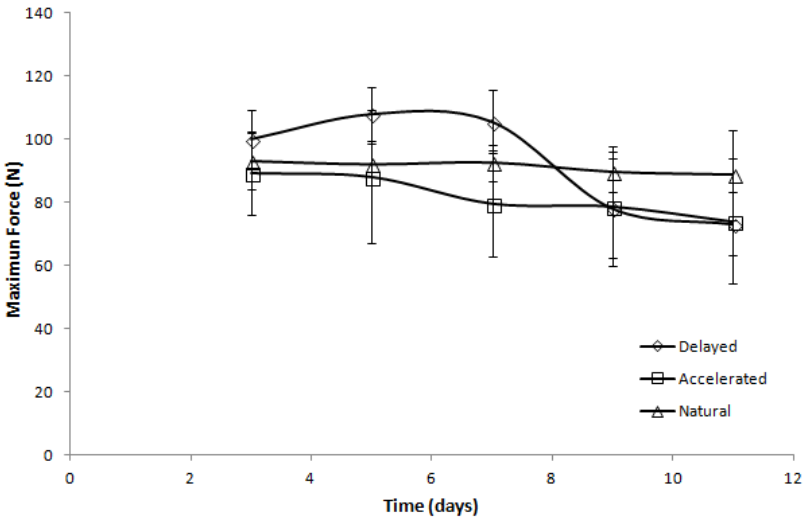

Fig. 1 – Evolution of the Maximum Force (N) from texture test with storage time after astringency removal post-harvest treatment.

As was expected, CI was higher in the apical than in the equatorial zone, since fruit begins to change color from the bottom, as can be observed in Figure 2.

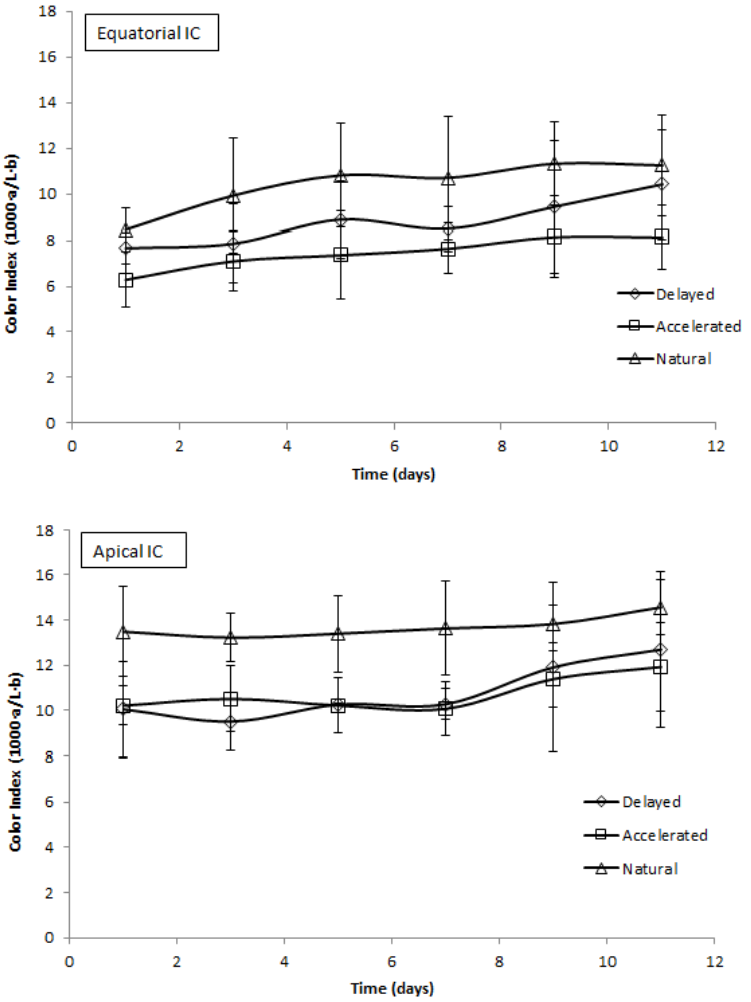

Fig. 2 – Evolution of the Color Index (CI) with storage time after astringency removal post-harvest treatment.

Furthermore, changes in CI throughout storage time were not significant in the equatorial zone. However, the apical zone of the naturally ripened persimmons reported higher CI values during the first week of storage, reaching similar values to the other fruits after that time. Consequently, significant differences between samples with different preharvest treatments were

found from the statistical analysis (Fig. 3). Lower CI values were reported for fruits treated to delay or accelerate ripening than for fruit that had not been pretreated probably due to the retarded effect of GA_3 and Etephon in the development of new carotenoids. Nevertheless, initial harvest time color shown by preharvest treated and untreated samples resulted in CI values that were within the range considered as being commercial for this cultivar (Novillo et al., 2014). Since this cultivar is marketed when orange, all treatments showed a commercially acceptable color, even samples from the accelerated treatment, which had the lowest CI values at harvest.

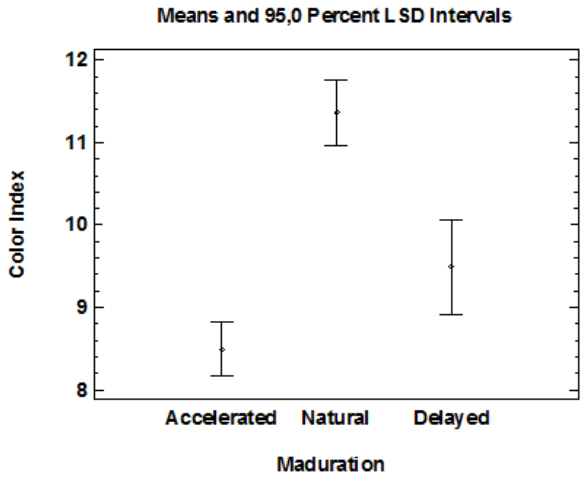

Fig. 3 – LSD interval results of Color Index (CI) as a function of the moment of harvest (accelerated, natural or delayed).

Table 2. Parameters of first-order kinetic model for total phenolic content and antioxidant activity adjustments.

	P_0	k_P	r^2	AA_0	K_{AA}	r^2
Natural	15.2 ± 0.5	-0.095 ± 0.004	0.90 ± 0.02	32 ± 2	-0.082 ± 0.008	0.95 ± 0.01
Accelerated	36.7 ± 0.7	-0.192 ± 0.002	0.96 ± 0.02	51 ± 4	-0.20 ± 0.02	0.96 ± 0.02
Delayed	36 ± 4	-0.11 ± 0.01	0.94 ± 0.04	52 ± 4	-0.09 ± 0.01	0.95 ± 0.06

The total polyphenols content and the antioxidant activity were also measured throughout the storage time and the evolution is shown in Figure 4.

The degradation kinetics of persimmon fruit phenolic compounds and antioxidant activity was analyzed by fitting a first-order kinetic model (Eq. II and IIII) to the experimental data (Martins et al., 2000; Gonçalves et al., 2010).

$$P = P_0 - k_P t \quad \text{(Eq II)}$$

$$AA = AA_0 - k_{AA} t \quad \text{(Eq IIII)}$$

Here, P and AA are the natural logarithm of phenol content and the antioxidant activity respectively, the sub index 0 indicates the initial value of the parameter, t is the stored time and k_P and k_{AA} are the model parameters for phenols and antioxidant activity respectively (Table 2). Thus, the slopes (K_p and K_{AA}) indicate the speed of degradation, whereas the y-intercepts (P and AA) represent the initial concentration of the antioxidant. This fitting was used based on the results obtained by Jaiswal & Abu-Ghannam (2013) who reported that first-order reaction model showed a good fit for the degradation of polyphenols and the antioxidant capacity of York cabbage after microwave processing. It is observed that the preharvest treated fruits initially reported greater polyphenol concentration and therefore greater antioxidant activity than the untreated samples, at least during the first days of storage; however the rate of degradation of phenolic compounds as well as antioxidant activity is greater in the case of the pretreated samples (Figure 4). Samples treated to accelerate ripening reached similar values to the untreated fruit at the end of the storage period, while fruits treated to retard ripening showed higher values of polyphenols and antioxidant activity after 11 days of storage (Figure 4). This trend is also reflected in the values obtained for the model parameters (Table 2) in terms of the initial values of polyphenols (P_0) and antioxidant activity (AA_0) along with the rate of degradation (k_P and K_{AA}).

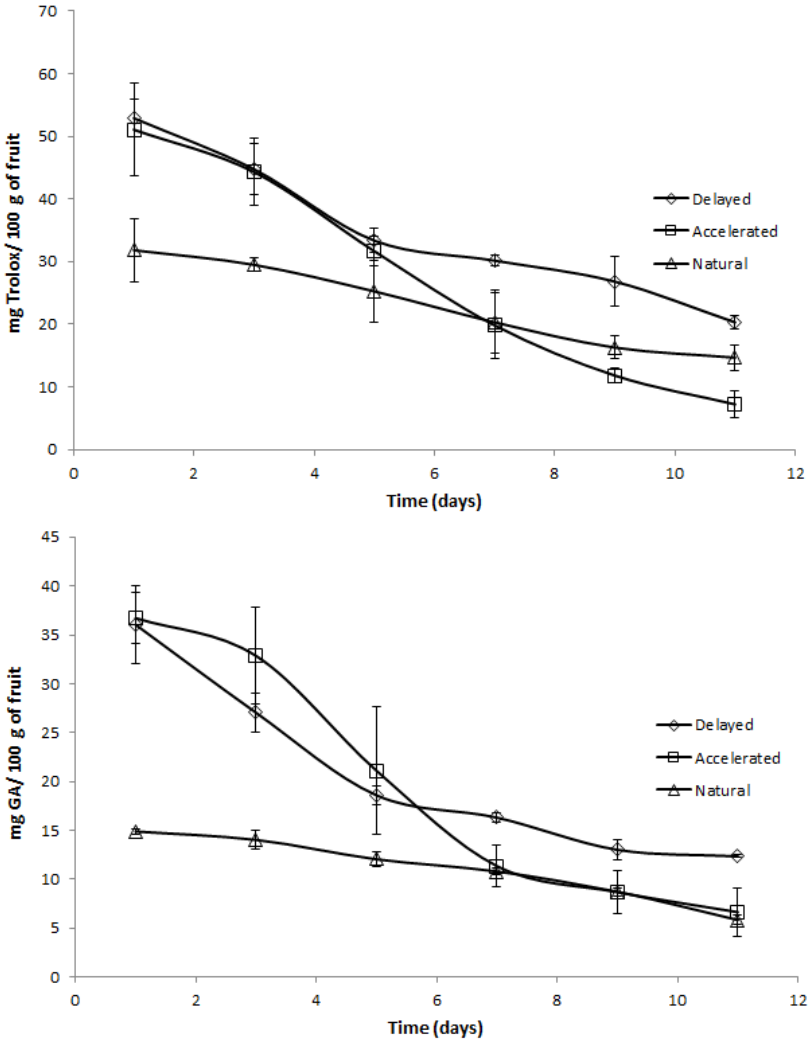

Fig. 4 – Evolution of the total phenolic content (mg GA/100 g of fruit) and antioxidant activity (mg Trolox/100 g of fruit) with storage time after astringency removal post-harvest treatment.

4. CONCLUSIONS

Preharvest treatments, both to accelerate or to retard ripening, applied to persimmon trees to extend the harvesting period mainly imply a reduction in the size of fruits and an increase in the antioxidant properties at the beginning of the storage period. Furthermore, first-order kinetic models have been fitted to predict the phenolic content and AA versus time depending on the moment of harvest of fruits (accelerated, natural or delayed ripening).

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IV. CONCLUSIONES

IV. CONCLUSIONES

IV.1.1 APROVECHAMIENTO DE LAS HOJAS COMO FUENTE DE ANTIOXIDANTES

(i) Los extractos de hojas secadas por aire caliente a 100ºC presentaron la mayor concentración de fenoles, seguidos por los extractos de hojas liofilizadas, los extractos de hojas secadas por aire caliente a 180ºC y, finalmente, los de hojas secadas a la sombra. La misma tendencia se observa para los flavonoides y la capacidad antioxidante. En cuanto a la influencia del tiempo y la temperatura de infusión en la extracción de compuestos antioxidantes, los resultados mostraron que la cinética de extracción de los fenoles y flavonoides fue muy rápida en ambos casos, teniendo lugar en los primeros minutos. Adicionalmente, los resultados obtenidos permiten afirmar que las hojas de menor tamaño presentaron un 7% más de capacidad antioxidante y fenoles totales, y un 9% más de flavonoides que las hojas de mayor tamaño.

(ii) Las isotermas de sorción obtenidas corresponden a isotermas Tipo II. Los modelos de Halsey, Smith, GAB y BET fueron los que mejor describieron los datos experimentales, mientras que el modelo de Henderson presentó los peores ajustes. El calor isostérico de sorción se determinó aplicando el concepto de Clausius-Clapeyron y se estableció una ecuación empírica para predecir el valor de este parámetro en un rango entre 0.5 y 14 % de humedad (en base seca).

Los resultados de este trabajo constituyen una herramienta útil para seleccionar los materiales y diseño del envase adecuado para garantizar la calidad del producto durante su almacenamiento y vida útil.

(iii) Ha sido posible la identificación en la hoja de caqui de 41 compuestos fenólicos por espectrometría de masas de alta resolución, y la mayoría de ellos han sido identificados por primera vez en este trabajo. Entre los polifenoles identificados se encuentran: 9 ácidos benzoicos, 5 ácidos hidroxicinámicos, 11 flavanoles, 13 flavonoles, 1 flavanona, 1 flavona y 1 tirosol. Estos resutados demuestran que las hojas de caqui son ricas en compuestos fenólicos y sus extractos podrían ser añadidos como ingrediente funcional en otras matrices alimentarias como bebidas, galletas, bizcochos, etc., con fines terapéuticos y/o funcionales.

IV.1.2 APROVECHAMIENTO DE LOS RESIDUOS DE INDUSTRIALIZACIÓN DEL CAQUI

(i) Los resultados de caracterización de la fibra de caqui permiten afirmar que tanto la fibra obtenida a partir de la piel como la fibra obtenida a partir del bagazo presentan propiedades de hidratación, emulsificación y antioxidantes similares, y en algunos casos superiores a las propiedades analizadas en distintas fibras comerciales procedentes de otras frutas. Se trata por tanto de un ingrediente que puede contribuir a valorizar el cultivo del caqui en momentos de excedentes de producción. En cuanto al proceso de obtención de este producto, el uso de la liofilización para la estabilización de las fibras mejora en muchos casos sus propiedades y en algunos casos incluso por encima de las comerciales, aunque el coste de operación de esta técnica no resulta competitivo por lo que para un producto de estas características se recomienda el secado por aire caliente.

(ii) Los resultados de la simulación in vitro de la digestión gastrointestinal de los distintos productos

evaluados: extracto de hoja, fruto y fibra extraída de la piel o bagazo, revelan la influencia de la matriz sobre los cambios experimentos por polifenoles, flavonoides y actividad antioxidante, así como sobre su bioaccesibilidad final. Concretamente, los compuestos antioxidantes del extracto acuoso de hoja de caqui resultaron ser más vulnerables a las condiciones gastrointestinales que los del resto de matrices evaluadas a pesar de ser la muestra con mayor concentración de antioxidantes. El patrón de cambio de los compuestos antioxidantes a lo largo de la etapa oral y gástrica fue muy similar con independencia del tipo de muestra. Las condiciones propias de la etapa oral fueron las que mayores pérdidas promovieron en todos los compuestos, manteniéndose dichas pérdidas, o aumentándose ligeramente, durante la etapa gástrica. En cambio, las condiciones propias del ambiente intestinal aumentaron la fracción soluble de los polifenoles procedentes del fruto y fibra extraída de la piel y de los flavonoides presentes en todas las muestras.

Es posible concluir finalmente que si bien la bioaccesibilidad de los compuestos antioxidantes resultó ser superior en el fruto de caqui o fibras extraídas del mismo que en el extracto acuoso de las hojas, la ingesta de una infusión (1.5 g en 110 mL de agua) de hoja de caqui y de un fruto de 200 g aportan al final de la digestión, la misma actividad antioxidante total, si bien el fruto aportaría 2 veces más polifenoles pero 3 veces menos flavonoides que una infusión de hojas de caqui.

IV.1.3 TRATAMIENTOS EN CAMPO COMO ESTRATEGIA PARA AMPLIAR LA RECOLECCIÓN DEL FRUTO DEL CAQUI

Los resultados mostraron que los frutos madurados de forma natural presentan un mayor tamaño que los frutos que fueron sometidos a pretratamientos en campo. Mientras que los frutos que maduraron de forma natural mantuvieron constante su textura a lo largo del almacenamiento postcosecha, los frutos sometidos a tratamientos en campo sufrieron una reducción de su firmeza a lo largo de este periodo. Respecto al color, los frutos que maduraron sin aplicación de ningún tratamiento, presentaron índices de color inferiores que aquellos procedentes de árboles tratados, si bien estos índices se igualaron después de una semana de almacenamiento. Por otro lado, se modelizó la cinética de degradación de compuestos fenólicos y de la actividad antioxidante, y se observó que los frutos pretratados en campo presentaron una mayor concentración inicial de polifenoles y, por lo tanto, una mayor actividad antioxidante que los no tratados. Este patrón se observó al menos durante los primeros días de almacenamiento si bien la tasa de degradación de compuestos fenólicos, así como su actividad antioxidante, también fue mayor en estos casos. Al final del periodo de almacenamiento los frutos que maduraron de forma natural y los tratados para acelerar la maduración alcanzaron valores similares de antioxidantes. Los frutos tratados para retrasar la maduración fueron los que después de 11 días de almacenamiento, presentaron mayor contenido en polifenoles y por tanto mayor actividad antioxidante.