UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE TECNOLOGÍA DE ALIMENTOS

OBTENCIÓN DE INGREDIENTES FUNCIONALES PARA LA FORMULACIÓN DE ALIMENTOS ENRIQUECIDOS CON EXTRACTOS VEGETALES. INFLUENCIA DEL TRATAMIENTO DE CONSERVACIÓN SOBRE ALGUNOS COMPUESTOS BIOACTIVOS

TESIS DOCTORAL

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HACEN CONSTAR QUE:

El trabajo de investigación **"Obtención de ingredientes funcionales para la formulación de alimentos enriquecidos con extractos vegetales. Influencia del tratamiento de conservación sobre algunos compuestos bioactivos"** que presenta Dña. María Hernández Carrión por la Universitat Politècnica de València, y que ha sido realizado bajo nuestra dirección en el Grupo de Investigación de Microestructura y Química de Alimentos de la Universitat Politècnica de València, reúne las condiciones para optar al grado de Doctor.

Valencia, Diciembre de 2014

Fdo. Dra. Isabel Hernando Hernando

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Para empezar un gran proyecto hace falta valentía.

Para terminarlo, perseverancia.

Índice

Resumen

La presente tesis doctoral se centra en la obtención de nuevos ingredientes ricos en compuestos bioactivos a partir de tejidos vegetales (**caqui** y **pimiento**) sometidos a distintos tratamientos de conservación como las altas presiones hidrostáticas (APH) y la pasteurización, con la finalidad de formular nuevos alimentos funcionales.

Se estudió el efecto de un tratamiento específico de APH (200 MPa/6 min/25 ºC) y otro de pasteurización (70 ºC/15min) sobre la estructura y el contenido en algunos compuestos bioactivos del **caqui**. Tanto las APH como la pasteurización causaron cambios estructurales en el tejido parenquimático, favorecieron la precipitación de taninos y la formación de células tánicas, lo que podría relacionarse con la pérdida de astringencia del fruto. Las APH mejoraron la extractabilidad de compuestos carotenoides y mantuvieron las propiedades antioxidantes del fruto. Esta técnica podría ser una alternativa al tratamiento de pasteurización convencional. Asimismo el **caqui** tratado por APH podría ser empleado en la formulación de nuevos alimentos funcionales, tales como bebidas lácteas enriquecidas con **caqui**.

Las nuevas bebidas lácteas, con idéntico contenido en carotenoides, se formularon haciendo uso de **caqui** no tratado, sometido a APH y pasteurizado; y tres matrices lácteas diferentes: leche entera, semi-desnatada y desnatada. Las bebidas elaboradas con **caqui** tratado por APH presentaron unas adecuadas propiedades reológicas ya que ni gelificaron como las elaboradas con **caqui** no tratado, ni sedimentaron como las formuladas con **caqui** pasteurizado. Los consumidores percibieron las nuevas bebidas lácteas enriquecidas con **caqui** como bebidas altamente antioxidantes. Las que más gustaron fueron las elaboradas con **caqui** tratado por APH independientemente del tipo de leche utilizada y las elaboradas con **caqui** no tratado y leche entera. Por tanto, el tratamiento por APH permite formular bebidas lácteas enriquecidas con **caqui** con alto valor nutricional, variable contenido graso y elevada aceptabilidad independientemente de la estacionalidad del fruto.

Por otro lado, se cuantificaron y localizaron algunos compuestos bioactivos y se determinaron algunas propiedades fisicoquímicas en tres tipos de **pimiento**: rojo, verde y amarillo. El contenido en compuestos bioactivos de cada tipo de **pimiento** estuvo condicionado por su estructura. El tipo de **pimiento** más adecuado para obtener extractos ricos en compuestos carotenoides sería el **rojo**, mientras que el **amarillo** sería apropiado para obtener extractos ricos en compuestos fenólicos con elevada actividad antioxidante. Por último, si se pretende obtener extractos con elevado contenido en fibra dietética el más adecuado sería el **pimiento verde**.

Se estudió el efecto de diferentes tratamientos de APH (100, 200, 300 y 500 MPa/15 min/25 ºC) y de un tratamiento de pasteurización (70 ºC/10 min) sobre la estructura de **pimiento rojo**. Además, se determinó el efecto de dichos tratamientos sobre el contenido en algunos compuestos bioactivos y textura. Tanto las APH como la pasteurización provocaron cambios microestructurales, aunque los tratamientos que menos impacto tuvieron fueron las APH a 500 MPa y la pasteurización. Estos tratamientos fueron a su vez los que menos afectaron al contenido en compuestos bioactivos y textura del **pimiento rojo**. Las APH podrían ser una alternativa a la pasteurización convencional dado que el contenido en compuestos bioactivos y la textura fue similar en ambos casos. Asimismo, podrían desarrollarse nuevos alimentos funcionales mediante el uso de tejido de **pimiento rojo** sometido a APH a 500 MPa y/o pasteurización.

Las modificaciones microestructurales causadas en el tejido de **pimiento rojo** como consecuencia de la aplicación de APH y pasteurización, provocaron variaciones en los parámetros morfométricos y de textura de la imagen. La dimensión fractal de textura, el contraste, el momento de diferencia inversa y la entropía fueron parámetros de textura apropiados para caracterizar el efecto de las APH y la pasteurización sobre la textura de **pimiento rojo**. El daño celular causado por los tratamientos de conservación se observó mejor a escalas bajas.

Para el desarrollo de las nuevas salsas bechamel enriquecidas con **pimiento rojo** se emplearon dos tipos de almidón de maíz (nativo y modificado) a dos concentraciones diferentes (4 y 6 g/100 g) y diferentes cantidades de **pimiento** (0, 5 y 15 g/100 g). Se estudiaron sus propiedades reológicas, microestructura y características sensoriales. El efecto de la incorporación de **pimiento** sobre las propiedades reológicas dependió del tipo de almidón utilizado. Las salsas presentaron una considerable auto-fluorescencia intrínseca debido al elevado contenido en carotenoides del **pimiento**. Las salsas que más gustaron a los consumidores fueron las elaboradas con almidón modificado, más cremosas y consistentes. Los consumidores las encontraron beneficiosas para la salud ya que el **pimiento rojo** proporciona antioxidantes y valor nutricional y mejora el sabor de la salsa. Así, sería posible formular nuevas salsas bechamel, funcionales, cremosas, con alto valor nutricional, elevada aceptabilidad, buenas propiedades reológicas y estabilidad con **pimiento** y almidón modificado.

Resum

La present tesi doctoral es centra en l'obtenció de nous ingredients rics en compostos bioactius a partir de teixits vegetals (**caqui** i **pebrot**) sotmesos a distints tractaments de conservació com les altes pressions hidrostàtiques (APH) i la pasteurització ,amb la finalitat de formular nous aliments funcionals.

Es va estudiar l'efecte d'un tractament específic de APH (200 MPa/6 min/25 ºC) i un altre de pasteurització (70 ºC/15min) sobre l'estructura i el contingut en alguns compostos bioactius del **caqui**. Tant les APH com la pasteurització van causar canvis estructurals en el teixit parenquimàtic, van afavorir la precipitació de tanins i la formació de cèl·lules tàniques, la qual cosa podria relacionar-se amb la pèrdua de astringència del fruit. Les APH van millorar la extractabilitat de compostos carotenoides i van mantenir les propietats antioxidants del fruit. Aquesta tècnica podria ser una alternativa al tractament de pasteurització convencional. Així mateix el **caqui** tractat per APH podria ser emprat en la formulació de nous aliments funcionals, tals com a begudes làctiques enriquides amb **caqui**.

Les noves begudes làctiques, amb idèntic contingut en carotenoides, es van formular fent ús de **caqui** no tractat, sotmès a APH i pasteuritzat; i tres matrius làctiques diferents: llet sencera, semi-descremada i descremada. Les begudes elaborades amb **caqui** tractat per APH van presentar unes adequades propietats reològiques ja que ni van gelificar com les elaborades amb **caqui** no tractat, ni van sedimentar com les formulades amb **caqui** pasteuritzat. Els consumidors van percebre les noves begudes làctiques enriquides amb **caqui** com a begudes altament antioxidants. Les que més van agradar van ser les elaborades amb **caqui** tractat per APH independentment del tipus de llet utilitzada i les elaborades amb **caqui** no tractat i llet sencera. Per tant, el tractament per APH permet formular begudes làctiques enriquides amb **caqui** amb alt valor nutricional, variable contingut gras i elevada acceptabilitat independentment de la estacionalitat del fruit.

D'altra banda, es van quantificar i van localitzar alguns compostos bioactius i es van determinar algunes propietats fisicoquímiques en tres tipus de pebrot: roig, verd i groc. El contingut en compostos bioactius de cada tipus de **pebrot** va estar condicionat per la seua estructura. El tipus de **pebrot** més adequat per a obtenir extractes rics en compostos carotenoides seria el **roig**, mentre que el **groc** seria apropiat per a obtenir extractes rics en compostos fenòlics amb elevada activitat antioxidant. Finalment, si es pretén obtenir extractes amb elevat contingut en fibra dietètica el més adequat seria el **pebrot verd**.

Es va estudiar l'efecte de diferents tractaments de APH (100, 200, 300 i 500 MPa/15 min/25 ºC) i d'un tractament de pasteurització (70 ºC/10 min) sobre l'estructura de **pebrot roig**. A més, es va determinar l'efecte d'aquests tractaments sobre el contingut en alguns compostos bioactius i textura. Tant les APH com la pasteurització van provocar canvis microestructurals, encara que els tractaments que menys impacte van tenir van ser les APH a 500 MPa i la pasteurització. Aquests tractaments van ser al seu torn els que menys van afectar al contingut en compostos bioactius i textura del **pebrot roig**. Les APH podrien ser una alternativa a la pasteurització convencional atès que el contingut en compostos bioactius i la textura va ser similar en tots dos casos. Així mateix, podrien desenvolupar-se nous aliments funcionals mitjançant l'ús de teixit de **pebrot roig** sotmès a APH a 500 MPa i/o pasteurització.

Les modificacions microestructurals causades en el teixit de **pebrot roig** com a conseqüència de l'aplicació de APH i pasteurització, van provocar variacions en els paràmetres morfomètrics i de textura de la imatge. La dimensió fractal de textura, el contrast, el moment de diferència inversa i l'entropia van ser paràmetres de textura apropiats per a caracteritzar l'efecte de les APH i la pasteurització sobre la textura de **pebrot roig**. El dany cel·lular causat pels tractaments de conservació es va observar millor a escales baixes.

Per al desenvolupament de les noves salses beixamel enriquides amb **pebrot roig** es van emprar dos tipus de midó de dacsa (nadiu i modificat) a dues concentracions diferents (4 i 6 g/100 g) i diferents quantitats de **pebrot** (0, 5 i 15 g/100 g). Es van estudiar les seues propietats reològiques, microestructura i característiques sensorials. L'efecte de la incorporació de **pebrot** sobre les propietats reològiques va dependre del tipus de midó utilitzat. Les salses van presentar una considerable auto-fluorescència intrínseca a causa de l'elevat contingut en carotenoides del **pebrot**. Les salses que més van agradar als consumidors van ser les elaborades amb midó modificat, més cremoses i consistents. Els consumidors les van trobar beneficioses per a la salut ja que el **pebrot roig** proporciona antioxidants i valor nutricional i millora el sabor de la salsa. Així, seria possible formular noves salses beixamel, funcionals, cremoses, amb alt valor nutricional, elevada acceptabilitat, bones propietats reològiques i estabilitat amb **pebrot** i midó modificat.

Abstract

The research of this doctoral thesis has focused on the obtaining of new ingredients, rich in bioactive compounds using plant tissues (**persimmon** and **sweet pepper**) subjected to different preservation treatments such as high hydrostatic pressure (HHP) and pasteurization. The main aim of this thesis was to formulate new functional foods.

The effect of specific HHP (200 MPa/6 min/25 ºC) and pasteurization (70 ºC/15min) treatments on structure and content of some bioactive compounds of **persimmon** was studied. Both HHP and pasteurization treatments caused structural changes in the parenchymal tissue, as well as precipitation of tannins and formation of tannin cells, which could be related to the loss of astringency in **persimmon**. HHP processing improved the extraction of carotenoids and maintained the antioxidant properties of the fruit. HHP technique could be an alternative to pasteurization. HHP-treated persimmon could be used in the formulation of new functional foods such as milk-based beverages enriched with **persimmon**.

The new milk-based beverages, with the same carotenoid content, were formulated using untreated **persimmon**, HHP-treated **persimmon** and pasteurized **persimmon**, and three different milk matrixes: whole milk, semi-skimmed milk, and skimmed milk. Milk-based beverages elaborated using HHP-treated **persimmon** presented the best rheological properties because unlike the untreated and pasteurized **persimmon** milk-based beverages, they did not form a gel-like structure or separate out. Consumers perceived **persimmon** beverages as high antioxidant foods. The beverages with HHP-treated **persimmon**, regardless the type of milk, and the ones enriched with untreated **persimmon** and whole milk were the consumers' favourites. Therefore, HHP treatment could be an ideal method for the formulation of milk-based persimmon beverages with high nutritional value, variable fat content and high acceptability, regardless the seasonality of the fruit.

The location and content of some bioactive compounds and the analysis of some physicochemical properties were carried-out in three different **sweet pepper** types: red, green and yellow. The content of bioactive compounds in each type of **sweet pepper** was conditioned by their structure. **Red peppers** could be suitable for obtaining extracts rich in carotenoids, while **yellow peppers** would provide extracts rich in phenolic compounds with high antioxidant activity. Regarding extracts with high dietary fibre content, **green peppers** would be the most suitable ones.

The effect of different HHP (100, 200, 300 and 500 MPa/15 min/25 ºC) and pasteurization (70 °C/10 min) treatments on structure, some bioactive compounds content and texture of **red sweet pepper** was studied. Both HHP and pasteurization treatments caused structural modifications in **red sweet pepper** tissue. However, HHP at 500 MPa and pasteurization were the treatments with least impact on the microstructure. These same treatments were also the ones with least effect on bioactive compound content and texture of **red sweet pepper**. HHP treatment could be an alternative to pasteurization for sweet pepper preservation, since the texture properties and bioactive compound content were found to be similar. New functional foods could be developed using **red sweet pepper** tissues treated with HHP at 500 MPa or pasteurization as well.

Microstructural alterations in **red sweet pepper** tissues caused by HHP and pasteurization led to variations in the morphometric and texture image parameters. Fractal dimension texture, contrast, inverse difference moment and entropy were suitable texture parameters for characterizing the effect of HHP and pasteurization on **red sweet pepper** texture. Cellular damage was best observed at low magnifications.

In order to formulate new white sauces enriched with **red sweet pepper**, two different types of waxy starch (native and modified) at two different concentrations (4 and 6 g/100 g) and different amounts of **sweet pepper** (0, 5, and 15 g/100 g) were used. Rheological properties, microstructure, and sensory characteristics were studied. The effect of incorporating **sweet pepper** on the rheological properties depended upon the type of starch used. The sauces exhibited considerable intrinsic auto-fluorescence due to the presence of carotenoids from the **sweet pepper**. The sauces prepared with modified starch, which were creamier and more consistent, were the most liked. Consumers also found these sauces beneficial for health because red pepper provides antioxidants and nutritional value and improves the sauces' taste. Therefore, novel, functional, and creamy white sauces with high nutritional value, high acceptability, good rheological properties and stability could be formulated using sweet pepper and modified starch.

Introducción

1. Compuestos bioactivos

Debido a la estrecha relación que existe entre la alimentación y la salud, en la actualidad el consumidor valora de forma muy positiva aquellos alimentos que, además de proporcionar nutrientes esenciales para su vida (vitaminas, hidratos de carbono, lípidos, proteínas…), poseen sustancias con posibles efectos saludables a largo plazo, como los compuestos bioactivos, en concreto los fitoquímicos o fitonutrientes (carotenoides, fibra y flavonoides, entre otros) (Drago Serrano et al., 2006 ; Kapsak et al., 2011). Estas sustancias biológicamente activas que confieren al alimento color, aroma y sabor, y que se degradan fácilmente por la luz, el calor o las enzimas (Ferrari et al., 2010) proporcionan significativos efectos beneficiosos, entre ellos una importante actividad antioxidante (Araya et al., 2006). Numerosas publicaciones (Figuerola et al., 2008 ; Chang & Liu, 2009 ; Trinidad et al., 2009 ; Muzquiz et al., 2012 ; Abugri et al., 2013) evidencian que el consumo de alimentos ricos en compuestos bioactivos también llamados ***alimentos funcionales***, disminuye el riesgo de padecer enfermedades cardiovasculares, renales, obesidad, degeneración macular, y cáncer de colon y recto. El consumo de estos fitoquímicos además parece mitigar los efectos de la diabetes, reducir el nivel de colesterol sérico y favorecer la evacuación intestinal. Es por ello que en los últimos años la búsqueda y obtención de compuestos bioactivos para la elaboración de nuevos alimentos ha sido objeto de múltiples investigaciones (Almeida et al., 2011 ; Fuentes-Alventosa et al., 2013).

El concepto de alimento funcional tiene su origen en Japón en la década de los 80 cuando el gobierno financió un proyecto para desarrollar alimentos que mejorasen la calidad de vida de una población que había incrementado su esperanza de vida y cuyos gastos en sanidad estaban aumentando. De esta forma aparecieron alimentos desarrollados específicamente para mantener la salud y prevenir algunas enfermedades (Ohama et al., 2006). A continuación, los Estados Unidos y la Unión Europea se interesaron por los beneficios que los alimentos funcionales podrían proporcionar a personas con una alimentación desequilibrada basada en grasas saturadas y poco aporte de vitaminas y fibra (Arias-Aranda & Romerosa-Martínez, 2010). Un alimento se considera funcional si ha demostrado satisfactoriamente que tiene efectos beneficiosos en el organismo, como mejorar el estado de salud y bienestar, y reducir el riesgo de padecer algunas enfermedades, mayores de los que proporcionan los

nutrientes básicos. Para que se considere funcional, el alimento debe demostrar sus efectos en las cantidades que normalmente se consumen en la dieta (Roberfroid, 2000).

Así, alimentos tradicionales como algunas frutas y verduras han pasado a considerarse alimentos con importantes componentes bioactivos (alimentos funcionales) beneficiosos para la salud (Turner et al., 2003; Santiago-Silva et al., 2011; Martínez et al., 2012). El **caqui** (*Diospyros kaki* L. f.) es la especie frutal más importante dentro del género *Diospyros*, de la familia *Ebanaceae* (Giordani et al., 2011) y es una de las frutas con mayor contenido en compuestos bioactivos (Jung et al., 2005). En cuanto a su contenido en vitaminas y minerales, destaca la provitamina A o β-caroteno, la vitamina C y el potasio (Wright & Kader, 1997; De Ancos et al., 2000). Además del β-caroteno, el caqui contiene también otros compuestos carotenoides de importante actividad antioxidante. También presenta un alto contenido en ácidos fenólicos así como niveles elevados de fibra diétetica (Gorinstein et al., 2001). Destaca también por su importante contenido en taninos, de carácter antioxidante. La sensación de astringencia producida en algunos frutos se debe a su contenido en taninos solubles; la intensidad de esta percepción depende de la concentración de taninos solubles en el fruto. En los cultivares no astringentes el contenido en taninos solubles disminuye hasta alcanzar niveles no detectables, sin embargo en los cultivares astringentes los niveles de taninos solubles permanecen elevados hasta en fases avanzadas de desarrollo. La astringencia es una sensación de sequedad intensa y amargor que se produce en la boca al entrar en contacto ciertas sustancias químicas con la saliva (Bacon & Rhodes, 2000). Concretamente, la variedad 'Rojo Brillante' es astringente y presenta un elevado contenido en taninos solubles que va disminuyendo a medida que se alcanza la madurez. Existen numerosos estudios acerca del caqui (Vázquez-Gutiérrez et al., 2012) y su contenido en diferentes compuestos bioactivos (Gorinstein et al., 2001; Giordani et al., 2011; Vázquez-Gutiérrez et al., 2013a). Asimismo, existen investigaciones relacionadas con los beneficios para la salud que supone el consumo de caqui (Gorinstein et al., 1998; 2011). También hay estudios sobre el efecto que tienen diferentes procesados, por ejemplo las altas presiones hidrostáticas (APH) sobre la microestructura y contenido en compuestos bioactivos del caqui (De Ancos et al., 2000; Vázquez-Gutiérrez et al., 2011).

Por otro lado, el **pimiento** - *Capsicum annuum* L.- es uno de los alimentos naturales funcionales de origen vegetal, mejor evaluado y más consumido (Eggink et al., 2012 ; Zhuang et al., 2012). Además de ser una buena fuente de nutrientes esenciales, tales como hidratos de carbono, vitaminas y minerales (Nuez et al., 1996 ; Faustino et al., 2007), es rico en fibra y en otros compuestos bioactivos, como los carotenoides (capsantina, β-caroteno, β-criptoxantina, luteína, zeaxantina), con actividad antioxidante y antiinflamatoria (Duma & Alsina, 2012) y algunos compuestos fénolicos (Deepa et al., 2007 ; Serrano et al., 2010 ; Zhuang et al., 2012). El pimiento se considera un alimento de bajo aporte calórico (Elias Tierrablanca et al., 2010). Al pimiento se le atribuyen propiedades beneficiosas y su consumo parece mejorar los procesos de cicatrización, prevenir la aterosclerosis y las hemorragias, evitar el aumento de los niveles de colesterol en sangre, y mejorar la resistencia física (Faustino et al., 2007). Además de por sus propiedades nutritivas, el pimiento forma parte importante de la dieta diaria del ser humano debido a que presenta una gran versatilidad en cuanto a su uso, ya que se puede consumir tanto en fresco, como en conserva o deshidratado. En la literatura se pueden encontrar numerosas publicaciones acerca del pimiento y su contenido en compuestos bioactivos (Hornero-Méndez & Mínguez-Mosquera, 2001 ; Serrano et al., 2010 ; Zhuang et al., 2012).

Los importantes beneficiosos para la salud derivados del consumo de alimentos ricos en compuestos bioactivos son los que han motivado tanto al colectivo científico como a la industria hacia la búsqueda de nuevos alimentos funcionales más saludables (Arias-Aranda & Romerosa-Martínez, 2010; Lamberti & Lettieri, 2011).

1.1. Carotenoides

Los carotenoides son pigmentos que se encuentran en las plantas. En una dieta estándar se consumen cantidades significativas de compuestos carotenoides, pues son los responsables del color amarillo, naranja y rojo de la mayoría de frutas y hortalizas. La síntesis de carotenoides se ve influenciada por el genotipo, la zona de cultivo, las prácticas agrícolas, el almacenamiento, el procesado y la preparación del fruto (Deepa et al., 2007; Rodríguez-Amaya et al., 2008). Los carotenoides, al ser compuestos coloreados, contienen un gran número de dobles enlaces conjugados que se oxidan fácilmente, siendo éste su principal mecanismo de degradación y

produciéndose como consecuencia una pérdida del color. En los tejidos vegetales, los carotenoides están protegidos de la oxidación, pero la lesión de estos tejidos o la extracción de los mismos aumenta la susceptibilidad a la oxidación. Son compuestos lipófilos, solubles en aceites y disolventes orgánicos. Es por ello que los procedimientos de extracción para la determinación cuantitativa de los carotenoides utilizan disolventes orgánicos que penetran en la matriz hidrófila. Su color varía del amarillo al rojo, por lo que las longitudes de onda para su detección oscilan en el intervalo 430-480 nm (Fennema, 2000).

Los carotenoides tienen acción antioxidante y actúan neutralizando los radicales libres que se encuentran en el cuerpo humano (Fernández-García et al., 2012). Dichas propiedades antioxidantes tienen un papel importante en la prevención del cáncer, las cataratas, la ateroesclerosis y el proceso de envejecimiento (Krinsky & Johnson, 2005; Hu et al., 2009). Numerosos estudios (Cantuti-Castelvetri et al., 2000; Yamaguchi & Uchiyama, 2003; Rao & Rao, 2007; Xu et al., 2013) han demostrado una alta correlación entre el consumo de carotenoides y un menor riesgo de padecer ciertas enfermedades, tales como el cáncer, enfermedades cardiovasculares, la aterogénesis, la calcificación de los huesos, la degeneración ocular y el daño neuronal. Algunos de los carotenoides, aproximadamente el 10% de los más de 600 identificados en la naturaleza, son precursores de la vitamina A. Los principales carotenoides responsables de la coloración del caqui y el pimiento con actividad antioxidante son el β-caroteno, capsantina, licopeno, β-criptoxantina, zeaxantina y luteína. El β-caroteno y la β-criptoxantina además son precursores de la vitamina A (De Ancos et al., 2000; Guil-Guerrero et al., 2006; O'Sullivan et al., 2010).

1.2. Compuestos fenólicos

Los compuestos fenólicos son metabolitos secundarios de las plantas que poseen en su estructura al menos un anillo aromático al que está unido uno o más grupos hidroxilo. Son esenciales en la alimentación y de gran interés debido a sus propiedades antioxidantes (Tapsell et al., 2006). El consumo de frutas y verduras es la mayor fuente de estos antioxidantes naturales, siendo por lo tanto su consumo altamente beneficioso para la salud. Son sustancias biológicamente activas con propiedades antialérgicas, antiinflamatorias, antiaterogénicas, antimicrobiales, antioxidantes,

antitrombóticas, cardioprotectores y con efectos vasodilatadores (Middleton et al., 2000; Manach et al., 2004). Asimismo debido a sus propiedades antioxidantes que protegen del daño oxidativo celular incluyendo la peroxidación lipídica, presentan potencial antimutagénico (Chung et al., 1998; Royer et al., 2011).

Los compuestos fenólicos se clasifican en ácidos fenólicos, flavonoides y taninos. Estos últimos son compuestos con un peso molecular relativamente alto y se dividen en dos grandes categorías en función de su estructura: taninos hidrolizables y no hidrolizables (condensados). El **caqui** es rico en taninos condensados (proantocianidinas del grupo B), tienen la propiedad de formar complejos estables con metales y proteínas, siendo por ello responsables de la astringencia característica del fruto (Santos-Buelga & Scalbert, 2000; Nakatsubo et al., 2002). La conversión de taninos solubles (astringentes) a insolubles (no astringentes) se produce durante la maduración o por determinados tratamientos, como la aplicación de atmósferas modificadas con etanol o CO_2 (Arnal & Del Río, 2003). De esta forma se produce la reducción de la sensación de astringencia durante el crecimiento y la maduración de las variedades astringentes y la desaparición de ésta en las no astringentes. Por otro lado, el **pimiento** es rico en ácidos fenólicos, flavonoides, hidroxicinamatos y flavonas (Marin et al., 2004). Los compuestos fenólicos del pimiento, en particular los flavonoides, son conocidos por sus propiedades antioxidantes y secuestradoras de radicales libres. Gracias a estas propiedades antioxidantes, los flavonoides protegen contra enfermedades oxidativas y cardiovasculares (Gülçin, 2012).

1.3. Fibra diétetica

Según la American Association for Clinical Chemistry (AACC, 2001), la fibra dietética se define como la parte comestible de la planta, principalmente hidratos de carbono, resistente a la digestión y absorción en el intestino delgado, con fermentación completa o parcial en el intestino grueso. La fibra dietética incluye polisacáridos, oligosacáridos, lignina y otras sustancias asociadas a estos componentes de los tejidos vegetales (Selvendran & MacDougall, 1995), y promueve efectos fisiológicos beneficiosos en el organismo, como el laxante, y/o la atenuación de niveles de colesterol y de glucosa en sangre. En función de su solubilidad en agua, la fibra se divide en insoluble y soluble; la fracción insoluble de la fibra parece estar relacionada

con la regulación del tracto intestinal, mientras que la fibra soluble está relacionada con la disminución en los niveles de colesterol en sangre y de absorción de glucosa intestinal (Ramulu & Udayasekhara, 2003). Según Rodríguez et al. (2006), la fibra dietética puede influir en la biodisponibilidad de los hidratos de carbono en el tracto gastrointestinal, este efecto ha sido confirmado en pacientes con diabetes cuyos niveles de glucosa en sangre disminuyeron al tener una dieta rica en fibra. Además, los polisacáridos no almidonados que forman parte de la fibra tienen alta capacidad antioxidante (Zha et al., 2009). El procesado puede afectar a los carbohidratos que forman parte de la fibra dietética. Está demostrado que procesos como la cocción o el calentamiento por microondas generan variaciones importantes en el contenido de fibra dietética y cambios estructurales en los tejidos (Zia-ur-Rehman et al., 2003). Por otro lado los procesos térmicos convencionales ocasionan una degradación importante de polisacáridos pécticos. Esta degradación produce una menor adhesión intercelular y mayor ablandamiento, originando un fruto menos atractivo para el consumidor. Sería importante comprobar y estudiar si otros tipos de procesados, no tan convencionales y más novedosos, como las APH producen algún tipo de modificación en la fracción de fibra dietética.

1.4. Capacidad antioxidante

Los estudios relacionados con los radicales libres, especies químicas con uno o más electrones desapareados, ha permitido conocer su funcionamiento y efectos sobre el organismo. Numerosos trabajos de investigación señalan que el oxígeno derivado de los radicales libres podría ser dañino para la salud (Dormandy, 1980; Slater, 1984). Desde entonces, la búsqueda de agentes capaces de proteger frente a la acción de los radicales libres (compuestos antioxidantes) ha sido el objetivo de muchas investigaciones. Se conoce como antioxidante a aquella molécula que es capaz de inhibir la oxidación de otras moléculas. En términos alimentarios, se denomina antioxidante a cualquier sustancia que, estando presente en bajas concentraciones en comparación con los sustratos oxidables, retrasa o previene de forma importante la oxidación de dichos sustratos. El término "sustrato oxidable" hace referencia a diferentes componentes como proteínas, lípidos, hidratos de carbono y ADN, entre otros (Halliwell et al., 1995; Gülçin, 2012).

En las células existe un balance pro-oxidante-antioxidante, que puede desplazarse hacia el lado de los pro-oxidantes cuando la producción de oxígeno aumenta o los niveles de antioxidantes disminuyen, apareciendo así un estado conocido como estrés oxidativo. Todos los organismos aerobios disponen de defensas antioxidantes para eliminar o reparar las moléculas dañadas. Sin embargo, la incorporación de más compuestos con acción antioxidante mediante la alimentación, mejora estas defensas (Gülçin, 2012).

Muchas enfermedades crónicas, como el cáncer o enfermedades cardiovasculares, están relacionadas con el daño producido por la oxidación celular. Existen estudios que demuestran que ciertos fitonutrientes presentes en frutas y verduras son beneficiosos para la salud ya que protegen al cuerpo humano frente a los daños producidos por especies de oxígeno reactivo (Hu et al., 2009; Fernández-García et al., 2012). Existe, por tanto una relación entre el consumo de alimentos con elevada capacidad antioxidante y sus beneficios para la salud y se ha demostrado que cuanto mayor es su consumo menor es la incidencia de enfermedades (Ramarathnam et al., 1995; Chang & Liu, 2009).

Los principales compuestos antioxidantes presentes en los alimentos son la vitamina C, la vitamina E, los carotenoides y los compuestos fenólicos que protegen a las células del estrés oxidativo (Podsędek, 2007). De estos últimos, los más importantes son los tocoferoles, flavonoides y ácidos fenólicos (Gülçin, 2012). El caqui y el pimiento, destacan por su alto contenido en carotenoides y compuestos polifenólicos, principalmente taninos en el caso del caqui y flavonoides en el del pimiento, que por ser importantes antioxidantes protegen frente a los radicales libres y previenen del riesgo de padecer enfermedades cardiovasculares, diabetes y cáncer (George & Redpath, 2008; Serrano et al., 2010).

2. Métodos de conservación de alimentos

La necesidad de aumentar la vida útil de los alimentos ha sido la causante del desarrollo de los métodos de conservación. Los alimentos vegetales que se consumen en fresco pierden calidad desde el momento que son cosechados como consecuencia de cambios físicos, químicos y/o microbiológicos. Los microorganismos y las enzimas

son los principales agentes responsables del deterioro de los alimentos. Hasta ahora, el método de conservación más comúnmente utilizado para preservar los alimentos ha sido el tratamiento térmico o pasteurización debido a su capacidad para inhibir el crecimiento microbiano e inactivar enzimas (polifenoloxidasa, pectinmetilesterasa, etc.). Sin embargo, el calor puede inducir cambios químicos y físicos que pueden dañar las propiedades organolépticas del alimento así como reducir la biodisponibilidad y contenido en ciertos compuestos bioactivos, especialmente si se aplica bajo condiciones severas (Patras et al., 2009; Rawson et al., 2010). El hecho de que no sólo la vida útil del alimento, sino también su calidad sean importantes para los consumidores, ha llevado al nacimiento del concepto de conservación de alimentos usando métodos no térmicos. Por ello, la investigación en la industria alimentaria se ha dirigido a reemplazar los métodos de conservación tradicionales por nuevas metodologías, entre ellas las altas presiones hidrostáticas (APH), que se adapten mejor al tipo de alimentos que los consumidores demandan.

2.1. La pasteurización como método tradicional de conservación de alimentos

La aplicación industrial de conservación de alimentos por calor comenzó tras la investigación de Nicolas Appert, quien demostró la posibilidad de conservar durante un largo periodo de tiempo diferentes tipos de alimentos tras calentarlos en recipientes herméticamente cerrados. Posteriormente, tras conocer que el deterioro de los alimentos era de origen microbiano, empezaron los estudios cinéticos como base para el diseño de los tratamientos térmicos. El calor se utiliza para frenar la actividad enzimática y bacteriana reduciendo de este modo el deterioro del alimento y previniendo su pérdida de calidad.

Los métodos de conservación por calor son los más utilizados en la industria agroalimentaria por su gran efectividad, y según la intensidad del proceso se clasifican en dos categorías, esterilización y pasteurización (Berk, 2009). En la pasteurización, el alimento se somete a temperaturas entre 70-100 ºC durante el tiempo necesario para destruir microorganismos y enzimas específicos. La pasteurización es un tratamiento efectivo en la inactivación de microrganismos como la *Salmonella* (Silva & Gibbs, 2012)

aunque no actúa sobre patógenos en forma de espora. El almacenamiento refrigerado y valores bajos de pH contribuyen a mejorar la efectividad de este tratamiento.

La principal desventaja de esta tecnología es que el calor aplicado produce alteraciones en la composición de los alimentos, dando lugar en muchos casos a pérdidas de calidad sensorial (productos reblandecidos, pérdida de sabor y color) y de calidad nutritiva como las vitaminas principalmente. Existen estudios que demuestran la pérdida de color en zumo de naranja pasteurizado, como consecuencia de la disminución del contenido en carotenoides (Torres-Gama & De Sylos, 2007). Asimismo, según Rawson et al. (2011), muchos autores han observado como la pasteurización conduce a una disminución del contenido en bioactivos (carotenoides, compuestos polifenólicos y actividad antioxidante) de algunas frutas y verduras tales como frutas exóticas (Elez-Martínez et al., 2006; Chin et al., 2010), mangos (Vásquez-Caicedo et al., 2007, Djioua et al., 2009; Kim et al., 2009), extracto de fruta de mora (Aramwit et al., 2010), zumo de piña (Rattanathanalerk et al., 2005) y zumo de manzana y anacardo (Zepka & Mercadante, 2009).

En lo relativo al contenido en fibra, Kutoš et al. (2003) estudiaron el efecto del procesado térmico a elevadas temperaturas en frijoles enlatados, y comprobaron que el tratamiento térmico producía solubilización de algunos polisacáridos (hemicelulosas y sustancias pécticas) y disminución en el contenido en fibra total, causado principalmente por las pérdidas de fibra soluble. Por otra parte, Elleuch et al. (2011), concluyeron que las modificaciones en el contenido en fibra total producidas por el tratamiento térmico dependían del tipo de material celular y de las condiciones del tratamiento.

El efecto negativo del tratamiento térmico sobre la calidad y los compuestos bioactivos de los alimentos es lo que ha llevado a la búsqueda de otros tratamientos de conservación en los que no se aplique calor y que sirvan de alternativa a la pasteurización convencional con la finalidad de conseguir alimentos con mejores propiedades organolépticas y nutritivas.

2.2. Las altas presiones hidrostáticas (APH) y su relación con el contenido en compuestos bioactivos

La demanda por parte de los consumidores de alimentos de conveniencia, fáciles de preparar o de consumir, seguros, con características sensoriales de frescura, y con propiedades biológicas más allá de las nutricionales ha motivado a los investigadores e industriales a desarrollar nuevas tecnologías de procesado y conservación. Así, tecnologías no-térmicas como la aplicación de pulsos eléctricos (Saldaña et al., 2011), la luz ultravioleta (Bintsis et al., 2000) y el calentamiento óhmico (Louarme & Billaud, 2012), entre otras, han sido las más estudiadas en los últimos años para obtener alimentos seguros, manteniendo sus propiedades organolépticas y sin afectar al contenido en compuestos bioactivos (Oms-Oliu et al., 2012). De entre estas nuevas tecnologías, el procesado por APH es una de las que ha recibido mayor atención por parte de investigadores e industriales (Devlieghere et al., 2004 ; Rendueles et al., 2011). Varios autores (Estrada-Girón et al., 2005 ; Rastogi et al., 2007 ; Buzrul, 2012) consideran a la tecnología de las APH la más viable comercialmente entre las tecnologías no-térmicas estudiadas tras los buenos resultados obtenidos al aplicar altas presiones sobre diferentes productos.

El procesado de alimentos por APH consiste en la aplicación de presión al alimento con una intensidad entre 50 y 1000 MPa. Este tratamiento puede efectuarse solo o en combinación con otras técnicas como tratamientos térmicos suaves, ultrasonidos, CO_2, etc. El principal objetivo de esta técnica es la inactivación de microorganismos y enzimas a temperaturas lo suficientemente bajas que eviten los efectos producidos por los tratamientos térmicos tradicionales, permitiendo obtener de esta manera alimentos seguros y saludables, manteniendo su calidad sensorial y organoléptica. Además, al preservar el contenido en compuestos bioactivos, la aplicación de APH resulta idónea durante la producción de alimentos funcionales (Ferrari et al., 2010). La principal ventaja de las APH frente a la pasteurización es que la presión aplicada es la misma en todas las direcciones y se transmite uniformemente al producto independientemente de su tamaño y geometría (Oey et al., 2008).

El potencial y las limitaciones del procesado de alimentos mediante APH ha sido extensamente revisado y la gran mayoría de los estudios realizados se han centrado en su efecto sobre la inactivación microbiana y enzimática. Sin embargo, el efecto que

ejerce esta tecnología sobre la microestructura del alimento y las consecuencias de los cambios microestructurales ocasionados por el tratamiento sobre el contenido en compuestos nutricionales y bioactivos, ha sido menos estudiado. En este sentido, Vázquez-Gutiérrez et al. (2013b) estudiaron los cambios en la microestructura y en las propiedades antioxidantes de cebolla tratada por APH, y comprobaron que las APH producían cambios estructurales y potenciaban la extractabilidad de fenoles y otros compuestos con actividad antioxidante. Por otro lado, Vázquez-Gutiérrez et al. (2012) al estudiar el efecto de las APH en la estructura, difusión de compuestos solubles y propiedades texturales del caqui, concluyeron que las APH favorecían la difusión y extractabilidad de taninos y otros compuestos solubles a los espacios intercelulares al mismo tiempo que disminuían la firmeza y la cohesividad del caqui. Asimismo, Vázquez-Gutiérrez et al. (2011) observaron cómo disminuía el contenido en taninos solubles del caqui al tratarlo por APH debido a la precipitación de taninos que el tratamiento provoca.

En cuanto al contenido en carotenoides, diversos autores al estudiar el efecto de las APH en tomate (Butz et al., 2002), zumo de naranja, limón y zanahoria (Butz et al., 2003), gazpacho (Plaza et al., 2006), y zanahoria y brócoli (McInerney et al., 2007), no encontraron diferencias en el contenido en carotenoides entre las muestras tratadas por APH y las no tratadas. Barba et al. (2010), al estudiar el efecto de las APH (100, 200, 300 y 400 MPa) sobre el contenido en carotenoides totales en una bebida a base de vegetales, establecieron que tratamientos a 100 y 400 MPa produjeron una disminución significativa en el contenido de carotenoides. Resultados similares encontraron Patras et al. (2009) en purés de tomate al aplicar presiones de 400 y 500 MPa durante 15 min. Por otro lado, Plaza et al. (2012) obtuvieron un aumento significativo en la extractabilidad de carotenoides al someter al caqui a diferentes tratamientos de APH. Del mismo modo, Sánchez-Moreno et al. (2005; 2006) observaron un aumento en la extractabilidad de carotenoides al aplicar APH en zumo de naranja y zumo de tomate, respectivamente.

En relación a la capacidad antioxidante, Clariana et al. (2011), al estudiar el efecto de las APH en nabo, obtuvieron que al aumentar la presión de tratamiento, la disminución en la capacidad antioxidante fue menor, llegando a no observarse diferencias significativas con respecto a la muestra no tratada cuando la presión fue de

600 MPa. Butz et al. (2003) no encontraron cambios significativos en la capacidad antioxidante de varias muestras (naranja, zanahoria, manzana, tomate, zumo) tratadas por APH comparadas con las no tratadas. Por otro lado, Sánchez-Moreno et al. (2005) al comparar los efectos de las APH (400 MPa) y pasteurización (70 ºC) sobre la capacidad antioxidante de zumo de naranja no observaron cambios significativos. McInerney et al. (2007) observaron cómo, en función del vegetal en estudio, las APH ejercen un efecto diferente sobre la capacidad antioxidante; así mientras que no afectaron significativamente a la capacidad antioxidante del brócoli, sí provocaron una disminución significativa de ésta en zanahoria al trabajar a presiones inferiores a 400 MPa.

3. Estructura del alimento y extractabilidad de compuestos bioactivos

Está demostrado que cuando se consumen productos naturales, la asimilación de algunos compuestos bioactivos es relativamente baja respecto a la cantidad ingerida (Boileau et al., 1999). Los compuestos bioactivos presentes en los alimentos vegetales se encuentran más o menos accesibles en el tracto gastrointestinal dependiendo de muchos factores, entre ellos, la variedad, el estado de maduración, la estructura de la matriz vegetal, la interacción con otros componentes de la matriz vegetal y el procesado del alimento (Parada & Aguilera, 2007). El efecto beneficioso de los nutrientes y de los compuestos bioactivos depende de su bioaccesibilidad y biodisponibilidad (Rein et al., 2013). La bioaccesibilidad hace referencia a la fracción de un nutriente que se libera de la matriz de un alimento en el tracto gastrointestinal. Por otro lado, la biodisponibilidad es la fracción de un compuesto que se absorbe durante el proceso digestivo completo. La biodisponibilidad de compuestos bioactivos, como la fibra, los fenoles y los carotenoides parece depender de factores relacionados con la matriz alimentaria, y con el estado nutricional y el perfil genético del individuo (Maiani et al., 2009). La absorción de compuestos bioactivos requiere la liberación de éstos desde la matriz vegetal; en este aspecto el procesado del alimento puede tener cierta influencia, ya que puede afectar a la estructura del material favoreciendo en primer lugar su extractabilidad, y por tanto su bioaccesibilidad y biodisponibilidad. En el caso concreto de los carotenoides, su biodisponibilidad puede ser baja y muy variable, a pesar de encontrarse en alta concentración en las materias

primas (Faulks & Southon, 2005), ya que pueden estar fuertemente asociados a paredes celulares e incluidos en orgánulos celulares (Fernández-García et al., 2012). En este sentido, la caracterización microestructural de los tejidos ayuda a comprender los mecanismos que pueden influir en la extractabilidad y biodisponibilidad de estos compuestos bioactivos. Entender estos mecanismos es un paso previo fundamental en el conocimiento de su bioaccesibilidad, e imprescindible para optimizar el aprovechamiento de los efectos beneficiosos que presentan estos componentes para la salud, entre ellos su poder antioxidante.

Está demostrado que algunos tratamientos de conservación (deshidratación osmótica, atmósferas modificadas, fritura, microondas, congelación, etc.) a los que se someten los alimentos producen modificaciones a nivel estructural (Llorca et al., 2003; Soliva-Fortuny et al., 2003; Quiles et al., 2004; Guardeño et al., 2011; Hernández-Carrión et al., 2011), y podrían influir en la fracción de nutrientes que se libera desde la matriz alimentaria y por lo tanto en la fracción que se absorbe durante la digestión. Por otro lado, estudios previos han mostrado un aumento significativo en la extracción de los carotenoides en caqui y puré de caqui tratados por APH (De Ancos et al., 2000; Plaza et al., 2012) lo que podría indicar que este tratamiento no térmico puede favorecer la extractabilidad de compuestos bioactivos y su mayor aprovechamiento en el momento de la ingesta del alimento. Se ha observado también como la aplicación de las APH parece aumentar la biodisponibilidad de vitaminas y otros componentes de bajo peso molecular en zumo de naranja y gazpacho (Oey et al., 2008). Además, algunos autores han afirmado que la aplicación de APH a alimentos ricos en micronutrientes y fitoquímicos, tales como minerales, carotenoides y otros compuestos antioxidantes podría resultar útil para el desarrollo de productos más saludables por un posible aumento en su bioaccesibilidad (Sánchez-Moreno et al., 2009; Briones-Labarca et al., 2011). Es por ello que la caracterización microestructural de estos alimentos ayudaría a dilucidar si determinadas formas de actuación sobre el alimento podrían influir en la extractabilidad de estos componentes desde la matriz alimentaria.

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Objetivo

El objetivo de la presente tesis doctoral es obtener nuevos ingredientes ricos en compuestos bioactivos a partir de tejidos vegetales (caqui y pimiento) sometidos a distintos tratamientos de conservación como las altas presiones hidrostáticas y la pasteurización. Estos tratamientos podrían favorecer la extractabilidad de los compuestos bioactivos lo que permitiría la formulación de nuevos alimentos funcionales. Para ello se evaluará la microestructura de los tejidos vegetales seleccionados, su contenido en algunos compuestos bioactivos (contenido en carotenoides, compuestos fenólicos, fibra dietética y capacidad antioxidante), textura y propiedades fisicoquímicas. Asimismo, se estudiará la estructura y reología de los nuevos alimentos funcionales obtenidos y se llevará a cabo un análisis sensorial con el fin de determinar sus propiedades sensoriales y valorar la aceptabilidad de los mismos.

Estructura de la tesis

La presente tesis doctoral se enmarca dentro del proyecto del Ministerio de Ciencia e Innovación titulado "Efecto del procesado por altas presiones hidrostáticas sobre la microestructura de vegetales con alto contenido en carotenoides". En este proyecto se ha pretendido avanzar en el estudio microestructural de tejidos vegetales sometidos a diferentes tratamientos de conservación, altas presiones hidrostáticas y pasteurización, y relacionar los cambios sufridos en la microestructura con la extractabilidad de ciertos componentes bioactivos. Se ha estudiado el efecto de dichos tratamientos de conservación sobre la textura y propiedades fisicoquímicas de los tejidos vegetales seleccionados así como las propiedades estructurales, reológicas y sensoriales de los nuevos alimentos funcionales formulados a partir de dichos tejidos vegetales. El proyecto aborda el estudio de dos tejidos vegetales con alto contenido en compuestos bioactivos: una fruta, el caqui, y una verdura, el pimiento.

En el caso del caqui dado el conocimiento previo del que se dispone sobre el efecto de las altas presiones en caqui, el primer objetivo consiste en el estudio de un tratamiento específico de altas presiones sobre la microestructura y contenido en compuestos bioactivos y la comparación de los resultados obtenidos con un tratamiento térmico de pasteurización. El tratamiento, tanto por altas presiones hidrostáticas como por pasteurización, permite prolongar la vida útil del caqui y evitar el problema de su estacionalidad. A continuación, el trabajo se centra en la formulación de nuevos alimentos funcionales elaborados con caqui. Los nuevos alimentos funcionales se formulan de manera que el contenido en carotenoides sea el mismo en todos los casos y se estudia su microestructura y propiedades reológicas. Por último, se lleva a cabo un completo análisis sensorial haciendo uso de un panel semi-entrenado de jueces y consumidores con el que se logran definir las propiedades sensoriales de los nuevos alimentos funcionales elaborados con caqui así como conocer la aceptabilidad de los mismos.

En el caso del pimiento, el primer objetivo consiste en la selección del tipo de pimiento más adecuado para la formulación de alimentos funcionales. Para ello, se estudia la microestructura y contenido en compuestos bioactivos de tres tipos diferentes de pimiento (rojo, verde y amarillo). Una vez seleccionado el tipo de pimiento, el rojo debido a su mayor contenido en compuestos bioactivos, el trabajo se centra en el estudio del efecto de diferentes tratamientos de altas presiones

hidrostáticas y pasteurización sobre su microestructura, textura y compuestos bioactivos. A continuación, el trabajo consiste en la cuantificación, haciendo uso de herramientas de análisis de imagen, del efecto de dichos tratamientos de conservación sobre la microestructura de pimiento rojo, en concreto sobre los parámetros morfométricos y de textura de la imagen. Por último, el trabajo se centra en la formulación de nuevas salsas bechamel enriquecidas con pimiento rojo. Las salsas se formulan utilizando dos tipos de almidón de maíz, nativo y modificado, a dos concentraciones diferentes y haciendo uso de dos concentraciones diferentes de pimiento rojo. Se estudia su comportamiento reológico, microestructura, propiedades físicas y sensoriales así como la aceptabilidad de las nuevas salsas enriquecidas con pimiento.

El contenido de la tesis se divide en dos capítulos que recogen los objetivos principales de este trabajo, el primero de ellos dedicado al caqui y el segundo al pimiento. Las publicaciones científicas derivadas de esta tesis se presentan a lo largo de los capítulos en el siguiente orden:

Capítulo 1:

Impact of high hydostratic pressure and pasteurization on the structure and the extractability of bioactive compounds of persimmon "Rojo Brillante". María Hernández Carrión, José Luis Vázquez-Gutiérrez, Isabel Hernando and Amparo Quiles. Journal of Food Science (2014) 79 (1), C32-C38.

High hydrostatic pressure treatment provides persimmon good characteristics to formulate milk-based beverages with enhanced functionality. María Hernández Carrión, Amparo Tárrega, Isabel Hernando, Susana Fiszman and Amparo Quiles. Food & Function (2014) 5 (6), 1250-1260.

Persimmon milkshakes with enhanced functionality: understanding consumers' perception of the concept and sensory experience of a functional food. María Hernández Carrión, Paula Varela, Isabel Hernando, Susana Fiszman and Amparo Quiles. LWT-Food Science and Technology. DOI: 10.1016/j.lwt.2014.10.063.

Capítulo 2:

Tissue microstructure, physicochemical properties, and bioactive compound locations in different sweet pepper types. María Hernández Carrión, Isabel Hernando and Amparo Quiles. Food Science and Technology International. DOI: 10.1177/1082013213501167.

High hydrostatic pressure treatment as an alternative to pasteurization to maintain bioactive compound content and texture in red sweet pepper. María Hernández Carrión, Isabel Hernando and Amparo Quiles. Innovative Food Science and Emerging Technologies. DOI: 10.1016/j.ifset.2014.06.004.

Use of image analysis to evaluate the effect of high hydrostatic pressure and pasteurization as preservation treatments on the microstructure of red sweet pepper. María Hernández Carrión, Isabel Hernando, Indira Sotelo-Díaz, María Ximena Quintanilla-Carvajal and Amparo Quiles. Innovative Food Science and Emerging Technologies. DOI: 10.1016/j.ifset.2014.10.011.

New formulations of white sauces with enhanced functionality. A rheological, microstructural and sensory study. María Hernández Carrión, Teresa Sanz, Isabel Hernando, Empar Llorca, Susana Fiszman and Amparo Quiles. European Food Research and Technology. En revisión.

Resultados y discusión

Capítulo 1

Impact of high hydrostatic pressure and pasteurization on the structure and the extractability of bioactive compounds of persimmon 'Rojo Brillante'

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Abstract

'Rojo Brillante' is an astringent oriental persimmon variety with high levels of bioactive compounds such as soluble tannins, carotenoids, phenolic acids, and dietary fibre. The purpose of this study was to investigate the effects of high hydrostatic pressure (HHP) and pasteurization on the structure of the fruit and on the extractability of certain bioactive compounds. The microstructure was studied using light microscopy, transmission electron microscopy, and low temperature scanning electron microscopy, and certain physicochemical properties (carotenoid and total soluble tannin content, antioxidant activity, fibre content, colour, and texture properties) were measured. The structural changes induced by HHP caused a rise in solute circulation in the tissues that could be responsible for the increased carotenoid level and the unchanged antioxidant activity in comparison with the untreated persimmon. In contrast, the changes that took place during pasteurization lowered the tannin content and antioxidant activity. Consequently, HHP treatment could improve the extraction of potentially bioactive compounds from persimmons. A high nutritional value ingredient to be used when formulating new functional foods could be obtained using HHP.

Keywords: bioactive compounds, high hydrostatic pressure, microstructure, pasteurization, persimmon

1. Introduction

Oriental persimmons or kakis (*Diospyros kaki* L. f.) are among the fruits with the highest levels of bioactive compounds (Jung et al., 2005). They contain vitamins and minerals, particularly provitamin A (β-carotene), vitamin C, and potassium (Wright & Kader, 1997; De Ancos et al., 2000). As well as β carotene, they contain other carotenoid compounds with considerable antioxidant activity. They also have high phenolic acid and dietary fibre contents (Gorinstein et al., 2001) and large quantities of tannin, an antioxidant that is responsible for the fruit's astringency. The 'Rojo Brillante' variety, specifically, is an astringent type persimmon (Tárrega et al., 2013). This variety has a high-soluble tannin content that gradually falls as the fruit ripens.

The greater or lesser extent to which the bioactive compounds in fruit and vegetables are accessible in the digestive tract depends on many factors, including the variety, stage of ripeness, structure of the plant matrix, interaction with other components of the plant matrix, and how the food has been processed (Parada & Aguilera, 2007). Previous studies have shown a significant increase in carotenoid extraction from persimmons and persimmon puree subjected to high-pressure treatment (De Ancos et al., 2000; Plaza et al., 2012), which could indicate that this non-thermal treatment could favour the extractability of bioactive compounds when the food is ingested. High hydrostatic pressure (HHP) treatments also seem to increase the bioavailability of vitamins and other low molecular weight compounds in orange juice and gazpacho (a cold tomato soup) (Oey et al., 2008b).

The main aim of HHP processing is to obtain healthy and suitable foods of high sensory quality. HHP facilitates the production of food products that have the quality of fresh foods but the convenience and profitability associated with shelf life extension (McClements et al., 2001). HPP can be applied to a range of different foods, including juices and beverages, fruits and vegetables, meat-based products, fish and precooked dishes, with meat and vegetables being the most popular applications (Norton & Sun, 2008). The potential and limitations of processing foods with HHP have been reviewed extensively (Hendrickx et al., 1998; Oey et al., 2008a; 2008b). Most of the studies of this method have focused on its microbe and enzyme inactivating effects. The effects of this technology on nutritional and bioactive compounds and on the microstructure of the food have received less attention. To

understand the bioavailability of certain nutritional components of foods such as carotenoids, it is essential to characterize the microstructure of plant tissues and the changes that take place during their industrial processing.

The aim of the present study was to compare the effects of an emerging non-thermal treatment such as HHP and of a conventional thermal treatment on the structure of persimmons and the extractability of certain bioactive compounds. In this way, improving their nutritional properties, it would be possible to make use of astringent persimmon varieties in functional food formulations.

2. Materials and methods

2.1. Sample preparation

Persimmon fruits cv. 'Rojo Brillante' were harvested in Carlet (Valencia, Spain) at the beginning of November of 2011. The maturity index was selected following the method of Salvador et al. (2007) where 6 maturity stages are accordingly defined, ranging from I (yellow green) to VI (orange red). Stage IV of this scale was studied in this work. Fruit was not treated for astringency. Cubes (15 mm) were taken from the equatorial area and heat-sealed in 110 × 220 mm plastic bags (Doypack type®, Amcor, Spain). Each bag contained approximately 80 g of sample. One third of the bags was placed inside a hydrostatic pressure unit (HHP-treated samples) with a 2350 mL capacity and water was used as the pressure medium (GEC Alsthom ACB 900 HP®, type ACIP 665, Nantes, France). The pressure employed in the treatment was 200 MPa during 6 min at 25 ºC, based on previous studies (Plaza et al., 2012). Another third of the bags was submitted to a pasteurization process (pasteurized samples) in a water bath at 70 ºC during 15 min. The final third of the bags was not submitted to treatment (untreated samples). Then, the bags were stored at 4 ºC until their analysis. Microstructure, colour, and texture properties were analyzed within 24 h after the treatment.

2.2. Microstructural analysis

2.2.1. Light Microscopy (LM)

For the LM, samples were fixed with a 25 g L^{-1} glutaraldehyde solution (0.025 M phosphate buffer, pH 6.8, at 4 ºC, 24 h), postfixed with a 20 g L^{-1} OsO_4 solution (1.5 h), dehydrated using a graded ethanol series (300, 500, and 700 g kg^{-1}), contrasted in 20 g L^{-1} uranyl acetate dissolved in ethanol (2 h) and embedded in epoxy resin (Durcupan® ; Sigma-Aldrich, St. Louis, Mo., U.S.A.). The samples were cut using a Reichert Jung ultramicrotome (Leica Mycrosystems®, Wetzlar, Germany). Semithin sections (1.5-µm-thick) were stained with 2 g L^{-1} toluidine blue and examined in a Nikon Eclipse 80i® light microscope (Nikon, Tokyo, Japan).

2.2.2. Transmission Electron Microscopy (TEM)

The samples followed the same protocol of fixation, dehydration, and infiltration as for LM. Ultramicrotomy was carried out in the same equipment, but in this case 0.05-µm-thick sections were collected. Ultrathin sections were stained with 40 g L^{-1} lead citrate and 20 g L^{-1} uranyl acetate and observed in a Philips EM 400® (Philips, Eindhoven, Holland) transmission electronic microscope at 80 kV.

2.2.3. Low temperature scanning Electron Microscopy (CryoSEM)

A JSM5410® SEM microscope (JEOL, Tokyo, Japan) was used with a Cryo CT500 C® unit (Oxford Instruments, Witney, U.K.) for the CryoSEM observation. Samples (1-mm-thick) were placed in the holder, fixed with nitrogen slush ($T \leq -210$ ºC), transferred frozen to the Cryo unit, fractured, etched (−90 ºC), and gold-coated (10^{-2} bar and 40 mA). Samples were then transferred to the microscope and examined at 15 kV,−130 ºC, and at a working distance of 15 mm.

2.2.4. Image analysis

The image analysis was carried out using ImageJ software (Rasband, W.S., ImageJ v. 1.43s, Natl. Inst. of Health, Bethesda, Md., U.S.A.). The area of the cells was determined using LM images, while the thickness of the cell walls was determined

using TEM images. Both area and thickness were assessed from at least 6 randomly acquired LM and TEM images, respectively. The cells and cells walls were manually labelled and their area (μm^2) and thickness (μm) measured from each image.

2.3. Physicochemical analysis

2.3.1. Persimmon purée preparation

A total of 120 g of cubes of persimmon was homogenized during 90 s. The persimmon purée was then stored in hermetically sealed glass jars at –40 ºC in a deep freezer until further analysis, and it was thawed at room temperature to determine the bioactive compounds content.

2.3.2. Extraction and quantification of carotenoids

Total carotenoids were extracted according to Hornero-Méndez & Mínguez-Mosquera (2001) with modifications. Persimmon purée (5 g) was extracted 5 times with 25-mL cool acetone using an Ultraturrax® (IKA Ultraturrax T25 Basic) and vacuum filtered, until no more colour was extracted. The extract was added gradually over 50 mL ethyl ether contained in a decanting funnel. With each addition of extract, enough NaCl solution (100 g L^{-1}) was added to separate the phases and to transfer the pigments to the ether, and the aqueous phase was removed. The process was carried out in several steps to ensure the highest elimination of aqueous phase. The organic phase was treated several times with anhydrous Na_2SO_4 (20 g L^{-1}) to remove residual water and evaporated to dryness in a rotary evaporator (model RII; Büchi Labortechnik, Flawil, Switzerland) at a temperature lower than 35 ºC. Finally, the pigments were collected with acetone to a volume of 100 mL and the absorbance was measured at 450 nm using a spectrophotometer (model Helios Zeta UV Visible; Thermo Fisher Scientific Inc., Cambridge, U.K.). The calibration curve was performed with different concentrations of β−carotene in acetone. Results were expressed as mg β carotene/100 g of fresh weight. Carotenoid extractions were made 3 separate times and measurements were performed in triplicate.

2.3.3. Total soluble tannin content

Total soluble tannin content of the samples was determined with a spectrophotometer (Helios Zeta UV Visible) using the Folin Denis colorimetric method as described by Arnal & Del Río (2004). Persimmon purée (5 g) was homogenized in an Ultraturrax with 25 mL of 800 g kg^{-1} methanol. Homogenates were centrifuged (14500 rpm, 20 min, 4 ºC) and filtered. The supernatant was kept. More supernatant was extracted from the pellet with 25 mL of 800 g kg^{-1} methanol and added to the 1st supernatant. The total supernatant was brought to 100 mL with 800 g kg^{-1} methanol. In a test tube, 1 mL of the extract and 6 mL distilled water were mixed and vortexed. Thereafter, 0.5 mL of Folin Ciocalteu reagent was added. After 3 min, 1 mL saturated Na_2CO_3 was added, vortexed, and 1.5 mL distilled water was added. Absorbance was measured after 90 min at 725 nm. The calibration curve was performed with different concentrations of gallic acid in 800 g kg^{-1} methanol. Results were expressed as g gallic acid/100 g of fresh weight. Total soluble tannin extractions were made 3 separate times and measurements were performed in duplicate.

2.3.4. Antioxidant activity

Antioxidant activity was measured by ferric reducing antioxidant power assay (FRAP). Extracts were obtained in the same way as for total soluble tannin content determination but using 960 g kg^{-1} ethanol. Distilled water (30 µL), sample (30 µL), and FRAP reagent (900 µL) were placed in each cuvette. Cuvettes were incubated during 30 min in a water bath at 37 ºC and the absorbance was measured at 595 nm. The calibrated curve was performed using different concentrations of Trolox in 960 g kg^{-1} ethanol. Results were expressed as µmol Trolox/g of sample. Extracts were made 3 separate times and measurements were performed in triplicate.

2.3.5. Total and insoluble dietary fibre

Total dietary fibre (TDF) and insoluble dietary fibre (IDF) were determined according to AOAC official method 991.43 (AOAC, 1992) using Fibertec E system® (model TM1023, Foss Analytical AB, Höganäs, Sweden). For this purpose, 1 g lyophilized sample was used. Duplicate samples underwent sequential enzymatic digestion by heat stable α amylase, protease, and amyloglycosidase to remove starch

and protein. For TDF, enzyme digestate was treated with ethanol to precipitate soluble dietary fibre before filtering, and TDF residue was washed with ethanol, dried and weighed. For IDF, enzyme digestate was filtered, and residue (IDF) was washed with warm water, dried and weighed. TDF and IDF residue values were corrected for protein, ash, and blank. Results were expressed as g/100 g of dry weight.

2.3.6. Colour

The measurements were carried out with a Chroma meter CR400® (Konica Minolta Sensing Americas, Inc., Ramsey, N.J., U.S.A.). The results were expressed in accordance with the CIELAB system with reference to illuminant C and a visual angle of 2º. The colorimeter was calibrated with a white standard pattern (Y = 92.9; x = 0.3137; y = 0.3198). The parameters determined were: lightness (L*), a* (green red hue), and b* (blue yellow hue). Hue (h_{ab}) and chroma (C_{ab}*) were determined using eq. 1 and 2, respectively.

$$h_{ab} = \arctan(b^*/a^*) \quad (1)$$

$$C_{ab}^* = (a^{*2} + b^{*2})^{1/2} \quad (2)$$

2.3.7. Texture

Flesh firmness, cohesiveness and shear force were determined at room temperature with a TA.XTplus Texture Analyzer® (Stable Micro Systems, UK). Flesh firmness was expressed as the load in newtons (N) required breaking the flesh of the persimmon cubes with a 4-mm diameter flat tipped cylindrical probe at 1 mm s^{-1} test speed. A texture profile analysis was performed to determine cohesiveness. Cohesiveness was calculated as the ratio of the area under the 2nd curve to the area under the 1st curve. The samples were axially compressed in 2 consecutive cycles at 1 mm s^{-1} test speed and 75% compression, 3 s apart, with a 50-mm diameter flat plunger. Shear force was determined as the load in newtons (N) needed to cut the persimmon cubes with a knife blade at 1 mm s^{-1} test speed. Firmness, cohesiveness, and shear force values were an average of the measurements from 10 cubes.

2.4. Statistical analysis

Data were subjected to variance analysis (ANOVA), using the least significant difference (LSD) test with a 95% confidence interval for the comparison of the test averages (Statgraphics Plus 5.1, Manugistics, Inc., Rockville, Mass., U.S.A.).

3. Results and discussion

3.1. Microstructural study

The parenchymal tissue of untreated 'Rojo Brillante' persimmons is made up of turgid cells with a rounded appearance measuring 21792.9 ± 6270.2 μm^2 in close contact with each other. The tissue contains intercellular spaces, mostly triangular (Figure 1A). The cell walls, approximately 0.700 ± 0.026 μm thick, stained uniformly (Figure 1D) and well-bundled cellulose fibrils (Figure 1E) and an unbroken continuous middle lamella (Figure 1B) can be seen. The cell membranes (plasmalemma and tonoplast) remain close to the cell wall in most of the cells (Figure 1B and 1E). A dense eutectic artifact can be seen in the parenchymal cell interiors, indicating high soluble matter content (Figure 1C and 1F). Precipitated solutes can be observed in some cells (Figure 1A). These are probably tannins which were beginning to turn insoluble, a natural effect of ripening in this fruit. The presence of tannin cells can also be seen with CryoSEM (Figure 1C). Most of the intercellular spaces appear to be empty although solutes can be seen in some, generally in larger spaces than the triangular ones (Figure 1C, 1D, and 1F). These persimmons appear to possess an active apoplastic pathway.

Treating persimmons with HHP causes structural modifications. In general, the parenchymal tissues of the persimmons subjected to HHP treatment display a more compact structure containing little air (Figure 2C). The cells have a mean surface area of 22110.977 ± 5723.972 μm^2, their perimeters are deformed, and they are spaced further apart from each other than in the untreated persimmon, so large intercellular spaces can be seen (Figure 2A). The cell walls are approximately 0.604 ± 0.026 μm thick, their cellulose fibrils present less bundling (Figure 2B) than in the untreated persimmon and their middle lamella is thicker and has broken down in some areas (Figure 2E). Breakdown of the cellulose "cements" encourages the walls of adjoining

cells to separate (Figure 2A and 2B). Despite the HHP treatment, the cell membranes have remained intact and are still close to the cell wall in many areas (Figure 2B and 2E). Eutectic artifacts indicating the presence of solutes can be observed both in the interior of the cells and in practically all the intercellular spaces (Figure 2C and 2 D). The tannin cells appear to be filled with a compact mass of insoluble matter (Figure 2C) indicating that HHP could encourage tannin precipitation and, therefore, tannin cell formation. The small triangular air-filled spaces that predominated in the untreated persimmon have disappeared with the HHP treatment, giving rise to large solute filled intercellular spaces (Figure 2D and 2F). The structural changes brought about by HHP treatment favour solute movement at cell level, probably using the apoplastic, symplastic, and transmembrane transport routes, which could influence the extractability of some bioactive compounds by encouraging their diffusion from the interior to the exterior of the cell.

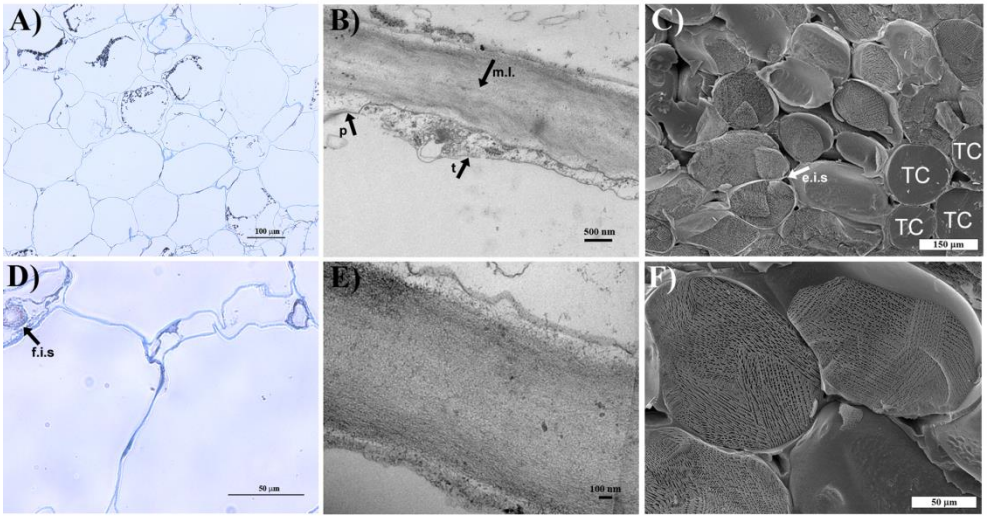

Figure 1. Light microscopy (A, D), transmission electron microscopy (B, E), and CryoSEM (C, F) micrographs of untreated persimmon. m.l., middle lamella; p, plasmalemma; t, tonoplast; TC, tannin cell; e.i.s., empty intercellular space; f.i.s., full intercellular space.

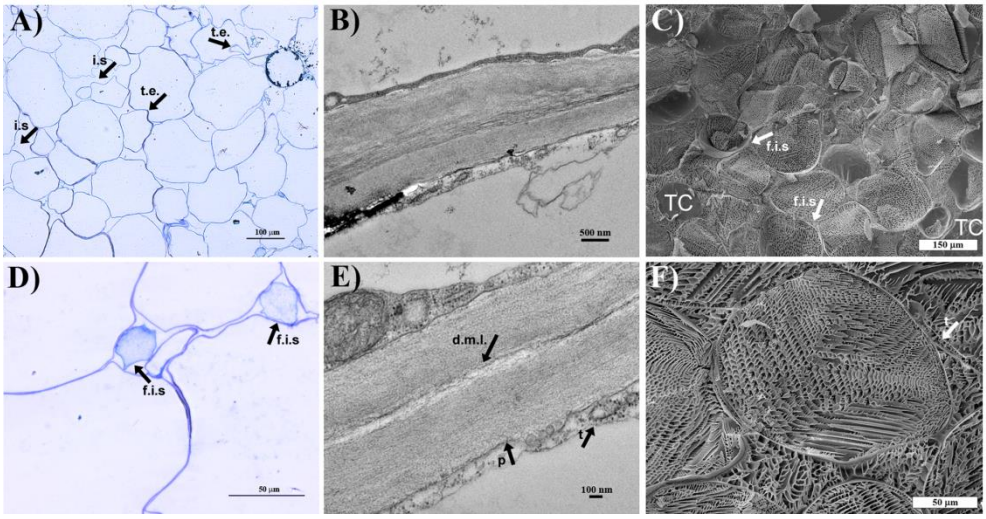

Figure 2. Light microscopy (A, D), transmission electron microscopy (B, E), and CryoSEM (C, F) micrographs of HHP-treated persimmon. t.e., twisted edges; i.s., intercellular space; d.m.l., dissolved middle lamella; p, plasmalemma; t, tonoplast; TC, tannin cell; f.i.s., full intercellular space.

Pasteurizing the persimmons also gave rise to changes in the parenchymal tissue microstructure in comparison with untreated persimmons and ones subjected to HHP. The cells are smaller, with surface areas of 12545.163 ± 2863.148 μm^2, and the cell walls are more deformed (Figure 3A) than those of the untreated persimmons and those subjected to HHP. Adjoining cells have drawn apart from each other and the parenchyma presents large intercellular spaces (Figure 3D). The cell walls are approximately 0.511 ± 0.021 µm thick (Figure 3B and 3E), thinner than those of the untreated and HHP-treated fruit. The cell walls are generally more faintly stained (Figure 3A) and show a certain loss of fibril bundling, and the middle lamella has broken down in some areas (Figure 3E). Although intact, the cell membranes have drawn away from the cell wall and toward the middle of the cell (Figure 3B and 3E). In the pasteurized persimmon parenchyma, the eutectic artifact is mainly located in the cell interior (Figure 3C), most of the large intercellular space appear empty, as in the untreated persimmon (Figure 3F), and groups of tannin cells can be seen. As HHP treatment, pasteurization also would seem to favour tannin precipitation and tannin cell formation (Figure 3A and 3C).

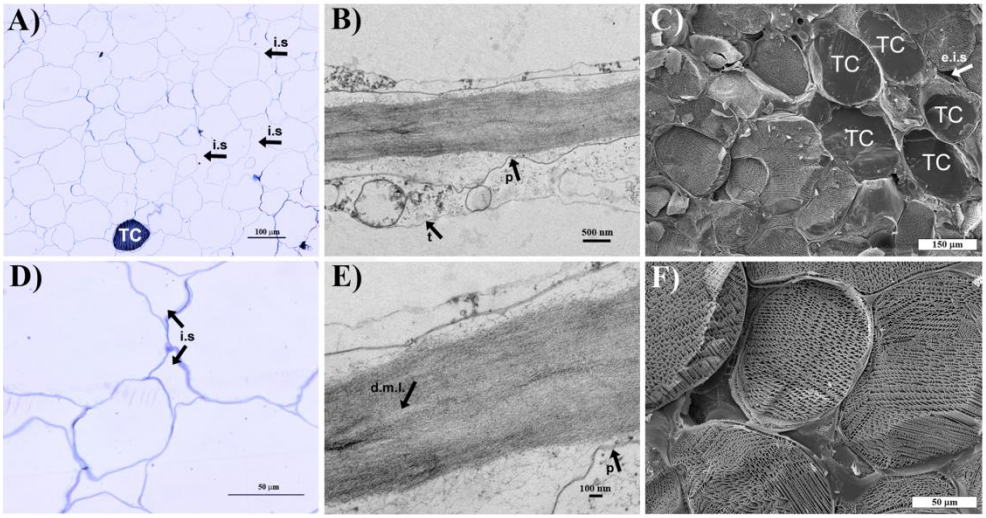

Figure 3. Light microscopy (A, D), transmission electron microscopy (B, E), and CryoSEM (C, F) micrographs of pasteurized persimmon. i.s., intercellular space; d.m.l., dissolved middle lamella; p, plasmalemma; t, tonoplast; e.i.s., empty intercellular space; TC, tannin cell.

3.2. Carotenoid content measurement

Table 1 shows the mean carotenoid contents of the 3 types of persimmon studied (untreated, HHP-treated, and pasteurized). It can be seen that the treated persimmons (HHP and pasteurization) had a significantly higher carotenoid content than the untreated fruit ($P < 0.05$). Of the 2 treatments studied, the rise in the carotenoid content was more significant with HHP ($P < 0.05$). Plaza et al (2012) obtained similar results on studying the influence of HHP treatment on the carotenoid content of persimmons. They showed that applying an HHP treatment at 200 MPa for 1, 3, or 6 min induced a significant increase in the total carotenoid content ($P < 0.05$). Of the 3 treatments they tested, 200 MPa for 6 min gave the highest level of carotenoid compound extraction.

Table 1. Carotenoid content, total soluble tannin content, and total antioxidant activity of untreated, HHP, and pasteurized persimmon.

	Carotenoid content (mg β-carotene/100 g f.w.)	Total soluble tannin content (g gallic acid/100 g f.w.)	Antioxidant activity [Trolox] (μmol/g)
Untreated	0.581[a] (0.130)	0.468[a] (0.059)	31.143[a] (0.165)
HHP	1.695[b] (0.046)	0.260[b] (0.031)	31.154[a] (0.135)
Pasteurized	1.237[c] (0.134)	0.251[b] (0.038)	25.445[b] (2.253)

f.w., fresh weight.

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

3.3. Total soluble tannin content measurement

No statistically significant differences ($P > 0.05$) were observed between the mean total soluble tannin content (Table 1) of the HHP-treated persimmon and pasteurized fruit, whereas the tannin content of the untreated sample was significantly higher ($P < 0.05$). The lower soluble tannin content of the HHP-treated and pasteurized persimmon could be due to the tannin insolubilization (tannin precipitation and tannin cell formation) already observed in the microstructural study (Figure 2 and 3), which could be related to the loss of astringency. These results are in agreement with previous studies (Vázquez-Gutiérrez et al., 2011) that established that the application of HHP provoked the precipitation of soluble tannins in 'Rojo Brillante' persimmons which could be related with the lower soluble tannin content detected in those samples.

3.4. Antioxidant activity measurement

The mean antioxidant activity values of the 3 types of persimmon analyzed are shown in Table 1. No statistically significant differences in antioxidant activity ($P > 0.05$) were found between the untreated persimmon and the fruit subjected to HHP. However, the thermal treatment led to a significant fall ($P < 0.05$) in the antioxidant activity of the pasteurized persimmons. Several researchers (Butz et al., 2002; 2003) have studied the influence of HHP on the antioxidant activity of very different foods

without finding any statistically significant differences ($P > 0.05$) between the controls and the samples treated with HHP. Other authors (Fernández-García et al., 2001; Sánchez-Moreno et al., 2005) established that for short treatment times (500 and 800 MPa/20 ºC/5 min or 400 MPa/40 ºC/1 min), no changes in antioxidant activity of orange juice and tomato purée were found after HHP treatments. The reduction in the antioxidant activity of the pasteurized persimmons could be related to the lower soluble tannin content of these samples and the degradation of other antioxidant compounds caused by thermal processing (Oey et al., 2008b). The HHP samples maintain a similar antioxidant activity to the untreated ones due to their high carotenoid content.

3.5. Total and insoluble dietary fibre content measurement

The results for TDF and IDF are shown in Table 2. No statistically significant differences in TDF and IDF values ($P > 0.05$) were found between the different types of persimmon under study. Consequently, it would appear that neither the HHP treatment nor pasteurization affected the dietary fibre content of the persimmons. So, persimmon seems to be a rich source of dietary fibre.

Table 2. Total dietary fibre (TDF) and insoluble dietary fibre (IDF) of untreated, HHP, and pasteurized persimmon.

	TDF (g/100 g d.w.)	IDF (g/100 g d.w.)	TDF (g/100 g d.w.)
Untreated	14.877[a] (2.751)	9.387[a] (1.735)	14.877[a] (2.751)
HHP	14.961[a] (2.845)	8.411[a] (1.600)	14.961[a] (2.845)
Pasteurized	15.308[a] (3.069)	8.744[a] (1.753)	15.308[a] (3.069)

d.w., dry weight.

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

3.6. Colour

Colour is an indicator of prime importance in relation to the different attributes that define the quality of plant products and is considered the major quality attribute that influences the consumer's choice (Quitão-Teixeira et al., 2008). Table 3 shows the colour parameters luminosity (L*), hue (h_{ab}), and chroma (C_{ab}*). With regard to L*, it may be seen that both HHP and pasteurization induced a significant reduction in luminosity ($P < 0.05$) and the non-thermal HHP treatment generated the significantly lowest values ($P < 0.05$). The lower L* values observed in HHP-treated and pasteurized persimmons could be associated with a higher browning reactions that could take place in these samples. Concerning hue (Table 3), statistically significant differences ($P < 0.05$) were found between the 3 types of persimmon. In this case, it was the thermal treatment, pasteurization, that led to the significantly lowest hue values ($P < 0.05$). Generally, hue values of the 3 types of persimmon were between 80 and 90º, corresponding to the yellow colouring of the samples due to carotenoid pigments of persimmon. The lower hue values of HHP-treated and pasteurized samples could be related to browning reactions, because the lower hue values, the higher redness the samples are. The decrease in the hue values was higher for pasteurization than for HHP-treated samples.

In the case of chroma (Table 3), no statistically significant differences ($P > 0.05$) between the untreated and pasteurized persimmons were observed but the persimmons treated with HHP registered significantly lower chroma values ($P < 0.05$).

So, both preservation treatments caused changes in the colour parameter values. Pasteurized samples showed higher L* and chroma values than HHP persimmons. These variations in the colour parameters of the treated (HHP and pasteurization) persimmons could be indicative of greater activity by the enzymes responsible for enzymatic browning, such as polyphenoloxidase and peroxidase (Quitão-Teixeira et al., 2008). The microstructural changes in the cell walls and membranes caused by the HHP and pasteurization treatments could favour contact between the enzyme and its substrates, which had previously remained separate in different compartments of the untreated persimmon cells (Rastogi et al., 2007). This contact could encourage browning reactions.

Table 3. Lightness, hue, and chroma force of untreated, HHP, and pasteurized persimmon.

	Lightness	Hue	Chroma
Untreated	67.584[a] (2.102)	84.446[a] (1.787)	46.474[a] (4.385)
HHP	48.839[b] (3.031)	82.721[b] (1.352)	31.856[b] (3.257)
Pasteurized	62.791[c] (3.486)	80.947[c] (1.488)	43.944[a] (3.446)

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

3.7. Texture properties

Table 4 shows the texture properties of firmness, cohesiveness, and shear force of the 3 types of persimmon under study. No statistically significant differences in these properties ($P > 0.05$) were observed between the untreated and pasteurized persimmons. However, the persimmons treated with HHP presented significantly lower firmness, cohesiveness, and shear force ($P < 0.05$). The structural modifications together with a greater movement of solutes at cell level could explain the lower texture parameter values of the HHP-treated persimmons. These results are in agreement with previous studies that observed lower firmness and cohesiveness in persimmons treated with HHP (Vázquez-Gutiérrez et al., 2012). Texture changes could be related to transformations in cell wall polymers due to enzymatic and non-enzymatic reactions (Sila et al., 2008). Due to cell structure changes, HHP processing facilitates the occurrence of enzymatic and non-enzymatic reactions. Substrates, ions, and enzymes which are located in different compartments in the cells can be liberated and interact with each other during and after HHP treatment. At the same time, pressure can enhance the action of pectinmethylesterase and polygalacturonase, causing the softening of persimmon and decrease of texture properties (Oey et al., 2008b).

Table 4. Firmness, cohesiveness, and shear force of untreated, HHP, and pasteurized persimmon.

	Firmness (N)	Cohesiveness	Shear force (N)
Untreated	5.915[a] (1.256)	0.084[a] (0.016)	11.959[a] (2.131)
HHP	3.526[b] (1.029)	0.059[b] (0.004)	10.118[b] (1.894)
Pasteurized	5.234[a] (1.329)	0.074[a] (0.014)	12.919[a] (1.884)

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

4. Conclusions

Both HHP treatment and pasteurization cause structural changes in the parenchymal tissues of persimmons. The fruit subjected to HHP presents a more compact structure containing little air and with intercellular spaces filled with cell material, indicating increased solute movement through the tissue. These microstructural changes could be responsible for the modifications in the bioactive compounds content of persimmon. Both preservation treatments lead to a fall in the total soluble tannin content and maintain the dietary fibre content of untreated persimmon. The decrease in the total soluble tannin content could be related to the loss of astringency and could make the persimmon more suitable for consumption. However, HHP processing improves the extraction of carotenoids and keeps the antioxidant properties of the fruit. Treating persimmon with HHP allows obtaining a high nutritional value ingredient to be used when formulating new functional foods.

5. Acknowledgements

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High hydrostatic pressure treatment provides persimmon good characteristics to formulate milk-based beverages with enhanced functionality

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Abstract

High hydrostatic pressure (HHP) applied during food processing can improve the retention of food quality attributes and nutritional values in comparison with pasteurization. Persimmon is a good source of bioactive compounds but it is a seasonal fruit that cannot be consumed throughout the year. The aim of this work was to compare the HHP and pasteurization treatments to formulate milk-based beverages containing this carotenoid rich ingredient and to evaluate their performance in these beverages. The carotenoid and tannin contents of persimmon were determined and the microstructure and rheology of the new milk-based persimmon beverages were examined. The results showed that HHP treatment favoured the release of carotenoids from the fruit matrix and precipitation of the tannins. The milk-based beverages prepared with the high-pressure persimmon presented the best rheological properties because unlike the untreated and pasteurized persimmon milk-based beverages, they did not form a gel-like structure or separate out.

Keywords: carotenoids, high hydrostatic pressure, microstructure, persimmon, rheological properties, tannins.

1. Introduction

Persimmon (*Diospyros kaki* L. f.) is an important and widespread fruit crop in China. In Europe, Spain is one of the major producers reaching in Valencia more than 130000 tonnes in 2012 (Conselleria de Presidencia y Agricultura, Pesca, Alimentación y Agua, 2013). Persimmons are among the fruits with the highest levels of bioactive antioxidant compounds (Jung et al., 2005), such as carotenoids and polyphenols. The main polyphenols in persimmons are tannins, which have degenerative disease prevention effects (Achiwa et al., 1997), but when they are in their soluble form they can impart astringency to persimmon fruits (Tárrega et al., 2013). Carotenoids such as lycopene, lutein, and zeaxanthin have considerable antioxidant potential (Shami & Moreira 2004; Stahl & Sies, 2005). Another important role of carotenes, especially β-carotene, is provitamin A activity (Rodríguez-Amaya, 1989; Miller et al., 1996). A major variety of persimmon grown in Spain is 'Rojo Brillante', an astringent cultivar which requires post-harvest deastringency treatment before the fruit can be marketed such as exposure to carbon dioxide in high concentrations, appropriate ethylene treatment or drying after peeling (Wei et al., 2014). Exposure to high levels of carbon dioxide (950 g kg^{-1} for 24 h at 20 ºC) has proven to be the most effective way to remove astringency while maintaining fruit firmness (Arnal & Del Río, 2003).

Persimmons are normally sold in fresh form but are seasonal fruits that cannot be consumed throughout the year. Therefore, it would be useful to develop industrial processes that prolong their shelf life and make it possible to produce persimmon derivatives for fruit juices mixtures, jams, yoghurts or ice creams from astringent varities, in order to obtain products with high nutritional value. Until now, thermal processing has been the method most commonly employed because of its ability to inactivate microorganisms and spoilage enzymes (polyphenoloxidase, pectinmethylesterase, etc.). However, heat may induce chemical and physical changes that damage organo leptic properties and may reduce the content or bioavailability of some bioactive compounds, particularly if applied under severe conditions (Patras et al., 2009; 2010; Rawson et al., 2010). According to Rawson et al (2011), most authors have reported that pasteurization leads to a decrease in the bioactive content (carotenoid content, phenolic content and antioxidant activity) of some fruits and vegetables such as exotic fruits (Elez-Martínez et al., 2006), mangos (Vásquez-

Caicedo et al., 2007; Djioua et al., 2009; Kim et al., 2009) and mulberry fruit extract, durian juice, pineapple juice, and cashew apple juice (Rattanathanalerk et al., 2005; Zepka & Mercadante, 2009; Aramwit et al., 2010; Chin et al., 2010).

High hydrostatic pressure (HHP) processing would appear to be a suitable alternative to thermal processing as it is considered one of the most economically viable non-thermal technologies and makes it possible to obtain products with high nutritional and quality parameters compared to conventional thermal processing (Norton & Sun, 2008; Rawson et al., 2011). HHP treatment is expected to be less harmful to low molecular weight food compounds such as flavouring agents, pigments and vitamins and preserves the nutritional value of treated food better than heat due to its limited effects on covalent bonds (Oey et al., 2008; Rawson et al., 2011). HHP treatment at ambient temperature is reported to have a minimal effect on the bioactive content of various fruits and vegetables (Oey et al., 2008).

Both HHP treatment and pasteurization can cause changes in the structure of the plant tissue which could be related to modifications in the bioavailability of some bioactive compounds. So, the microstructural study of food products could clarify the relationship between structure and functionality. In this sense, confocal laser scanning microscopy (CLSM) could be a suitable technique to study the interactions among components using specific dyes and their fluorescent excitation–emission features. CLSM has been employed to study the microstructure of some dairy products such as the interaction between carrageenan and milk proteins (Arltoft et al., 2007), the distribution of fat and protein in different dairy products (Auty et al., 2001), the structure of dairy products with different compositions (Panouillé et al., 2011), or the effect of whey protein addition on the structural properties of stirred yoghurt systems at different protein and fat contents (Krzeminski et al., 2011).

The aim of this work was to study the effect of HHP and pasteurization processes on some bioactive compounds of the persimmon, such as carotenoids and tannins, in order to formulate milk-based beverages with improved nutritional and functional properties. The microstructure and rheological behaviour of these milk-based beverages were also examined.

2. Materials and methods

2.1. Material and milk based beverages

Persimmon fruits (cv. 'Rojo Brillante') were harvested in Carlet (Valencia, Spain) at the beginning of November 2012. The maturity index was selected following the method reported by Salvador et al. (2007), which defines six maturity stages based on the external colour ranging from I (yellow green) to VI (orange red); fruits in their commercial maturity stage (stage IV) were used in the present work. Cubes (15 mm) were taken from the equatorial area of the fruits and heat-sealed in 110 x 220 mm plastic bags (Doypack type, Amcor, Spain). Each bag contained approximately 80 g of persimmon. One-third of the bags were not subjected to any treatment (untreated persimmon). Another third of the bags (HHP-treated persimmon) were placed inside a hydrostatic pressure unit with a 2350 mL capacity (GEC Alsthom ACB 900 HP, type ACIP 665, Nantes, France) using water as the pressure medium; the pressure employed was 200 MPa for 6 min at 25 ºC (energy consumption 244 kJ kg^{-1}), based on previous studies where HHP treatment at 200 MPa was applied for 1, 3, and 6 min and the treatment at 200 MPa for 6 min gave the highest level of carotenoid compound extraction (Plaza et al., 2012). The last third of the bags were pasteurized in a water bath at 70 ºC for 15 min with an energy consumption of 3600 kJ kg^{-1} (pasteurized persimmon). Each type of persimmon was homogenized for 90 s and freeze-dried for 120 h at -45 ºC and 1.3 x 10-3 mPa in a freeze-drier (Lioalfa-6®, Telstar, Terrassa, Spain) before their use in milk based beverages.

Nine different milk-based persimmon beverages were studied by varying the type of milk and the treatment of the persimmon. Three different types of milk were used: whole milk (36 g L^{-1} fat content), semi-skimmed milk (15.5 g L^{-1} fat content), and skimmed milk (2.5 g L^{-1} fat content) from Central Lechera Asturiana, Siero, Spain. Three different types of freeze-dried persimmons were employed: untreated, HHP-treated and pasteurized. The quantity of persimmon used in the formulation of 1 L of milk-based persimmon beverage was calculated to have the same carotenoid content to that in 200 g of fresh persimmon (0.743 mg β-carotene per 100 g fresh weight).

The milk-based beverages were prepared by placing the corresponding amount of freeze-dried untreated persimmon (115.9 g kg^{-1}), freeze-dried HHP-treated persimmon (52.8 g kg^{-1}) or freeze-dried pasteurized persimmon (95.1 g kg^{-1}) in a food processor (Thermomix TM31, Wuppertal, Germany) and stirring at increasing agitation speeds (1100 rpm, 3250 rpm and 10 200 rpm), for 10 s at each speed, in order to reduce the particle size of the freeze-dried persimmon samples. The milk was added and stirred following the same procedure (1100 rpm, 3250 rpm and 10200 rpm, for 10 s at each speed). All the milk- based beverages were kept at 4–5 ºC until their analysis. The microstructure, rheological properties and loss of stability were analyzed within 24 h of milk-based beverage preparation.

2.2. Extraction and quantification of carotenoids

The total carotenoids were determined according to the method described by Hornero-Méndez & Mínguez-Mosquera (2001) with modifications. Freeze-dried persimmon (5 g) was extracted with 25-mL of cool acetone using a homogenizer (IKA T25 Basic Ultra-Turrax) and vacuum filtered until no more colour was extracted. The extract was added gradually to 50 mL ethyl ether in a decanting funnel. With each addition of extract, sufficient NaCl solution (100 g L^{-1}) was added to separate the phases and transfer the pigments to the ether phase; the aqueous phase was removed. This process was carried out in several steps to ensure maximum elimination of the aqueous phase. The ether phase was treated several times with anhydrous Na_2SO_4 to remove residual water and finally evaporated to dryness in a rotary evaporator (model RII; Büchi Labortechnik, Flawil, Switzerland) at a temperature below 35 ºC. Finally, the pigments were collected with acetone to a volume of 100 mL and the absorbance was measured at 450 nm using a spectrophotometer (model Helios Zeta UV Visible; Thermo Fisher Scientific Inc., Cambridge, U.K.). The calibration curve was constructed with different concentrations of β carotene (Sigma-Aldrich, Madrid, Spain) in acetone (Panreac, Barcelona, Spain). The results were expressed as mg β carotene per 100 g of dry weight freeze-dried persimmon. Three separate carotenoid extractions were made for each type of persimmon treatment and for untreated persimmon and the measurements were performed in triplicate.

2.3. Total soluble tannin content

The total soluble tannin content of the freeze-dried persimmons was determined with a spectrophotometer (Helios Zeta UV Visible) using the Folin Denis colorimetric method as described by Arnal & Del Río (2004). Freeze-dried persimmon (5 g) was homogenized with 25 mL of 800 g kg^{-1} methanol-water blend in a homogenizer (IKA T25 Basic Ultra-Turrax). The homogenate was centrifuged (14500 rpm, 20 min, 4 ºC) and filtered. The supernatant was kept. More supernatant was extracted from the pellet with 25 mL of 800 g kg^{-1} methanol and added to the first supernatant. The total supernatant was brought up to 100 mL with 800 g kg^{-1} methanol. In a test tube, 1 mL of the extract and 6 mL of distilled water were mixed and vortexed, then 0.5 mL of Folin Ciocalteu reagent (Panreac, Barcelona, Spain) was added. After 3 min, 1 mL saturated Na_2CO_3 was added, the mixture was vortexed, and 1.5 mL distilled water was added. Absorbance was measured after 90 min at 725 nm. The calibration curve was constructed with different concentrations of gallic acid (Panreac, Barcelona, Spain) in 800 g kg^{-1} methanol. The results were expressed as g gallic acid per 100 g of dry weight. Three separate total soluble tannin extractions were made for each persimmon treatment and for untreated persimmon and the measurements were performed in triplicate.

2.4. Confocal laser scanning microscopy (CLSM)

2.4.1. Equipment and dyes

CLSM was selected as the most appropriate microscopy technique for studying the microstructure of the freeze-dried persimmons and the milk-based beverages, due to the ability of carotenoid compounds to emit fluorescence when excited by a laser line. This makes it possible to locate them using CLSM without staining the sample. Moreover, protein and fat can also be identified by using specific dyes such as Rhodamine and Nile Red, respectively. A Nikon confocal microscope C1 unit fitted on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) was used. An Ar laser line (488 nm) was employed as the light source to excite the Rhodamine B and Nile Red fluorescent dyes. Rhodamine B (Fluka, Sigma-Aldrich, Missouri, USA) with $\lambda_{ex\ max}$ 488 nm and $\lambda_{em\ max}$ 580 nm was dissolved in distilled water at 2 g L^{-1}. This dye

was used to stain proteins and carbohydrates. Nile Red (Fluka, Sigma-Aldrich, Missouri, USA) with $\lambda_{ex\ max}$ 488 nm and $\lambda_{em\ max}$ 515 nm was dissolved in polyethylene glycol (PEG) 200 at 0.1 g L^{-1} and was used to stain fat. The auto-fluorescence of the samples was observed using the Ar laser line without any dye. A 60x/1.40NA/Oil/Plan Apo VC Nikon objective lens was used.

2.4.2. Sample viewing

A drop of freeze-dried persimmon or milk- based beverages was placed on a slide and 20 μL of Rhodamine B solution and 20 μL of Nile Red solution were added. The observations were made 10 min after diffusion of the dyes into the sample or beverage. The images were obtained and stored with 1024 x 1024 pixel resolution using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

2.5. Rheological measurements

Both the flow behaviour and the viscoelastic properties of each milk-based beverage were measured in triplicate. The measurements were carried out in a RS1 controlled stress rheometer (Thermo Haake, Karlsruhe, Germany), using a parallel plate geometry of 6 cm in diameter with a 1 mm gap, monitored using the RheoWin software package (version 2.93, Thermo Haake). A temperature of 10 ± 1 ºC was selected as representative of the usual consumption temperature of dairy desserts; it was maintained throughout the measurements by means of a Phoenix P1 Circulator device (Thermo Haake). The milk-based beverages were allowed to rest on the rheometer plate for 5 min before measurement and a fresh milk-based beverage was loaded for each measurement.

2.5.1. Flow behaviour

The flow of milk and milk-based beverages was measured by recording the shear stress values when shearing them with a linear increasing shear rate from 0 to 200 s^{-1} for a period of 60 s and in the reverse sequence for the same time. The areas under the upstream data point curve (A_{up}) and under the downstream data point curve (A_{down}), as well as the hysteresis area ($A_{thix} = A_{up} - A_{down}$), were obtained using

RheoWin Pro software (version 2.93, Thermo Haake). The data from the ascending segment of the shear cycle were fitted to the Ostwald–de Waele model (eq. 1) using RheoWin Pro software (version 2.93, Thermo Haake):

$$\sigma = K \cdot \gamma^{n} \quad (1)$$

where K (Pa s^n) is the consistency index and n is the flow index.

In addition, apparent viscosity values at 10 s^{-1} and 100 s^{-1} (η_{10} and η_{100}, respectively) were also calculated as follows.

$$\eta_{app} = K \cdot \gamma^{n-1} \quad (2)$$

2.5.2. Viscoelastic properties

In order to determine the linear viscoelastic region (LVR), stress sweeps (0.01–100 Pa) were run at 1 Hz. Frequency sweeps were then performed within the LVR over the range f = 0.01–10 Hz; the values of the storage modulus (G') and the loss modulus (G") as a function of frequency (mechanical spectra) were obtained using RheoWin Pro software (version 2.93, Thermo Haake).

2.6. Sedimentation

Sedimentation is very negative for the quality of food products and may cause consumers to reject the product. The loss of stability (sedimentation) of the milk-based beverages was measured by placing the milk-based beverages in 10 mL test tubes and leaving them to stand until the amount of sediment remained constant. This state was reached after 90 min. The % of sedimentation was calculated as (volume of sediment/total volume) x 100.

2.7. Statistical analysis

The effect of the persimmon treatment (untreated, HHP treatment and pasteurization) on the carotenoid and total soluble tannin contents was analyzed by one-way ANOVA. The effects of the type of milk (whole milk, semi-skimmed milk, and skimmed milk) on the flow parameters (K, n, η_{10}, η_{100}, and A_{thix}) and viscoelastic

parameters (G' and G") at 1 Hz were analyzed by one-way ANOVA for each type of milk-based beverage (prepared with freeze-dried untreated, freeze-dried HHP-treated, and freeze-dried pasteurized persimmon). The least significant difference (LSD) test with a 95% confidence interval was used to compare the mean values obtained. All the calculations were carried out with Statgraphics Plus 5.1 software.

3. Results and discussion

3.1. Carotenoid and total soluble tannin contents of the freeze-dried persimmons

Figure 1A shows the carotenoid content of the different persimmons analyzed in this study. The untreated and the pasteurized persimmons did not show statistically significant differences ($P > 0.05$) in the extracted carotenoid content. However, the HHP-treated persimmons presented a significantly higher extracted carotenoid content ($P < 0.05$). Similar results have been published for persimmon and orange juice, in which the authors reported increases in total carotenoids extracted after HHP processing (De Ancos et al., 2000; Sánchez-Moreno et al., 2003; Plaza et al., 2012).

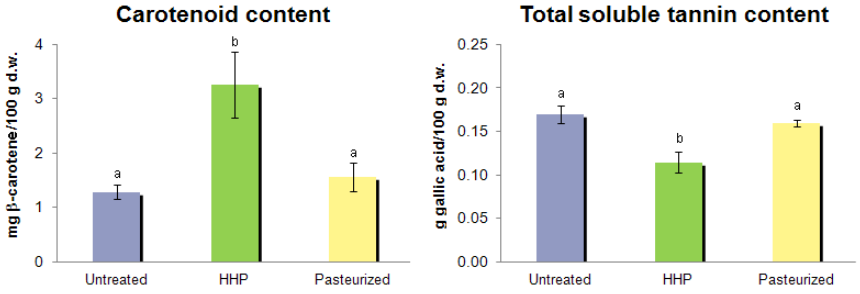

Figure 1. Carotenoid content and total soluble tannin content of untreated, HHP-treated and pasteurized freeze-dried persimmons different letters indicate significant differences ($P < 0.05$) between the samples.

No statistically significant differences in total soluble tannin content were found between the untreated and pasteurized persimmons ($P > 0.05$). The HHP-treated persimmons presented significantly lower total soluble tannin content ($P < 0.05$). It has been proved that this treatment decreases the total soluble tannin content in persimmon, as this treatment favours the precipitation of soluble tannins (Vázquez-Gutiérrez et al., 2013). The fact that HHP treatment seems to favour tannin precipitation could be related to decreased astringency of the HHP-treated fruit. So, this treatment could be used to develop new, less astringent and more versatile persimmon derivatives.

3.2. Confocal laser scanning microscopy (CLSM)

3.2.1. Freeze-dried persimmon

In order to understand the structure of the freeze-dried persimmon without the interference of the milk structure, samples were prepared by dispersing the corresponding amount of freeze-dried persimmon in deionized water and their microstructure was analyzed. Figure 2 shows the CLSM images of the freeze-dried persimmon dispersed in water. All the persimmon samples showed significant auto-fluorescence (Figures 2A, 2E, and 2I) due to the presence of carotenoids. According to Vázquez-Gutiérrez et al. (2011), carotenoids are associated mainly with cell walls, forming spherical bodies (chromoplasts). In the untreated samples (Figure 2A) most of the carotenoids tended to aggregate into interconnected clusters, forming a network. In the HHP-treated and pasteurized samples (Figures 2E and 2I) the carotenoid aggregates were smaller than those in the untreated ones, probably because the carotenoids were more dispersed throughout the plant tissue. HHP treatments are known to induce morphological changes in plant cells which result in the rupture of cell walls (Vázquez-Gutiérrez et al., 2011; 2012; 2013). These structural modifications could cause leaching of cellular constituents into the food matrix, and so the spread of carotenoids throughout the tissue. Other authors have established that thermal processing can affect functionalities such as carotenoid bioaccessibility due to its effect on the barrier properties of the cell wall polysaccharide network (Ribas-Agustí et al., 2014).

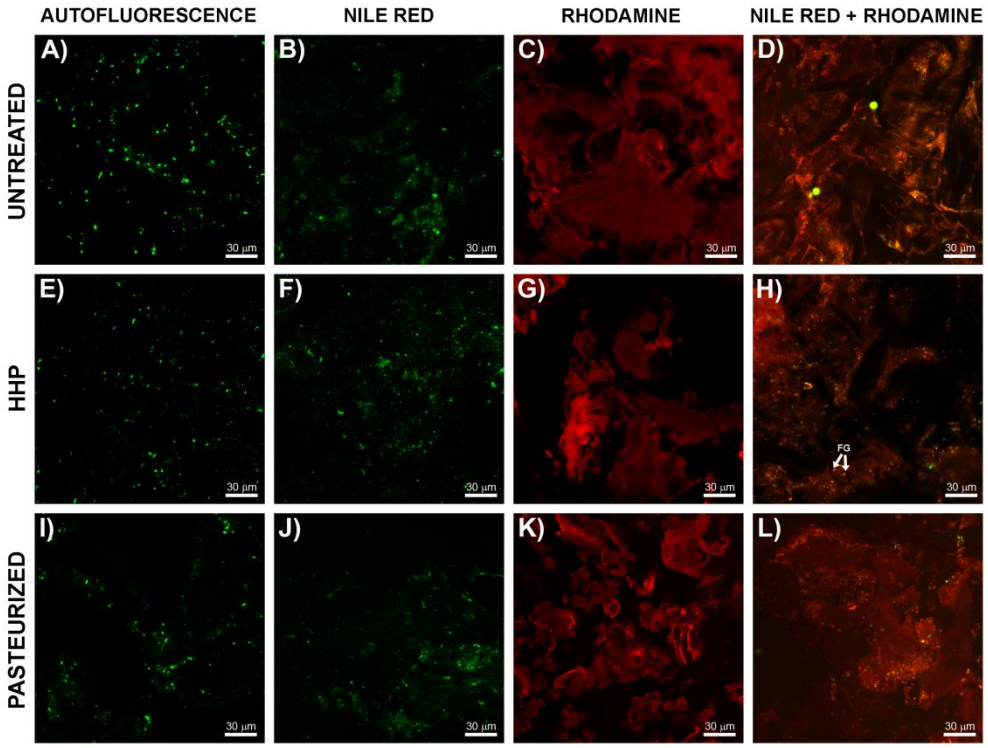

Figure 2. Confocal laser scanning microscopy (CLSM). Samples prepared with untreated, HHP-treated and pasteurized freeze-dried persimmons dispersed in water. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG, fat globules. Magnification: 60x.

When the samples were stained with Nile Red, a specific dye for fat (Figures 2B, 2F and 2J), a green network was observed, consisting of unstructured fat from the plant persimmon tissues and carotenoid compounds, as these pigments were excited by the same wavelength as Nile Red. This network was denser and more widely dispersed in the HHP and pasteurized samples (Figures 2F and 2J) than in the untreated ones (Figure 2B); this fact could be related to the structural modifications occurred on the treated tissue (HHP and pasteurized) in comparison with the untreated one. Staining with Rhodamine dye (Figures 2C, 2G and 2K) made it possible to view the plant persimmon structures in the samples because Rhodamine allows visualizing proteins and carbohydrates as those present in the cell wall. When Rhodamine and Nile Red (Figures 2D, 2H and 2L) were used to stain protein

and fat respectively, the samples showed a protein network (red) in which carotenoid pigments (green) were dispersed. The HHP-treated samples seemed to present the highest carotenoid content (Figure 2H), as reported above (Figure 1A).

3.2.2. Milk

Figure 3 shows the CLSM images of the three different types of milk (whole, semi-skimmed and skimmed) employed for formulating the milk-based persimmon beverages.

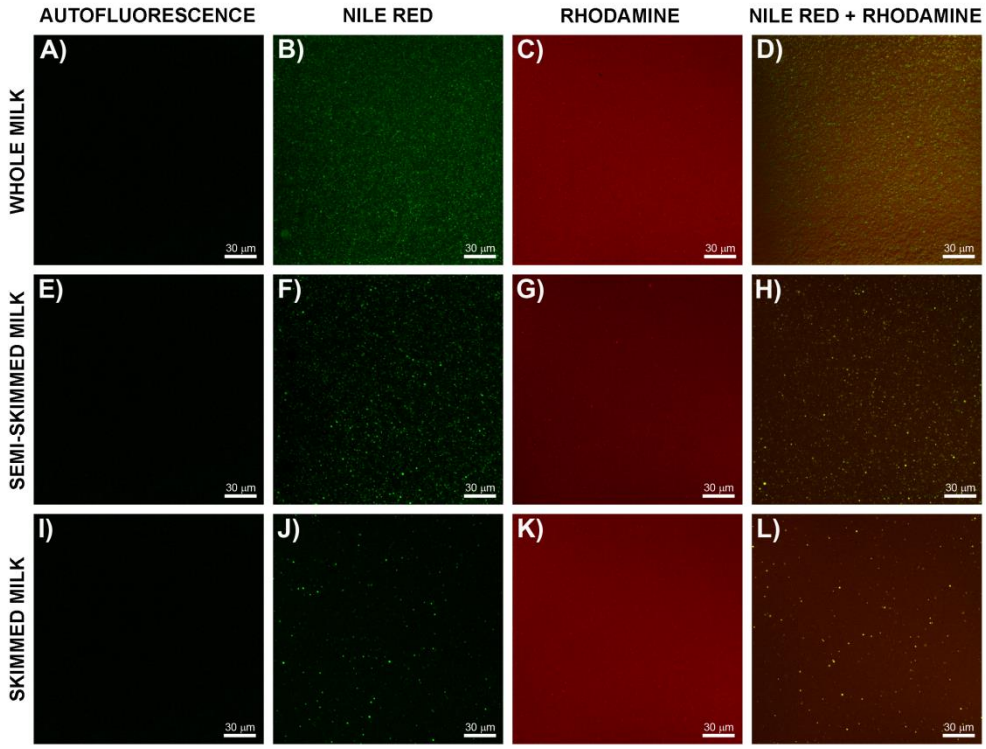

Figure 3. Confocal laser scanning microscopy (CLSM). Different milk matrices (whole, semi-skimmed, and skimmed milk) used in the formulation of the milk-based persimmon beverages. Green: carotenoids and fat globules. Red: proteins and carbohydrates. Magnification: 60x.

The fat globules did not display intrinsic fluorescence, despite β-carotene being normally present in their composition (Figures 3A, 3E and 3I). To study these different milk samples by fluorescence, an extrinsic fluorescent dye was therefore required (Gallier et al., 2010). Figures 3B, 3F and 3J show the fat globules coloured green due to Nile Red staining. In Figures 3C, 3G and 3K, the proteins can be seen as a continuous phase stained red by Rhodamine. Figure 3D shows how the protein and lipid phases interacted, forming a homogenous network in whole milk. In semi-skimmed milk (Figure 3H) this mixture was not as homogenous as in whole milk, as in these milks the protein acted as a continuous phase and the fat globules as a dispersed phase. In skimmed milk (Figure 3L) the distinction between the two phases was more evident.

3.2.3. Milk-based persimmon beverage

Figure 4 shows the CLSM images of the untreated persimmon milk-based beverages prepared with milk with different fat contents. All the beverages studied presented considerable intrinsic auto-fluorescence (Figures 4A, 4E and 4I) due to the presence of carotenoids, which appeared as groups of spherical bodies. These bodies appeared to be linked and arranged in a pattern. The milk-based beverages stained with Nile Red (Figures 4B, 4F and 4J) showed a green network made up of fat globules and unstructured fat from the milk and unstructured fat and carotenoid compounds from the persimmon. Consequently, the fat from the milk was found to form two networks: one made up of globules connected together forming bright green clusters and another made up of unstructured fat and carotenoids coloured dark green. In the milk-based beverages stained with Rhodamine (Figures 4C, 4G and 4K), a red protein network was observed. This distribution was similar in all the milk-based persimmon beverages studied. This protein network, in which part of the plant tissue is fused, seemed to behave as a continuous phase that kept some fragments of plant tissue dispersed throughout. In the micrographs of the milk-based persimmon beverages with a high fat content stained with Nile Red and Rhodamine (Figures 4D and 4H), two different phases could be observed: a continuous phase consisting of a dense lipoprotein network stained in yellow-green and a dispersed phase formed by green groups, probably carotenoids and fragments of plant tissue, with red contours. When

skimmed milk was used in the formulations (Figure 4L) the continuous phase was coloured orange and was mainly composed of protein, while the dispersed phase consisted of plant material, with red contours.

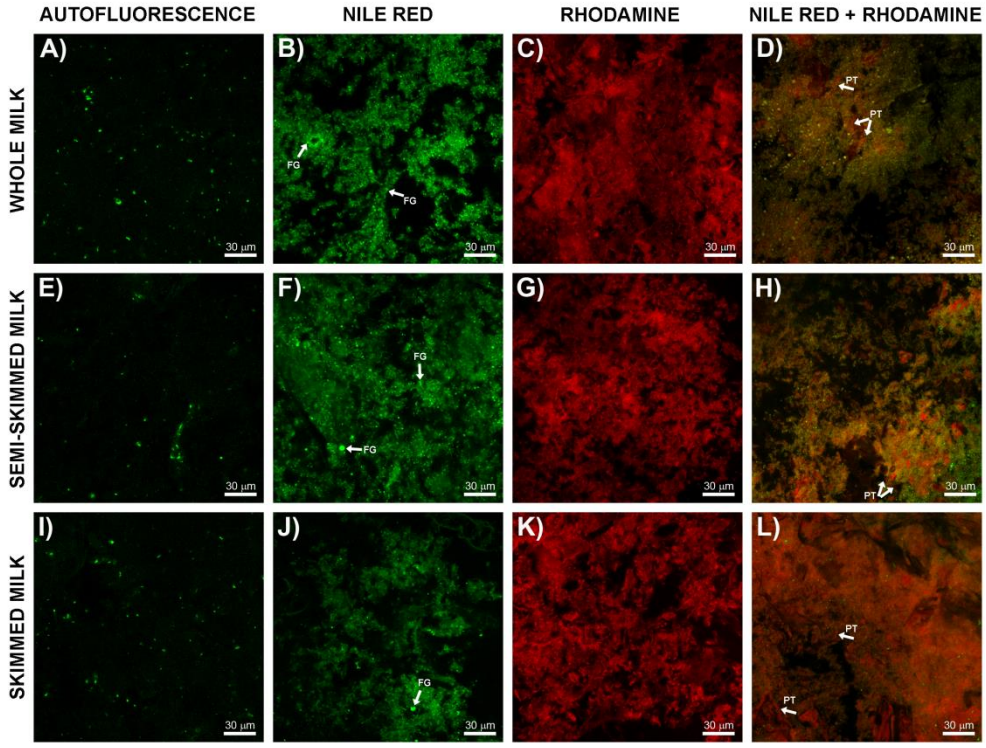

Figure 4. Confocal laser scanning microscopy (CLSM). Milk-based beverages prepared with freeze-dried untreated persimmon dispersed in whole, semi-skimmed and skimmed milk. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG, fat globules; PT, plant tissue. Magnification: 60x.

3.2.4. Effect of the persimmon treatment

The milk-based persimmon beverages prepared with freeze-dried HHP-treated persimmon (Figure 5) differed from the milk-based beverages prepared with freeze-dried untreated persimmon (Figure 4). The former presented higher auto-fluorescence (Figures 5A, 5E and 5I) than the latter despite containing a lower quantity of persimmon in their formulation. This high auto-fluorescence made it possible to see

fragments of persimmon, probably due to the dispersion of carotenoids throughout the tissue. Consequently, HHP treatment could encourage the release and extraction of carotenoids. Nile Red staining (Figures 5B, 5F and 5J) showed a network made up of fat from the milk matrix and fat and carotenoids from the plant material. This network formed a continuous film in the HHP-treated milk-based beverages and clusters in the untreated ones which could be related, again, to higher carotenoid extractability obtained in the HHP-treated persimmons compared to the untreated ones.

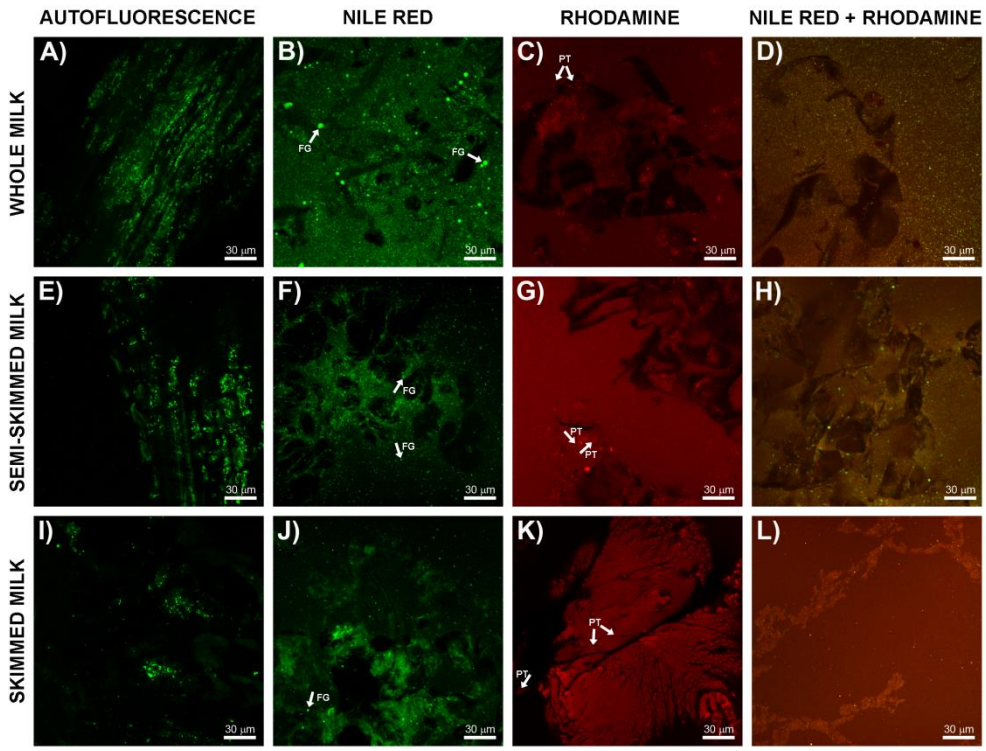

Figure 5. Confocal laser scanning microscopy (CLSM). Milk-based beverages prepared with freeze-dried HHP-treated persimmon dispersed in whole, semi-skimmed and skimmed milk. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG, fat globules; PT, plant tissue. Magnification: 60x.

When Rhodamine (Figures 5C, 5G and 5K) was used to stain the milk-based beverages a continuous and homogeneous red-coloured protein phase was observed, with red- and black-coloured fragments of plant tissue dispersed throughout the milk matrix. Milk-based beverages with a high fat content stained with Nile Red and Rhodamine (Figures 5D and 5H) showed two different phases, a continuous phase consisting of a dense lipoprotein network coloured yellow-green and a dispersed green-stained phase formed by carotenoids and fat with dark fragments of plant tissue. When skimmed milk was used in the formulation of the milk-based beverages (Figure 5L) the continuous phase was composed basically of protein. The carotenoid compounds were better identified in these milk-based beverages (Figures 5D, 5H and 5L) than in those made with freeze-dried untreated persimmon (Figures 4D, 4H and 4L).

When the milk-based beverages were prepared with freeze-dried pasteurized persimmon (Figure 6) considerable auto-fluorescence was also observed (Figures 6A, 6E and 6I), although less than that in the milk-based beverages prepared with freeze-dried HHP-treated persimmon (Figures 5A, 5E and 5I). It should be noted that the formulation of these milk-based beverages contained less persimmon than those made with freeze-dried untreated persimmon, but more than those prepared with the freeze-dried HHP-treated persimmon, although containing the same carotenoid content. The milk-based beverages stained with Nile Red (Figures 6B, 6F and 6J) showed a less dense network of fat and carotenoids than those with the freeze-dried HHP-treated persimmon. When Rhodamine was used to stain the milk-based beverages (Figures 6C, 6G and 6K), red- and black- coloured fragments of plant tissue were observed as a dispersed phase. When the milk-based beverages were stained with Nile Red and Rhodamine (Figures 6D, 6H and 6L), the carotenoids appeared as a green dispersed phase. They were observed more clearly than in the beverages with freeze-dried untreated persimmon (Figures 4D, 4H and 4L) but less than in those made with the freeze-dried HHP-treated persimmon (Figures 5D, 5H and 5L).

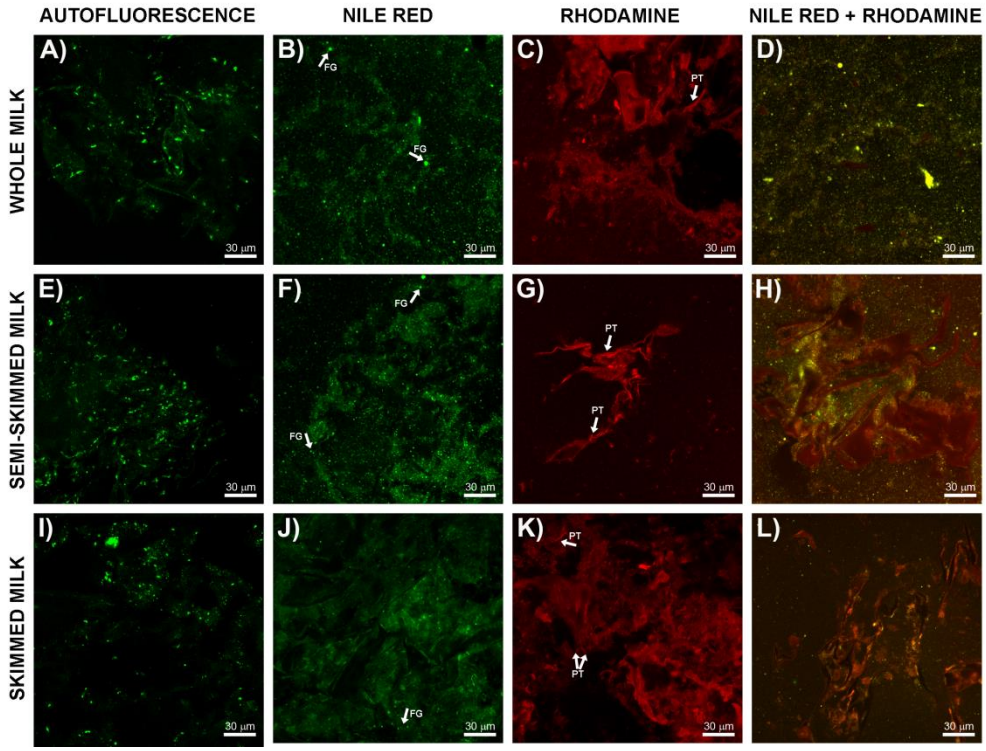

Figure 6. Confocal laser scanning microscopy (CLSM). Milk-based beverages prepared with freeze-dried pasteurized persimmon dispersed in whole, semi-skimmed and skimmed milk. Green: carotenoids and fat globules. Red: proteins and carbohydrates. FG, fat globules; PT, plant tissue. Magnification: 60x.

3.3. Rheological behaviour of milk-based persimmon beverages

3.3.1. Flow behaviour

Figure 7 shows the flow curves for the different milk-based persimmon beverages analyzed in this study. The milk-based beverages prepared with the different types of persimmon presented different flow behaviour.

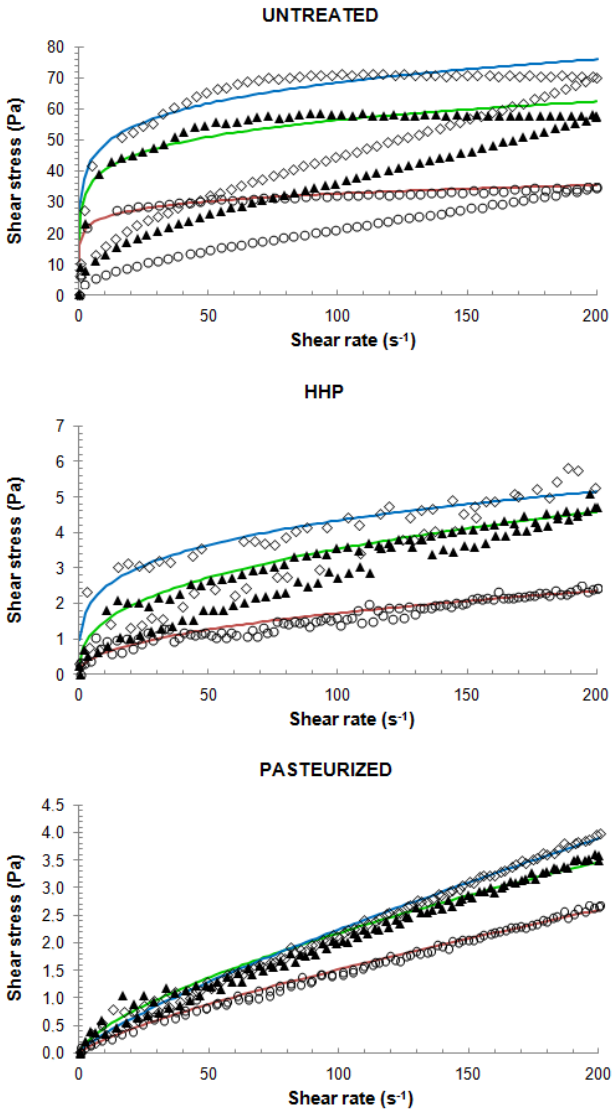

Figure 7. Flow behaviour of milk-based persimmon beverages prepared with freeze-dried untreated persimmon, freeze-dried HHP-treated persimmon, and freeze-dried pasteurized persimmon. Whole milk (◊), semi-skimmed milk (▲) and skimmed milk (○). Lines represent the curve fit to Ostwalds models.

Those made with freeze-dried untreated persimmon were semisolid systems which could be attributed to the gelation of pectins in the presence of divalent ions like Ca^{2+} from the persimmon fruit and the milk. The gelation mechanism of persimmon has been studied previously in acidified and natural persimmon purée with and without the addition of ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA) (Tárrega et al., 2013). Tables 1 and 2 show the flow parameters of milk without persimmon and milk-based persimmon beverages, respectively. The flow curves for the milk-based beverages made with freeze- dried untreated persimmon, regardless of the type of milk used, showed pseudoplastic, time-dependent flow behaviour that was characterized by determining Ostwald–de Waele parameters and the thixotropic area (Table 2). The milk-based beverages prepared with freeze-dried untreated persimmon presented higher consistency, pseudoplasticity and thixotropy than milk without persimmon (Table 1). No statistically significant differences ($P > 0.05$) were observed in the consistency index (K), flow index (n), apparent viscosity at 10 s^{-1} (η_{10}), or thixotropic area (A_{thix}) when different types of milk were used (Table 2). It would therefore seem to be the addition of persimmon rather than the fat content of the milk that defines the flow behaviour of the milk-based beverages. However, the apparent viscosity at 100 s^{-1} (η_{100}) decreased significantly when skimmed milk was used ($P < 0.05$).

Table 1. Ostwald–de Waele fit of milks used to formulate the milk-based persimmon beverages ($R^2 > 0.9979$).

	K (Pa·s^n)	n	η_{10} (Pa·s)
Whole milk	0.0079 (0.0002)	0.8254 (0.0005)	0.0053 (0.0001)
Semi-skimmed milk	0.0066 (0.0010)	0.8459 (0.0260)	0.0046 (0.0004)
Skimmed milk	0.0046 (0.0004)	0.9015 (0.0191)	0.0037 (0.0001)

Values in parentheses are the standard deviations.

Table 2. Ostwald–de Waele fit of milk-based persimmon beverages ($0.915 < R^2 < 0.9957$)

	Milk type	K (Pa·s^n)	n	η_{10} (Pa·s)	η_{100} (Pa·s)	A_{thix} (Pa·s^{-1})
Untreated	Whole milk	28.275^a (8.719)	0.191^a (0.058)	4.313^a (0.770)	0.664^a (0.030)	3860.500^a (962.372)
Untreated	Semi-skimmed milk	31.630^a (3.847)	0.146^a (0.001)	4.423^a (0.544)	0.619^a (0.077)	4258.500^a (656.902)
Untreated	Skimmed milk	20.710^a (2.093)	0.125^a (0.013)	2.764^a (0.362)	0.369^b (0.059)	2597.000^a (576.999)
HHP	Whole milk	1.258^a (0.177)	0.253^a (0.007)	0.225^a (0.028)	0.040^a (0.004)	148.850^a (2.333)
HHP	Semi-skimmed milk	0.730^b (0.139)	0.353^b (0.029)	0.164^a (0.020)	0.037^a (0.002)	139.700^a (26.022)
HHP	Skimmed milk	0.225^c (0.011)	0.458^c (0.012)	0.065^b (0.005)	0.019^b (0.002)	34.055^b (21.970)
Pasteurized	Whole milk	0.050^a (0.011)	0.813^a (0.023)	0.032^a (0.005)	0.021^a (0.002)	9.639^a (4.145)
Pasteurized	Semi-skimmed milk	0.090^a (0.001)	0.670^a (0.003)	0.046^a (0.001)	0.022^a (0.000)	27.675^a (2.001)
Pasteurized	Skimmed milk	0.065^a (0.032)	0.714^a (0.085)	0.032^a (0.010)	0.016^a (0.002)	17.620^a (7.750)

Values in parentheses are the standard deviations.

All values shown are averages of three measurements. For each milk-based beverage, values within a column with different letters are significantly different.

The HHP-treated persimmon milk-based beverages (Figure 7B) were liquid systems that also showed pseudoplastic and thixotropic behaviour. As in the case of freeze-dried untreated persimmon, when comparing the milks without persimmon (Table 1) with those of the milk-based beverage elaborated with freeze-dried HHP-treated persimmon, an increase in consistency, pseudoplasticity and thixotropy could be observed (Table 2). However, these effects were much smaller than those observed with the freeze-dried untreated persimmon, indicating that gelation did not occur or was less intense with the persimmon obtained by HHP treatment. So the freeze-dried HHP-treated persimmon was more appropriate for formulating this kind of product since a gel-like texture would be not adequate for beverages. The ANOVA results showed that with the HPP-treated persimmon the flow parameter values varied significantly depending on the type of milk. As expected, as the fat content of the milk decreased, the consistency index decreased significantly ($P < 0.05$) and the milk-based beverages formulated with skimmed milk presented significantly the lowest values ($P < 0.05$). The opposite occurred with the flow index: as the fat content decreased, the flow index increased significantly ($P < 0.05$), which corresponded to a more Newtonian flow. No significant differences ($P > 0.05$) in apparent viscosity at 10 s^{-1} and 100 s^{-1}, and the thixotropic area was found between the HHP-treated milk-based beverages made with whole and semi-skimmed milk, but the milk-based beverages formulated with skimmed milk presented significantly the lowest values ($P < 0.05$). It therefore seems that the flow behaviour of these milk-based beverages is governed by both the addition of persimmon and the fat content of the milk used in the formulation. Freeze-dried untreated persimmon has a very high gelling ability; so the presence of fat globules in the system structure does not significantly affect the response to deformation. In contrast, in the case of freeze-dried HHP-treated persimmon, which has less gelling ability, the presence or absence of fat in the structure, does influence its response to deformation.

When freeze-dried pasteurized persimmon (Figure 7C) was used in the formulation of the milk-based beverages, non-homogeneous dispersion of persimmon particles in a liquid matrix was obtained. This fact could probably correspond to the persimmon already being gelified and particles of persimmon gel remaining undissolved. As commented above, gelation of the persimmon could have taken place during pasteurization: the gel formation of pectins caused by the heating process has been

observed previously by other authors in olives (Galanakis et al., 2010) and persimmons (Tárrega et al., 2013). A noticeable sedimentation was observed in these milk-based beverages (43 ± 1% for whole milk; 45 ± 4% for semi-skimmed milk, and 42 ± 3% for skimmed milk). This sedimentation could be responsible for the lower consistency index (*K*) and apparent viscosity at 10 s^{-1} and 100 s^{-1} obtained for these milk-based beverages compared to the values obtained when freeze-dried untreated and HHP-treated persimmon were used to prepare the milk-based beverages (Table 2). Before flow measurement, the milk-based beverages that showed sedimentation were stirred manually to favour homogenisation. In these milk-based beverages, Newtonian-like flow was observed, with more linear flow curves, without thixotropy (Figure 7C) and flow index (*n*) values closer to 1 (Table 2) compared to the milk-based beverages prepared with freeze-dried untreated and HHP- treated persimmon. No statistically significant differences ($P > 0.05$) were observed (Table 2) in any of the flow parameters studied when different types of milk were used. The heterogeneous final product, with sedimented particles, could explain the fact that the type of milk used did not affect the rheological behaviour of the milk-based beverages. The flow response of the milk-based beverages made with freeze-dried pasteurized persimmon therefore seems to be mainly governed by the gelified persimmon particles.

3.3.2. Viscoelastic properties

Figure 8 shows the storage modulus (G') and loss modulus (G") values as a function of frequency. The mechanical spectra of the untreated persimmon milk-based beverages showed a response typical of weak gels, with G' higher than G" (Figure 8A). Although small differences in rheological parameters were noticed when using different types of milk (Table 3), these differences were not significant ($P > 0.05$). As with flow behaviour, it was observed that the fat content did not affect the viscoelastic properties of the milk-based beverages.

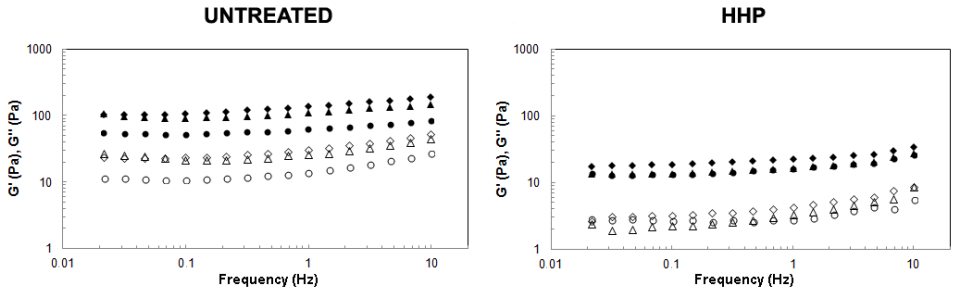

Figure 8. Viscoelastic properties of milk-based persimmon beverages prepared (G' full symbols, G" empty symbols, whole milk, ◊; semi-skimmed milk, Δ; skimmed milk, ○.

Table 3. Storage modulus (G') and loss modulus (G") values at 1 Hz for milk-based persimmon beverages.

	Milk type	G'(Pa)	G" (Pa)
Untreated	Whole milk	138.300[a] (31.961)	30.950[a] (8.910)
Untreated	Semi-skimmed milk	113.095[a] (22.210)	26.420[a] (2.291)
Untreated	Skimmed milk	62.435[a] (4.250)	14.220[a] (0.325)
HHP	Whole milk	23.230[a] (1.428)	4.360[a] (0.074)
HHP	Semi-skimmed milk	17.080[b] (0.792)	3.344[b] (0.227)
HHP	Skimmed milk	16.315[b] (0.884)	2.766[b] (0.346)

Values in parentheses are the standard deviations.

All values shown are averages of three measurements. For each milk-based beverage, values within a column with different letters are significantly different.

The mechanical spectra of the HHP-treated milk-based beverages (Figure 8B) also showed G' values higher than G" ones, but both of them were much lower than those obtained with freeze-dried untreated persimmon and G" showed higher frequency dependence, indicating a much weaker structure and therefore confirming the lower gelling ability of these milk- based beverages. No statistically significant differences ($P > 0.05$) in either G' or G" at 1 Hz were found between the milk- based beverages made with semi-skimmed and skimmed milk (Table 3). However, the milk-based beverages formulated with whole milk presented significantly the highest G' and G" values ($P < 0.05$).

Due to the heterogeneity and low viscosity of the milk-based beverages made with freeze-dried pasteurized persimmon, the linear viscoelastic region could be not found and viscoelastic properties for these milk-based beverages could not be determined.

4. Conclusions

HHP treatment would encourage the release of carotenoids from the plant material matrix and hence increase their extractability as could be seen via confocal microscopy and when quantifying the carotenoid content. In addition, HHP treatment seems to favour tannin precipitation and could therefore decrease the astringency of the fruit. Freeze-drying processing demonstrates to be useful to extend the shelf life of persimmon and to obtain derivatives that could be incorporated in formulations with new functional features. HHP treatment provides persimmon to formulate milk-based beverages with high carotenoid content using smaller quantities of freeze-dried persimmon. These beverages possess suitable rheological properties because they do not form a gel-like structure, unlike the milk-based beverages with freeze-dried untreated persimmon, and do not present sedimentation, unlike the milk-based beverages formulated with freeze-dried pasteurized persimmon. Further research should be conducted to evaluate the sensory properties and consumers' liking of these milk-based persimmon beverages prior to their commercial release.

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Persimmon milkshakes with enhanced functionality: understanding consumers' perception of the concept and sensory experience of a functional food

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Abstract

Persimmon is a good source of antioxidant compounds, such as carotenoids and tannins but it cannot be consumed throughout the whole year because it is a seasonal fruit. Therefore, it would be useful to prolong its shelf life producing food products with high nutritional value. High hydrostatic pressure (HHP) represents an alternative to pasteurization in order to preserve and extend the shelf life. In this work six different persimmon milkshakes were studied varying the type of milk (whole and skimmed) and the treatment of the persimmon (untreated, HHP-treated and pasteurized). The sensory characteristics of persimmon milkshakes with enhanced functional properties and their liking were studied, and these findings were related with the consumers' health and taste related attitudes. Results showed that consumers perceived persimmon milkshakes as a high antioxidant beverage. Milkshakes with HHP-treated persimmon, regardless the type of milk and with untreated persimmon and whole milk were scored with the highest overall liking. So, treating persimmon by HHP makes it possible to formulate persimmon milkshakes with high nutritional value and high acceptability despite of the seasonality of the fruit.

Keywords: flash profile, functionality, high hydrostatic pressure, persimmon, sensory properties.

1. Introduction

Persimmon (*Diospyros kaki* L.) is one of the fruits with the highest levels of bioactive compounds (Jung et al., 2005). In addition to β-carotene, persimmons have carotenoid compounds with considerable antioxidant activity, phenolic acids, dietary fibre (Hernández-Carrión et al., 2014b) and large quantities of tannin, an antioxidant that is responsible for their astringency.

High hydrostatic pressure (HHP) is a non-thermal technology for preserving and extending the shelf life and quality of foods (Ferrari et al., 2010; Nguyen et al., 2010).

Consumer perception of food products is a very complex phenomenon that is influenced by a wide range of characteristics (Vidal et al., 2013). The role of the consumer in determining the market success of a product is of maximum relevance. Consumers judge food quality on its sensory characteristics and on the nutritional value, health benefits, calorie content, vitamins and antioxidants, which determine the individual preferences for specific products (Norton & Sun, 2008). Recently, there has been an increased awareness of the importance of healthful food products among consumers (Santos et al., 2013). They appreciate the protective role that dietary antioxidants, like phenolics, have against the development of chronic diseases (Sanders III et al., 2014). When developing a new functional food, consumers' interest and liking has to be evaluated.

Identifying segments of consumers with different attitudes towards food and nutrition might allow the targeting of different types of products for each segment (Roininen et al., 1999). Several authors have reported that consumers more interested in health and nutrition related issues have a more positive attitude towards functional foods (Urala & Lähteenmäki, 2004; Ares et al., 2009).

The content of some bioactive compounds and the rheological properties of milkshakes elaborated using untreated, HHP-treated and pasteurized persimmon have been previously studied (Hernández-Carrión et al., 2014a) On this basis, in the present work, consumer perception toward them is studied. So, the aim of this work was to explore: 1) the concept of persimmon milkshakes with enhanced functional properties, 2) to know their sensory characteristics as perceived by consumers and their liking and 3) to relate these findings with consumers' health and taste related attitudes.

2. Materials and methods

2.1. Materials and sample preparation

Commercial maturity stage IV persimmon fruits (*Diospyros kaki* L., 'Rojo Brillante', astringent variety) were harvested in Carlet (Valencia, Spain). Bags containing persimmon cubes were prepared. One-third was not treated (Unt), one-third was HHP-treated (HHP) (200 MPa, 6 min, 25 ºC), and one-third was pasteurized (PAST) in a water bath at 70 ºC for 15 min (Hernández-Carrión et al., 2014b). Each type of persimmon was homogenized for 90 s and freeze dried for 120 h at -45 ºC and $1.3 \cdot 10^{-3}$ mPa in a freeze drier (Lioalfa-6®, Telstar, Terrassa, Spain) before their use in the milkshakes.

Six different milkshakes were studied, varying the type of milk: whole milk (36 g L^{-1} fat content, W) and skimmed milk (2.5 g L^{-1} fat content, S) from the Central Lechera Asturiana, Siero, Spain, and the treatment of the persimmon fruit. The quantity of freeze-dried persimmon used in the formulation, and the milkshakes preparation followed the methodology described by Hernández-Carrión et al. (2014a). For the samples used in sensory analysis, sugar (70 g kg^{-1}) from Azucarera Ebro (Madrid, Spain) and 1 mL of Allura Red AC stain (0.12 g L^{-1}) were added. The milkshakes were kept at 4 ºC until their analysis. The sensory analysis was carried out within 24 h of the milkshake preparation.

2.2. Theory and fundamentals of sensory methodologies

An increasing number of sensory techniques using panels with different degrees of training have been applied to food development in recent years (Varela & Ares, 2012). Dairou & Sieffermann (2002) suggested the use of flash profiling (FP) for sensory description, a combination of free choice terms selection with a ranking method based on the simultaneous presentation of the entire product set. A comparative evaluation of all the products is done to generate the attribute list, followed by a ranking on the intensity of each generated attribute (Albert et al., 2011; Santos et al., 2013). FP is a flexible method for rapidly profiling products according to their most salient sensory attributes, and it has been successfully applied to describe various foods (Valentin et al., 2012; Varela & Ares, 2012). It is particularly good as screening tool for

a new product set or category (Delarue & Sieffermann, 2004; Tarea et al., 2007). Sensory methodologies based on consumer responses can also be valuable for exploring the perception of foods, usually requiring only one consumer test (Cruz et al., 2013). In this context, "check all that apply" (CATA) questions are a good choice (Ares et al., 2010). They consist of a pre-selected list of terms from which respondents select all they consider appropriate to describe a product. Several studies have shown that the results from CATA questions used with consumers are very similar to the results obtained from trained panels (Ares et al., 2010; Dooley et al., 2010; Bruzzone et al., 2012); consumers generally consider that answering CATA questions is simple (Ares et al., 2011).

Word association (WA) is a qualitative exploratory methodology that is based on the assumption that providing a stimulus to a respondent and asking him/ her to freely associate the ideas that enter his/ her mind could provide a thorough unconscious response to the product (Ares et al., 2008). This procedure elicits more spontaneous reactions than ordinary interviews or closed questionnaires (Gámbaro et al., 2014), comprising a quick and useful method for exploring consumer perception of food products (Varela & Fiszman, 2013; Varela et al., 2014).

2.2.1. Sensory description via Flash Profiling (FP)

Panel

The FP process was conducted by 10 semi-trained assessors from the Institute of Agrochemistry and Food Technology who had experience in the sensory description of food products with no particular training in the description of persimmon milkshakes.

Procedure

The FP process consisted of one session with two steps, conducted in a standardized tasting room equipped with individual booths (ISO, 1988). The samples were presented all together, the six different persimmon milkshakes in the study and one additional repeated sample as the blind control in order to check the panel performance (7 samples in total), coded with three digit random numbers. Mineral water was provided for mouth rinsing. During the first step, the assessors were given

an explanation about the procedure, and each assessor tasted all the samples comparatively in order to generate a list of the descriptors that they considered appropriate for discriminating between the milkshakes. Each assessor generated his/her own set of attributes; no indication was given regarding the number of attributes (Lassoued et al., 2008; Moussaoui & Varela, 2010). In a second step, they assessed the seven samples, by ranking the products for all of the selected attributes from "low" to "high" (with ties allowed).

2.2.1. Consumers' perception study

The experiment consisted of four parts aiming at collecting information of the consumers' perception from different perspectives. First, a WA task was directed at exploring the concept of a persimmon milkshake with high natural antioxidant content. Then, the samples were tasted, and an acceptability study tested the overall hedonic appreciation and investigated the different attributes of the samples in relation to their overall liking. For unveiling the attributes underlying product preference, consumers answered a CATA question directed to describe the samples sensorially and to gather information about the uses and attitudes. Lastly, the consumers answered an attitudinal questionnaire on health and hedonics. Details of the procedure are below.

General procedure

Consumers were recruited among the employees and students of the Universitat Politècnica de València. A total of 100 consumers, aged 19-65, were recruited for the study, based on their willingness to participate and their consumption frequency of milkshakes (Table 1). All the consumers completed the four parts of the test.

The samples were assessed in a standardized tasting room equipped with individual booths (ISO, 1988). Each consumer received six samples of milkshake in a sequential monadic series in a single session, following a balanced complete block experimental design (William's design). The samples were served at 10 ºC in plastic glasses coded with three digit random numbers. The test was recorded on paper and self completed after instructions were given by an interviewer.

Table 1. Gender, frequency of consumption of milkshakes and drinking yoghurts, and level of education of the consumer samples.

Sex	Women	57%
	Men	43%
Frequency of consumption	Daily	25%
	Weekly	32%
	Sporadically	38%
	Never	5%
Level of education	None	0%
	Primary	2%
	Secondary	13%
	Universitary	85%

Word association (WA) task.

This part of the task was completed without the consumers having tasted the samples. The participants were shown the following statement: "Persimmon is a fruit with a high content of natural antioxidants", and they were asked to write down the first four words, descriptions, associations, thoughts or feelings that entered their minds when thinking about a "Persimmon milkshake".

Acceptability testing

The consumer acceptance test was performed using a nine-point hedonic scale (9 = like extremely and 1 = dislike extremely). For each persimmon milkshake, the consumers scored their degrees of liking in the following order: "overall liking", "appearance liking", "flavour liking" and "consistency liking".

CATA question

For each sample, the participants answered a CATA question featuring 33 attributes. The following instruction was given to participants: "Which of the following characteristics better describe this sample? Please check all that you think apply. You can re-taste the simple if desired". The terms had been previously selected on the basis of the available literature and an informal tasting by the researchers and a sensory descriptive panel (with training in descriptive analysis but not specifically on

persimmon drinks). They belonged to five groups; Appearance terms: *lumpy appearance, soft appearance, thick appearance, fluid appearance, dark colour, light colour; consistency terms: viscous, lumpy in mouth, soft in mouth, creamy, thick in mouth, fluid, fibrous, pasty; flavour terms: fruity, sweet, bitter, persimmon flavour, odd flavour, intense flavour*; mouthfeel and aftertaste terms: *astringent, mouth coating, aftertaste*; use & attitudes terms: *antioxidant, not antioxidant, healthy, I would consume, I would not consume, prevents illnesses, prevents ageing, nutritive, believable* and *not believable*. The 33 terms were presented following the "natural" sequence of evaluation in terms of sensory modality as follows: appearance, consistency, flavour, mouthfeel and aftertaste, followed by the non-sensory parameters. Within those groups the terms were randomized between the products and among the consumers so that the terms were not physically divided into groups and were presented as a continuous list in two columns.

Attitudinal questionnaire regarding health and hedonic characteristics

At the end of the interview, the participants were asked to answer a questionnaire (Table 2) regarding their attitudes towards health and hedonic characteristics of foods. The attitudinal questionnaire was based on one developed by Roininen et al. (1999). The participants endorsed their degree of agreement with 18 statements using a seven-box scale anchored with 'I completely disagree' on the left and 'I completely agree' on the right

2.3. Data analyses

FP: Data processing involved converting the ranking positions into scores (Delarue, 2014): the lowest intensity was given a 1 and the highest intensity was a 7. In case of ties, the mean rank was given to all tied samples. One table is then obtained for each panellist, with the 7 samples in rows and a varying number of attributes in columns. A Generalized Procrustes Analysis (GPA) was used to obtain the product and attribute configurations (Gower, 1975). The GPA reduces the scale usage effects, delivers a consensus configuration and allows a comparison of the proximity between the terms used by different assessors to describe products (Moussaoui & Varela, 2010); it is very suitable for this methodology.

WA: all of the terms provided by the participants were considered for the analysis. The frequency in which each association was mentioned was determined by counting the number of participants that mentioned that particular term. Then, the associations were grouped into different categories; finally, the categories were grouped into different dimensions. The grouping procedure was performed by two of the researchers who authored this study, considering the personal interpretation of the meaning of the words and word synonyms as determined by a Spanish dictionary. After individually evaluating the data, a meeting of the researchers was conducted to verify the agreement between their classifications. The final categories and their names were determined by consensus between the two researchers considering their two independent classifications and the discussion between them (Guerrero et al., 2010). At this stage of the analysis, only categories mentioned by more than 5% of the participants were considered.

CATA question: a chi-square test was used to study the differences in the persimmon milkshakes from the CATA responses. For each persimmon milkshake, the frequency of use of each sensory attribute was determined by counting the number of consumers that selected that term to describe each sample. Cochran's Q test (Manoukian, 1986) was performed on the data of the individual terms to identify the significant differences between the samples for each of them (one table per attribute, with the binomial data obtained from consumers for the 6 samples). A multiple factor analysis (MFA) was run on the table with the frequency of selection of CATA terms data to understand the comparative positioning of the six persimmon milkshakes, as perceived by the consumers (samples in columns, 33 attributes in rows). The overall liking was superimposed in the obtained CATA sensory space as a supplementary variable.

Liking: One way ANOVA was applied to study the differences between the formulations for the consumer acceptance test; the least significant differences were calculated by Fisher's test ($P < 0.05$).

Consumer's attitudes: To identify the groups of consumers with different attitudes towards the health and hedonic characteristics of foods, a hierarchical cluster analysis (HCA) was performed on the data from the attitudinal questionnaire.

All data analyses were performed using XLSTAT statistical software (Version 2010.5.02, Microsoft Excel®, Barcelona, Spain), except from acceptability data that were analyzed using the Statgraphics Plus 5.1 software package (Statistical Graph Co., Rockville, MD, USA).

3. Results and discussion

3.1. Sensory description via Flash Profiling (FP)

The assessors generated a total of 65 terms; the most frequently mentioned were *astringent, lumpy appearance, thick appearance, thick in mouth, creamy* and *sweet*. The first two dimensions of the FP (Figure 1A) accounted for 90.88% of the variance of the original dataset (68.02% and 22.87%, respectively). The first dimension (the X axis) was related to the consistency of the milkshakes, where *thick* and *viscous appearance*, were placed at the right of the axis, and the attributes *liquid* and *fluid appearance* were placed at the left of the axis.

Figure 1B shows that the participants were able to perceive the differences and similarities between the samples, and the milkshakes were clearly separated into three groups according to their sensory characteristics and the treatment. There was a clear differentiation between untreated persimmon (Unt_W and Unt_S) compared to HHP-treated (HHP_W and HHP_S) and pasteurized (PAST_W and PAST_S). The milkshakes Unt_W and Unt_S were placed very close together as were HHP_W and HHP_S and PAST_W and PAST_S that were placed at an intermediate position regarding the texture characteristics. PAST_W and its replica (PAST_Wrep) were located very closely showing the good performance of the semi trained panel in describing the samples. The joint study of the maps of the attributes and samples (Figure 1) enabled a better understanding of the sensory characterisation of each sample and of the groups of samples. Unt_W and Unt_S were characterized by having an *intense colour* and a *viscous appearance* and by having the attribute of *viscous in mouth* (right lower quadrant). The intense colour observed in them could be from the greater amount of persimmon in the formulation as compared to the rest, which was required to achieve the identical carotenoid content. The higher perceived viscosity could be attributed to the gelation of the pectins in the presence of divalent ions such Ca^{+2}

from the persimmon fruit and milk (Tárrega et al., 2013; Hernández-Carrión et al., 2014a). The attributes *sweetness, lumpy appearance, lumpy in mouth* and *astringency* predominantly defined the milkshakes PAST_W and PAST_S (the right and left upper quadrant). The gelation mechanism of the persimmon during pasteurization could explain the lumpy appearance due to the undissolved persimmon gel. Among pasteurized persimmon, those formulated with whole milk (PAST_W and PAST_Wrep) were perceived as *viscous* (right upper quadrant), whereas those prepared with skimmed milk (PAST_S) were characterized as *fluid appearance* (left upper quadrant). HHP_W and HHP_S were characterized by a less *lumpy appearance* and by being less *sweet* and less *astringent* than the pasteurized milkshakes (the left lower quadrant). Regarding the astringency, although its location for most of the semi trained assessors was related to the pasteurized persimmon milkshakes, some assessors placed this attribute in the quadrant corresponding to that of the HHP-treated. Gawel et al. (2000) defined astringency as a complex phenomenon that causes a wide range of oral sensations and obtained up to 33 descriptors to define the astringency in red wine. This complexity could explain the low agreement among the assessors for this attribute. The sensory characterisation with the use of rapid methods such as FP could be difficult for complex sensations in which more than one modality is involved (Ares & Varela, 2014). In this sense, the use of a semi-trained panel through a rapid method might have been a limitation of the present work, in comparison to the use of a classical descriptive approach (e.g. QDA) with a trained panel, which might have drawn a more accurate picture of the astringent character. However, it is noteworthy that astringency is challenging to evaluate even by trained panels, because of its complexity and its temporal aspects during consumption, particularly because of its enhanced carry-over effects reported to build up in time over repeated exposures (Ross et al., 2007; Lee & Vickers, 2010). On the other hand, the fast and flexible response FP delivered - given the overall good performance of the semi trained panel as reflected by the blind reference - somehow outweighed the limitations of having a less detailed picture. Flash profile is especially well suited for applications like the present one, when running a project with no pre-existing descriptive tool and no need for a trained panel after the project is completed (Delarue, 2014).

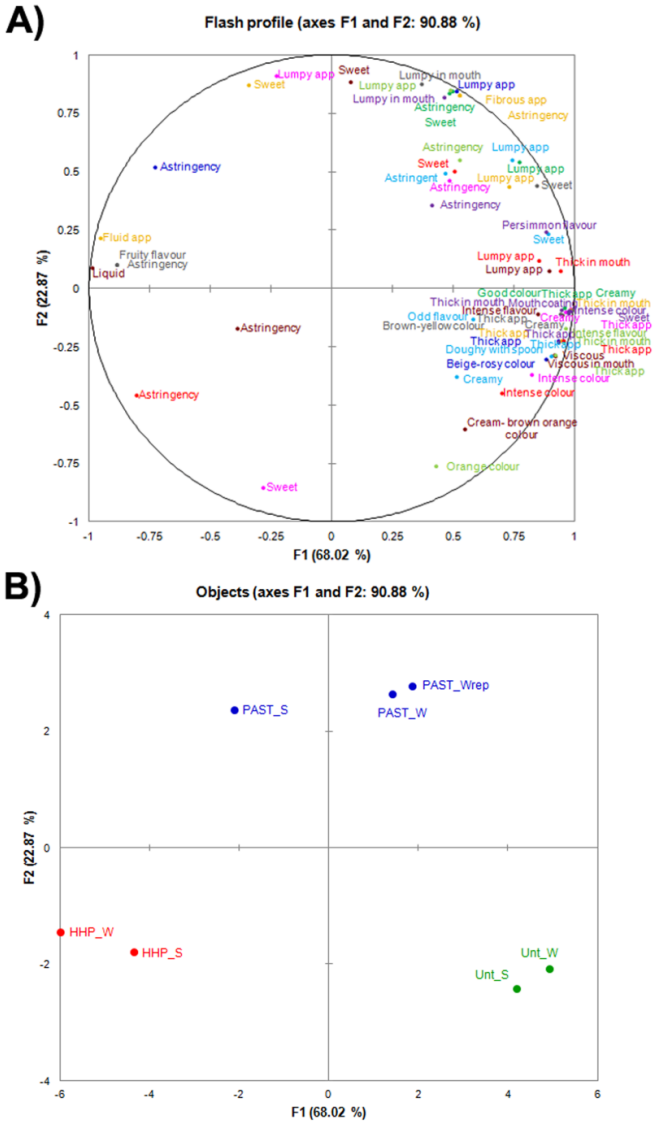

Figure 1. A) Representation of the terms from the flash profiling data, and B) representation of the six persimmon milkshakes and the replica, in the first two dimension of the Generalized Procrustes Analysis (GPA). Unt_W and Unt_S, untreated persimmon with whole and skimmed milk, respectively; HHP_W and HHP_S, HHP treated persimmon with whole and skimmed milk, respectively; PAST_W and PAST_S, pasteurized persimmon with whole and skimmed milk, respectively.

3.2. Consumers' perception study

3.2.1. Word association task

All of the participants completed the WA task, suggesting that they had a clear image of the stimulus. They were expected to think about a "Persimmon milkshake", keeping in mind the concept that "Persimmon is a fruit with a high natural antioxidant content". The participants generated 313 words. The data analysis process highlighted eight dimensions (Table 3). The most frequently mentioned dimension was *hedonics* (positive terms), and *refreshing* and *healthy* were the most frequently elicited terms. The relationship between persimmon milkshakes and expected pleasantness was promising for a new product in terms of the expected positive sensory experience. The other three most relevant sensory dimensions were the *flavour, texture* and *appearance* attributes. Thus, consumers appeared to associate persimmon milkshakes with specific flavour, texture and appearance sensations.

Regarding the *flavour attributes*, the consumers rated the persimmon milkshake predominantly on *sweetness*. Another relevant dimension was related to the *texture characteristics* comprised a relevant dimension, of which the most frequently elicited texture terms were *creamy, thick* and *astringent*, indicating the foremost tactile sensations the consumers expect to perceive when tasting a persimmon milkshake. *Creamy* and *thick* were two positive characteristics. *Astringency* could be seen in principle as a non-desired sensory property, but consumers might expect some astringency, most likely at a maximum optimal level. This finding is promising, particularly because the sensory description by the semi trained panel via FP highlighted this attribute for most of the tested samples. That consumers expected to find persimmon milkshakes astringent might be related to their past experience when consuming the fruit or with the fact that other foods high in natural antioxidants are normally astringent including wines, olive oil and dark chocolate (Misnawi et al., 2004; Bendini et al., 2007). The *Appearance attributes* were elicited by the participants, and *orange* was the major appearance attribute mentioned.

The participants associated milkshakes with different *emotion-related terms* such as *summer, winter, autumn, twilight* or *trees*. Despite the fact that this dimension was mentioned by approximately one-sixth of the participants, none of the categories within this dimension was mentioned by more than five participants. This suggests

that the associations were heterogeneous. *Fruit*-related associations and *consumption/use* associations were mentioned when consumers thought of persimmon milkshakes. The most salient term within the *fruit* dimension was *tropical fruit*, suggesting that consumers would relate milkshake to this type of fruit (possibly exotic fruit). Regarding the *consumption/use terms*, most of them were mentioned by only one participant (data not shown). These two dimensions were mentioned by a small number of participants, indicating that persimmon milkshakes predominantly generated expectations related to *hedonics*, *flavour, texture* and *appearance,*

Table 3. Frequency of mention of dimensions and categories determined by grouping elicited words.

Dimensions and categories	Percentage of mention (%)
Appearance characteristics	60
Orange	35
Red	8
Soft/Ripen	6
Others	11
Flavour characteristics	65
Sweet	50
Tasty	9
Others	6
Hedonics	74
Refreshing	28
Healthy	12
Natural	5
Vitamins	6
Others	23
Texture characteristics	62
Creamy	10
Thick/consistent	16
Astringent/Rough	22
Others	14
Consumption/use	11
Fruits	15
Emotionally related terms	15
Others	11

3.2.2. Attitudinal questionnaire regarding health and hedonic characteristics

A hierarchical cluster analysis (HCA) was performed on the attitudinal questionnaire (Table 2). Based on the dendrogram obtained, no differentiated patterns were highlighted (data not shown), suggesting that all of the participants showed similar attitudes towards food health and sensory characteristics.

The answers showed that participants were generally positive about food and health; they considered that diet was important for health (item 1). The consumers perceived that consuming certain food products could have a positive effect on health and prevent some diseases (items 3 and 4). They were interested in taking disease prevention measures (item 8), related antioxidant intake to a reduction of risk in some diseases (item 13) and were willing to consume products with a positive health effect (item 16). The consumers thought that food should have a taste that they like (item 10), were interested in enjoying the taste of foods (item 12) and disagreed with the statements "I take into account the appearance of food when deciding what to eat" and "I consume highly nutritious products that I don't like". These results suggest that although the participants consider their health to be important and are conscious of the importance of diet to good health, they usually eat what they like regardless of the health benefit of certain foods. This behaviour is in accordance with previous results showing that taste is a major condition for acceptance, in addition to the trustworthiness of health claims (Cox et al., 2004; Verbeke, 2005). According to Verbeke (2006), numerous consumer studies have pointed to the primary role of taste in directing the food choices of consumers (Urala & Lähteenmäki, 2003). This finding is in agreement with Carrillo et al. (2011) who showed that the most important factors conditioning consumer food choice attitudes were that the item "tastes good," "is good value for money" and "keeps me healthy"; however, the "health" factor was not the most important factor. They demonstrated that consumers do not necessarily recognize or know the health benefits associated with some individual components (proteins or fibre) or with reduced levels of others (fat and sugars). In the present study, the consumers seemed quite certain that "Antioxidant intake could decrease the risk of some diseases", which could be because antioxidants are frequently discussed in the media and presented in food claims. This finding could be positive in light of the development of a high antioxidant milkshake because the consumers' attitudes towards antioxidants were positive.

Table 2. Items of the attitudinal questionnaire about health and hedonic characteristics of foods.

Statements	Average score
1. Diet is important for my health	6.4 (1.0)
2. I always eat food products that I like	5.4 (1.4)
3. Consuming some food products could have a positive impact on my health	6.6 (0.9)
4. Consuming some food products could help prevent some diseases	6.4 (1.0)
5. Food products should not necessarily be a source of pleasure	4.4 (2.0)
6. I do all I can to keep myself healthy	5.4 (1.3)
7. I am willing to make sacrifices to keep myself healthy	5.3 (1.4)
8. I am interested in taking measures to prevent the occurrence of some diseases	5.8 (1.3)
9. I do not take into account the appearance of food when deciding what to eat	3.3 (1.9)
10. Food products should have a taste that I like	6.0 (1.2)
11. I am interested in losing weight	4.5 (2.2)
12. I am interested in enjoying the taste of foods	6.4 (0.8)
13. Antioxidants intake could decrease the risk of some diseases	6.2 (1.1)
14. Healthiness and nutritional content have a high impact on my food choices	5.1 (1.4)
15. I always follow a healthy and balanced diet	4.9 (1.4)
16. I am willing to consume products that have a positive impact on my health	6.0 (1.1)
17. I consume highly nutritious products that I don't like	3.0 (1.7)
18. I am interested in consuming products that have a positive impact on my health even if I don't like them as much as others	4.6 (1.6)

Values in parentheses are the standard deviations.

The fact that the consumers' primary interest was taste rather than health could be associated with the responses obtained in the word association task, which were predominantly related to sensory attributes rather than to those related to health.

In the present work health and taste attitudes were measured to check for the potential existence of groups of consumers that might differ in this point, which could be very relevant in the study of functional foods perception. The results did not show any segmentation in this sense, showing the homogeneity in the interviewed population; furthermore attitude measurement is a good way of characterizing them, helping to better understand their perception.

3.2.3. CATA questions

The first two factors of the attribute map (Figure 2A) accounted for 84.03% of the variance (51.40% and 32.63%, respectively). Most of the attributes were well represented in the perceptual space defined by the first two factors of the MFA. The first factor (X axis) was related to textural attributes; *fibrous, lumpy in mouth* and *lumpy appearance* as attributes were placed on the right of the axis and *soft in mouth* and *soft appearance were* placed on the left of the axis. This factor was related to the taste, and the attributes such as *odd flavour, aftertaste* and *mouth coating* were located on the right of the axis and *persimmon flavour, fruity* and *sweet* were placed on the left. Food claim attributes such as *nutritive, prevents illnesses, antioxidant, believable, healthy* and *I would consume* were placed on the left of the axis, whereas *not believable* and *I would not consume* were located on the right of the axis. The second factor (Y axis) was related to the textural attributes (*fluid, fluid appearance* and *astringent,* down; *pasty, viscous, creamy, thick in mouth* and *thick appearance* up); the colour (*light colour* down; *dark*, up) and the taste of the milkshakes (*intense flavour* and *bitter,* up). The Chi-square test ($P < 0.0001$) indicated statistically significant differences between the descriptions of the samples. Cochran's Q test was performed on the data to identify the significant differences between the samples for each of the terms included on the CATA question. The results (Table 4) showed that statistically significant differences ($P < 0.05$) were found between the samples for most of the analyzed attributes. No statistically significant differences ($P > 0.05$) were found for *bitter*, *intense flavour, aftertaste, not antioxidant* and *prevents illnesses* among the samples. This finding is in agreement with the MFA results

in reporting that the samples were well discriminated based on the CATA description. Figure 2B shows that milkshakes were clearly separated into three groups according to their sensory characteristics. Samples Unt_W and Unt_S were perceived as *pasty* and *viscous*, with the attributes of *thick appearance*, *thick in mouth*, *dark colour* and *intense flavour*. These results are consistent with those obtained by the semi trained assessors' panel that perceived these milkshakes as having the attributes of *viscous, viscous in mouth* and with *intense colour*. The milkshakes HHP-W and HHP_S were related to *fluid appearance* and *soft*, *sweet*, *persimmon flavour* and *light colour*. Similar results were obtained when using FP, in which assessors associated these milkshakes with *fluid appearance*. Finally, milkshakes PAST_W and PAST_S were mainly associated with *astringent*, *not antioxidant*, *lumpy, fibrous, not believable* and with *aftertaste*. In the same way, assessors defined these milkshakes as *astringents* and *lumpy*. With respect to the non-sensory parameters positive attributes as *nutritive, prevents illnesses, antioxidant, believable* and *healthy* were mainly correlated to the first factor of the MFA. Those characters were mainly associated to samples Unt_W and both HHP; also, those samples were described as with *persimmon flavour*. At the same time, this area of the perceptual space was associated to the highest overall liking (OL, superimposed as supplementary variable) and to the CATA parameter *I would consume*. It can be concluded that an enhanced persimmon flavour, together with the right sweetness and softness would drive the liking and the purchase intent in these category, fitting very well the intended concept of high antioxidant content.

The advantage of gathering direct feedback from consumers through the use of a consumer profiling method like CATA is clear in this study; consumer description of the samples was very much in line with the semi-trained panellists but also added further information regarding the drivers of liking and disliking associated with the consumption of such products. CATA permitted the association of concrete attributes of the product category to the willingness to consume, believability and healthiness perception, allowing a better understanding of which technologies would allow having a product better "fit to concept". The samples treated by HHP had better sensory appreciation but also were more believable and perceived as healthier, as compared to the other samples.

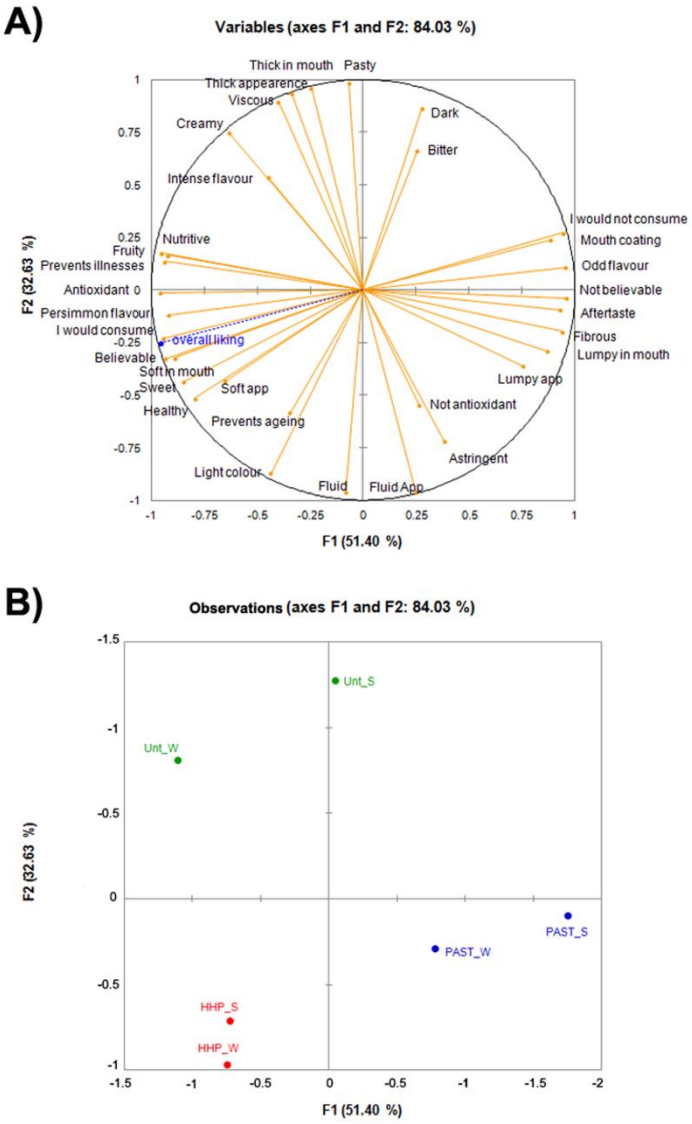

Figure 2. A) Attribute map from the check all that apply (CATA) questionnaire, and B) Representation of the six persimmon milkshakes, in the first two dimensions of the Multiple Factor Analysis (MFA) of the CATA counts. Unt_W and Unt_S, untreated persimmon with whole and skimmed milk, respectively; HHP_W and HHP_S, HHP treated persimmon with whole and skimmed milk, respectively; PAST_W and PAST_S, pasteurized persimmon with whole and skimmed milk, respectively.

Table 4. Frequency of selection of CATA terms and Cochran's Q test for significant differences between six persimmon milkshakes.

CATA Terms	Unt_W	Unt_S	HHP_W	HHP_S	PAST_W	PAST_S	Q
Lumpy app.	15	24	40	15	57	49	**<0.0001**
Soft app.	39	20	40	45	22	19	**<0.0001**
Thick app.	83	87	9	8	10	12	**<0.0001**
Fluid app.	4	2	64	67	53	57	**<0.0001**
Dark	16	33	2	11	9	20	**<0.0001**
Light colour	58	40	79	72	66	50	**<0.0001**
Viscous	34	32	6	6	1	7	**<0.0001**
Lumpy in mouth	13	20	39	10	73	78	**<0.0001**
Soft in mouth	31	23	43	45	10	6	**<0.0001**
Creamy	69	52	21	20	5	6	**<0.0001**
Thick in mouth	43	56	5	6	10	9	**<0.0001**
Fluid	4	1	55	61	31	31	**<0.0001**
Fibrous	8	11	13	9	36	44	**<0.0001**
Pasty	41	59	6	8	18	18	**<0.0001**
Fruity	43	35	34	41	28	19	**0.001**
Sweet	60	41	60	53	56	42	**0.001**
Bitter	5	8	4	6	5	6	0.801
Persimmon fl.	46	39	52	47	23	24	**<0.0001**
Odd fl.	14	26	13	18	27	48	**<0.0001**
Intense fl.	23	16	15	14	17	14	0.398
Astringent	19	27	35	36	28	37	**0.003**
Mouthcoating	27	29	20	23	30	46	**0.000**
Aftertaste	18	25	20	25	27	33	0.082
Antioxidant	18	15	16	19	13	9	**0.044**
Not antioxidant	0	2	5	2	1	4	0.149
Healthy	27	22	38	33	19	18	**<0.0001**
I would consume	36	16	39	30	12	5	**<0.0001**
I would not consume	31	52	24	35	55	68	**<0.0001**
Prevents illnesses	4	4	4	4	3	2	0.767
Prevents ageing	5	1	4	10	6	3	**0.000**
Nutritive	34	25	30	24	22	13	**0.001**
Believable	23	16	30	27	12	5	**<0.0001**
Not believable	9	12	11	8	17	19	**0.037**

Highlighted terms correspond to those for which significant differences between samples were identified according to Cochran's Q test ($P < 0.05$).

3.2.4. Acceptability study

For the six milkshakes, the overall liking (OL) scores were between 6.1 and 3.7 (Table 5). The liking of appearance, flavour and texture was in line with the OL, which indicated that the most liked samples were well liked in those parameters. The milkshake HHP_W obtained the maximum score for the OL, appearance and consistency liking, with no statistically significant differences ($P > 0.05$) in the OL between it, HHP_S and Unt_W.

The milkshake PAST_S had the significantly lowest ($P < 0.05$) OL and flavour liking scores. Regarding the appearance and consistency liking, the milkshakes Unt_S, PAST_W and PAST_S obtained the lowest significant ($P < 0.05$) scores, indicating that they were the least acceptable by the consumers on these modalities. The worst performance of Unt_S in comparison with its whole milk counterpart was because it was selected less for *soft in appearance* and *in mouth* and more for *dark in colour* (Table 4). The consumers rejected milkshakes that were related to *astringent*, *fibrous* and *lumpy* (PAST_W and PAST_S). Considering the frequency of the selection of *astringency* (Table 4), the consumers found most of the samples to be *astringent*, and astringency was not actually a driver of disliking; HHP_W was selected as *astringent* as many times as the PAST_W sample. It is likely that *lumpy appearance* and *mouth perception,* as well as the lower intensity of *persimmon flavour* drove the disliking of the pasteurized samples. Although astringency is expected (according to the word association results), there might be a maximum acceptable level of astringency, which this study does not uncover. This relationship merits further investigation.

As the most appreciated samples were the ones treated by HHP, although it was outside the scope of the present work, the perception of this preservation method in this category should be further investigated in the future. Consumers' perception of alternative processing techniques is usually challenging and could potentially determine some barriers against the consumption; however, HHP seem to be easier for consumers to understand and accept as compared to other new technologies (Olsen et al., 2010), particularly due to the better sensory features obtained, similar to those of natural products (Gomes da Cruz et al., 2010, as it was also highlighted for the products studied in this investigation.

Table 5. Scores for overall liking, appearance liking, flavour liking, and texture liking for persimmon milkshakes with enhanced functionality.

	Overall liking	Appearance liking	Flavour liking	Texture liking
Unt_W	6.0[a] (2.2)	5.5[a] (2.3)	6.3[a] (2.4)	5.3[a] (2.5)
Unt_S	4.6[b] (2.3)	4.2[b] (2.5)	5.2[b] (2.4)	4.0[b] (2.6)
HHP_W	6.1[a] (2.2)	6.0[a] (2.1)	6.2[a] (2.2)	5.9[a] (2.1)
HHP_S	5.7[a] (2.2)	5.7[a] (2.3)	5.9[a] (2.2)	5.7[a] (2.2)
PAST_W	4.6[b] (2.2)	4.8[b] (2.0)	4.9[b] (2.3)	4.0[b] (2.3)
PAST_S	3.7[c] (2.1)	4.6[b] (2.2)	4.1[c] (2.1)	3.5[b] (2.2)

Values in parentheses are the standard deviations.

In the same column, menas without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

4. Conclusions

This work highlighted that the formulation of persimmon milkshakes with high nutritional value, variable fat content and high acceptability could be possible, despite the seasonality of the fruit. It also illuminates consumers' positive perception of persimmon milkshakes as a high antioxidant source. The interviewed sample was a group of consumers generally interested in health, who were also perceptive and positive regarding the concept that consuming certain food products could have a positive effect on health.

The application of combined methods of consumer profiling to functional food development using an emerging technology proved to be a successful approach to better understand consumers' perception of a novel product.

In the present study, the milkshakes with HHP-treated persimmon and the ones enriched with untreated persimmon and whole milk were the consumers' favourites. The HHP treatment could be an ideal method because of its technological and sensory advantages, in line with the high consumer acceptance, however the perception of this emerging preservation method in this category should be further investigated in next steps of the development of this product, as it might imply some barriers to the consumption.

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Capítulo 2

Tissue microstructure, physicochemical properties and bioactive compound locations in different sweet pepper types

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Abstract

This article focuses on the location and content of some bioactive compounds in three different California sweet pepper types (red, green and yellow). The location was studied using different microscopic techniques, such as scanning electron microscopy at low temperatures (CryoSEM) and light microscopy. Several physicochemical properties of the samples (carotenoid content, total soluble phenol content, antioxidant activity, dietary fibre content, total soluble solids content, pH and textural properties) were also examined. The degree of compaction and structuring of the cell wall was found to be indirectly related to solute transport at the cellular level and directly related to total dietary fibre content. The three types of pepper displayed formation and accumulation of phenolic aggregates and an active circulation of solutes. Yellow pepper tissue had the most labile cell walls and the highest transport of solutes. Red peppers could be suitable for obtaining extracts rich in carotenoid compounds, yellow peppers for obtaining phenolic compounds with a high antioxidant activity and green peppers for extracts with high dietary fibre content.

Keywords: microstructure, sweet pepper, carotenoids, dietary fibre, phenolic compounds

1. Introduction

Consumers now appreciate foods rich in compounds with potential long-term health effects, including bioactive compounds, in particular phytochemicals or phytonutrients such as carotenoids, fibre and flavonoids (Drago-Serrano et al., 2006; Kapsak et al., 2011). These biologically active substances that give the food colour, aroma and taste are easily degraded by oxygen, light and especially pH, and temperature and provide significant benefits, including a substantial antioxidant activity (Araya et al., 2006; Ferrari et al., 2010).

Many publications show that consumption of foods rich in bioactive compounds also called functional foods reduces the risk of cardiovascular disease, kidney disease, obesity, macular degeneration and colon and rectal cancers. Moreover, the consumption of these phytochemicals appears to mitigate the effects of diabetes, reduce serum cholesterol level and promote bowel movement (Morais et al., 2002; Figuerola et al., 2008; Chang et al., 2009; Trinidad et al., 2009).

Due to the close relationship between food and health, it is very interesting to direct future research towards the study and use of foods rich in bioactive compounds and to the design of new functional foods. Microstructural characterisation of tissues helps us to understand the mechanisms that may influence the extractability and bioavailability of these components. Understanding these mechanisms is a fundamental step in the knowledge of their bioavailability and in the use of the benefits presented by these health components, including its antioxidant activity.

In recent years, the fresh-cut vegetable market has grown rapidly due to an increased consciousness of the importance of consuming fresh, healthy and convenient food (Das et al., 2011). Traditional foods such as some fruits and vegetables are now considered important bioactive foods with health benefits (Turner et al., 2003; Santiago-Silva et al., 2011). One of the most consumed functional food plants is pepper – *Capsicum annuum* L. (Eggink et al., 2012; Zhuang et al., 2012). As well as being a good source of essential nutrients, such as vitamins (A, C, B1, B2), pepper is rich in fibre and other bioactive compounds such as carotenoids (capsanthin, β-carotene, beta-cryptoxanthin, lutein, zeaxanthin), vitamin C and some phenolic compounds (Deepa et al., 2007; Serrano et al., 2010; Zhuang et al., 2012). Pepper is considered a low-calorie food (Elias Tierrablanca et al., 2010). Among the different

pepper varieties, California is well known by consumers; it is sweet, can be consumed as fresh and/or canned and is harvested throughout the year, but preferably in summer months.

Although numerous publications can be found about pepper where different bioactive characteristics, carotenoids and antioxidants constituents were determined (Hornero-Méndez & Mínguez-Mosquera, 2001; Acero-Ortega et al., 2005; Deepa et al., 2007; Zhuang et al., 2012), there are no studies that relate the microstructure of this vegetable with its content of bioactive compounds, such as carotenoids, phenolic compounds and dietary fibre. In this sense, the microstructural characterisation of the plant tissue, which can locate the bioactive compounds, is essential to study and improve the extractability and bioavailability of these components.

The aim of this study was to select the most suitable vegetable material in order to obtain pepper extracts, rich in bioactive compounds, for being used in food enrichment and/or the development of new functional foods such as *gazpacho* (a cold tomato soup) or sauces. For this purpose, the tissue microstructure and the physicochemical properties of three different California sweet pepper types (red, green and yellow) were related to the location and content of different bioactive compounds.

2. Materials and methods

2.1. Vegetable material and sample preparation

Three different types of California sweet pepper – red variety Melchor, green variety Alonso and yellow variety Deniro – were harvested in El Ejido (Almeria, Spain) in April 2012 and purchased in a local supermarket in their commercial maturity stage. Sweet peppers were washed, cut in pieces (15 mm side) and stored at 4 ºC until their analysis. Several batches of each type of sweet pepper in the same maturity state were studied.

2.2. Microstructural analysis

2.2.1. Low temperature scanning Electron Microscopy (CryoSEM)

A JSM-5410 SEM microscope (JEOL, Tokyo, Japan) was used with a Cryo CT-500 C unit (Oxford Instruments, Witney, U.K.) for the CryoSEM observation. Samples (1-mm-thick pieces from the pepper samples) were placed in the holder, fixed with nitrogen slush ($T \leq -210$ ºC), transferred frozen to the Cryo unit, fractured, etched (−90 ºC), and gold-coated (10^{-2} bar and 40 mA). Samples were then transferred to the microscope and examined at 15 kV, −130 ºC, and at a working distance of 15 mm.

2.2.2. Light Microscopy (LM)

For the LM, samples (1 cm^3) were fixed with a 25 g L^{-1} glutaraldehyde solution (0.025 M phosphate buffer, pH 6.8, at 4 ºC, 24 h), post-fixed with a 20 g L^{-1} OsO_4 solution (1.5 h), dehydrated using a graded ethanol series (300, 500 and 700 g kg^{-1}), contrasted in 20 g L^{-1} uranyl acetate dissolved in ethanol (2 h) and embedded in epoxy resin (Durcupan; Sigma-Aldrich, St. Louis, Mo., U.S.A.). The samples were cut using a Reichert Jung ultramicrotome (Leica Mycrosystems, Wetzlar, Germany). Semi-thin sections (1.5 μm) were stained with 2 g L^{-1} toluidine blue and examined in a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan).

The study using LM was also performed on fresh tissue cryostat sections. To obtain cryostat sections, pepper samples of 15 mm^2 were frozen at -40 ºC during 24 h in a deep freezer (Dairei Europe, Denmark) and were quickly transferred to the cryostat (CM1950; Leica Biosystems, Nussloch, Germany). The sections of 10 μm were obtained using a stainless steel blade, collected by a brush and placed in pre-cooled glass in the cryostat chamber. Phenolic compounds were localized using equal proportions of 50 mM ferric chloride in 0.1 M HCl and 8 mM $K_3Fe(CN)_6$. The cell walls were observed using calcofluor white stain (Sigma-Aldrich, Buchs, Switzerland). Some of the samples were not stained in order to study the presence of carotenoids. In these samples, carotenoids were identified in red colour on the red pepper and in yellow on the yellow pepper.

2.3. Image analysis

The image analysis was carried out using the software ImageJ (Rasband, W.S., ImageJ v. 1.43s, National Institute of Health, Bethesda, MD., U.S.A.). The diameter of the cells was determined using CryoSEM images, while the thickness of the cell walls was determined using LM images. Both diameter and thickness were assessed from at least 6 randomly acquired CryoSEM and LM images, respectively. The cells and cells walls were manually labelled and their diameter and thickness (μm) measured from each image. Diameter was considered as the largest segment length that crossed the cell.

2.3. Physicochemical analysis

2.3.1. Pepper purée preparation

One hundred and twenty grams of each type of pepper (red, green and yellow) was homogenized during 90 s. The sweet pepper purée was then stored in hermetically sealed glass jars at –40 ºC in a deep freezer until further analysis, and it was thawed at room temperature to determine the bioactive compounds content.

2.3.2. Extraction and quantification of carotenoids

Total carotenoids were extracted according to Hornero-Méndez & Mínguez-Mosquera (2001) with modifications. Pepper purée (5 g) was extracted five times with 25 mL cool acetone using an Ultraturrax (IKA Ultraturrax T25 Basic, Staufen, Germany) and vacuum filtered, until no more colour was extracted. The extract was added gradually over 50 mL ethyl ether contained in a decanting funnel. With each addition of extract, enough NaCl solution (100 g L^{-1}) was added to separate the phases and to transfer the pigments to the ether, and the aqueous phase was removed. The process was carried out in several steps to ensure the highest elimination of aqueous phase. The organic phase was treated several times with anhydrous Na_2SO_4 (20 g L^{-1}) to remove residual water and evaporated to dryness in a rotary evaporator (model RII; Büchi Labortechnik, Flawil, Switzerland) at a temperature lower than 35 ºC. Finally, the pigments were collected with acetone to a volume of 100 mL and the absorbance was measured at 450 nm using a spectrophotometer (model Helios Zeta UV-Visible;

Thermo Fisher Scientific Inc., Cambridge, U.K.). The calibration curve was performed with different concentrations of β−carotene in acetone. Results were expressed as milligrams of β carotene per 100 g fresh weight. Carotenoid extractions were made three separate times and measurements were performed in triplicate.

2.3.3. Total soluble phenol content

Total soluble phenol content of the samples was determined with a spectrophotometer (Helios Zeta UV-Visible) using the Folin Denis colorimetric method as described by Arnal & Del Río (2004). Pepper purée (5 g) was homogenized in an Ultraturrax with 25 mL of 960 g kg^{-1} ethanol. Homogenates were centrifuged (14500 rpm, 20 min, 4 ºC) and filtered. The supernatant was kept. More supernatant was extracted from the pellet with 25 mL of 960 g kg^{-1} ethanol and added to the first supernatant. The total supernatant was brought to 100 mL with 960 g kg^{-1} ethanol. In a test tube, 1 mL of the extract and 6 mL distilled water were mixed and vortexed. Thereafter, 0.5 mL of Folin Ciocalteu reagent was added. After 3 min, 1 mL saturated Na_2CO_3 was added, vortexed, and 1.5 mL distilled water was added. Absorbance was measured after 90 min at 765 nm. The calibration curve was performed with different concentrations of gallic acid in 960 g kg^{-1} ethanol. Results were expressed as grams of gallic acid per 100 g fresh weight. Total soluble phenol extractions were made three separate times and measurements were performed in duplicate.

2.3.4. Antioxidant activity

Antioxidant activity was measured by ferric reducing antioxidant power assay (FRAP). Extracts were obtained in the same way as for total soluble phenol content determination. Distilled water (30 µL), sample (30 µL), and FRAP reagent (900 µL) were placed in each cuvette. Cuvettes were incubated during 30 min in a water bath at 37 ºC and the absorbance was measured at 595 nm. The calibration curve was performed using different concentrations of Trolox in 960 g kg^{-1} ethanol. Results were expressed as µmol of Trolox per gram sample. Extracts were made three separate times and measurements were performed in triplicate.

2.3.5. Total and insoluble dietary fibre

Total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) were determined according to AOAC official method 991.43 (AOAC, 1992) using Fibertec E system (Fibertec TM1023, Foss Analytical AB, Höganäs, Sweden). For this purpose, 1 g lyophilized sample was used. Measurements were carried out in three different duplicate samples. Duplicate samples underwent sequential enzymatic digestion by heat stable α amylase, protease, and amyloglycosidase to remove starch and protein. For TDF, enzyme digestate was treated with ethanol to precipitate soluble dietary fibre before filtering, and TDF residue was washed with ethanol, dried and weighed. For IDF and SDF, enzyme digestate was filtered, and residue (IDF) was washed with warm water, dried and weighed. For SDF, combined filtrate and washes were precipitated with ethanol, filtered, dried and weighed. TDF, IDF and SDF residue values were corrected for protein, ash, and blank.

2.3.6. Total soluble solids content (TSS) and pH

TSS and pH were determined from three different pepper purées by duplicate. TSS was determined using a hand-held refractometer (model Pal-α; Atago, Tokyo, Japan) and the results were expressed in ºBrix. pH was determined using a pH-meter Basic 20+ Crison (Barcelona, Spain).

2.3.7. Textural properties

Flesh firmness were determined at room temperature with a TA.XTplus Texture Analyzer (Stable Micro Systems, UK). Flesh firmness was expressed as the load in newtons (N) required for breaking the flesh of the pepper samples with a 2 mm diameter flat-tipped cylindrical probe at 1 mm/s test speed. Firmness values were an average of the measurements from 12 pepper samples.

2.4. Statistical analysis

Data were subjected to analysis of variance (ANOVA), using the least significant difference test with a 95% confidence interval for the comparison of the test averages (Statgraphics Plus 5.1, Manugistics, Inc., Rockville, MA., U.S.A.).

3. Results and discussion

3.1. Microstructural study

3.1.1. CryoSEM

CryoSEM makes it possible to obtain general information on the microstructure of the plant tissue. Specifically, it shows the state or level of cell integrity and which intercellular spaces do or do not contain liquid. The parenchymal tissue of the three types of California sweet pepper studied (Figure 1A to 1C) is composed of round cells and small triangular intercellular spaces between adjoining cells. The mean diameter of the cells was 134.45 ± 33.12 μm in red peppers, 192.11 ± 39.57 μm in green peppers and 193.22 ± 51.05 μm in yellow peppers. In all three types of peppers (red, green and yellow), the cells were closely joined to each other over extensive areas and the cell walls were whole and smooth (Figure 1A to 1C). The cell membrane or plasmalemma was attached to the cell wall in most areas, which explains the high cell turgidity of the three tissue types. The interior of the cell was occupied by large vacuoles filled with soluble material, which was identified in the images as a network structure (Figure 1A to 1C). This structure is known as eutectic artefact or solute aggregation phenomenon and is inherent to the CryoSEM technique. It is caused by an accumulation of soluble material during etching of the sample (Neri et al., 2011). On comparing the cell interiors of the three types of sweet pepper (Figure 1A to 1C), yellow pepper can be seen to present the densest eutectic artefact.

In yellow pepper (Figure 1C), most of the triangular intercellular spaces can be seen to be filled with soluble material, which probably comes from the cell interior. This could indicate that the circulation and exchange of material is very active in this tissue, not only in the symplast and apoplast but also through the membrane. In green pepper (Figure 1B) and red pepper (Figure 1A), both solute-filled and empty intercellular

spaces can be seen. The degree of compaction of the cell wall; the soluble material content of the cell interior and the symplastic, apoplastic and transmembrane movement of soluble material would appear to be interrelated. These nutrient transport routes at the cellular level have been previously described in different tissues such as apple (Quiles et al., 2003) and potato (Marcotte et al., 1991).

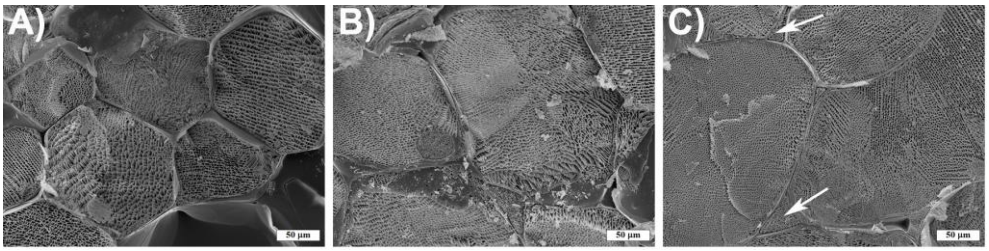

Figure 1. CryoSEM images of different types of California sweet pepper: red pepper (A), green pepper (B) and yellow pepper (C). Arrow: intercellular space filled with soluble material. Magnification: 350x

3.1.2. LM

LM provided more exact knowledge of the structure of the cell wall and membranes of the sweet pepper samples. Most of the cell walls in all three types of pepper (Figure 2) were approximately 1–1.5 μm thick. In green pepper parenchyma (Figure 2B and 2E), the cell walls were compact and uniformly stained and possessed a high degree of structural integrity.

In this tissue, the cellulose fibrils remained closely bound to each other by intact cellulose cements and the middle lamella was practically indistinguishable from the cell walls. In red pepper tissue (Figure 2A and 2D), the walls of adjacent cells were joined by an intact, continuous middle lamella throughout most of the sample but in some areas the adjoining walls had drawn apart, probably owing to dissolution of the middle lamella. The cellulose fibrils appeared to be less compacted in red pepper tissue than in green pepper. Comparing the images of the three types of sweet pepper (Figure 2), yellow pepper cell walls were observed to be more faintly stained and less compact and structured (Figure 2C and 2F). In this sample the middle lamella also presented

destructuring and in some areas had even dissolved, which is why the walls of adjacent cells can be seen to have separated from each other in some zones. In all three types of pepper (Figure 2), the plasmalemma remained in the vicinity of the cell wall, as was also found with CryoSEM. The lower degree of structuring and compaction of the cell wall of yellow pepper could favour greater cellular transport in this type of tissue.

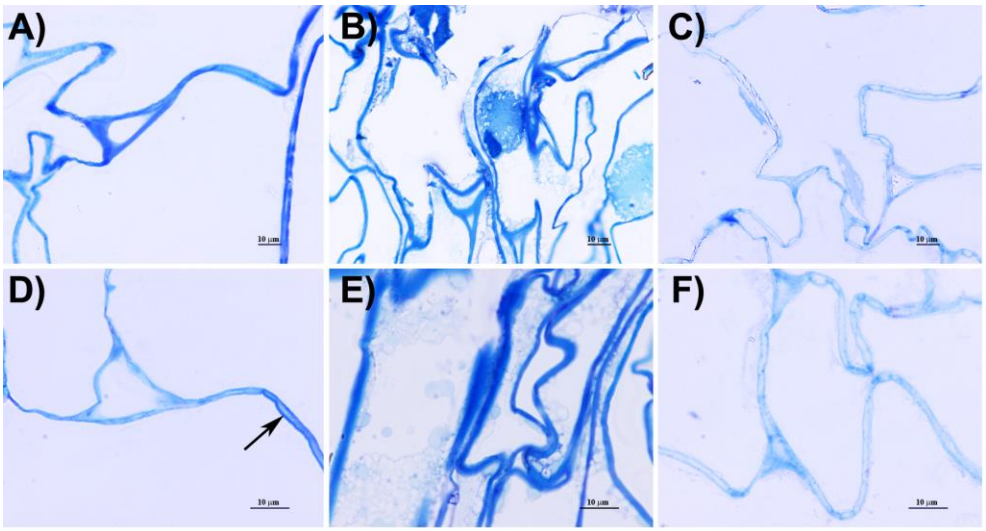

Figure 2. Light microscopy images of California sweet pepper tissues: red pepper (A, D), green pepper (B, E) and yellow pepper (C, F). Toluidine blue stain. Arrow: separation of adjacent cells. Magnification: 60x (A, B, C) and 100x (D, E, F).

Using cryostat sections to study the samples by LM makes it possible to confirm, contrast and expand the information obtained by CryoSEM as it facilitates identification of the chemical components of interest and their distribution at cellular level. In the unstained samples of the three types of pepper (Figure 3A to 3C), it can be seen that the pigment distribution was generally higher in the cell wall and its vicinity. Comparing the three types of parenchymatic tissues, red pepper (Figure 3A) was found to contain more carotenoid pigments (in red), mainly forming clusters, throughout the cell tissue. The carotenoid clusters seemed to be larger in the areas closest to the cell wall. Using the specific acidified $FeCl_3$ and $K_3Fe(CN)_6$ stain made it possible to observe phenolic compounds in the pepper tissues (Pearse et al., 2005),

phenols appear blue after a 15-min incubation at ambient temperature (Lavid et al., 2001). All three types of pepper appeared to be rich in phenolic compounds (Figure 3D to 3F), both localized in the cell walls and also scattered to a large extent throughout the plant tissue. This could indicate active transport of these components, both outside the cell (in the apoplast) and within the cell (in the symplast), as already found by CryoSEM. When the three types of pepper were compared, red pepper tissue (Figure 3D), which had strong, compact cell walls, showed cells with a strong tendency for phenolic compound clusters to form and accumulate. This could indicate a high content of these compounds in the interior of the cell and not very active cellular transport. Green pepper tissue (Figure 3E), the type that was found to have the most compacted, most structured and strongest cell walls, seemed to have cells with a tendency towards phenolic cluster formation and not very active cellular transport and exchange. Yellow pepper (Figure 3F) seemed to have cells with the highest quantity of phenolic compounds and very active transport of these compounds both within and outside the cell. Staining the different tissues with calcofluor white, which has the property of emitting fluorescence when subjected to ultraviolet radiation and has affinity for cellulose and chitin present in the cell walls (Ramos et al., 2006), made it possible to find and confirm that although the cells remained turgid and the cell walls intact in all three types of pepper – red (Figure 3G), green (Figure 3H) and yellow (Figure 3I) – the cell walls of red and green peppers withstood the sample preparation process better than in yellow pepper tissue, where greater gaps between adjacent cell walls could be observed. Of the three pepper types, yellow pepper tissues appeared to have the least compact and structured cell walls. This could be related to the greater cellular exchange of soluble material observed by CryoSEM.

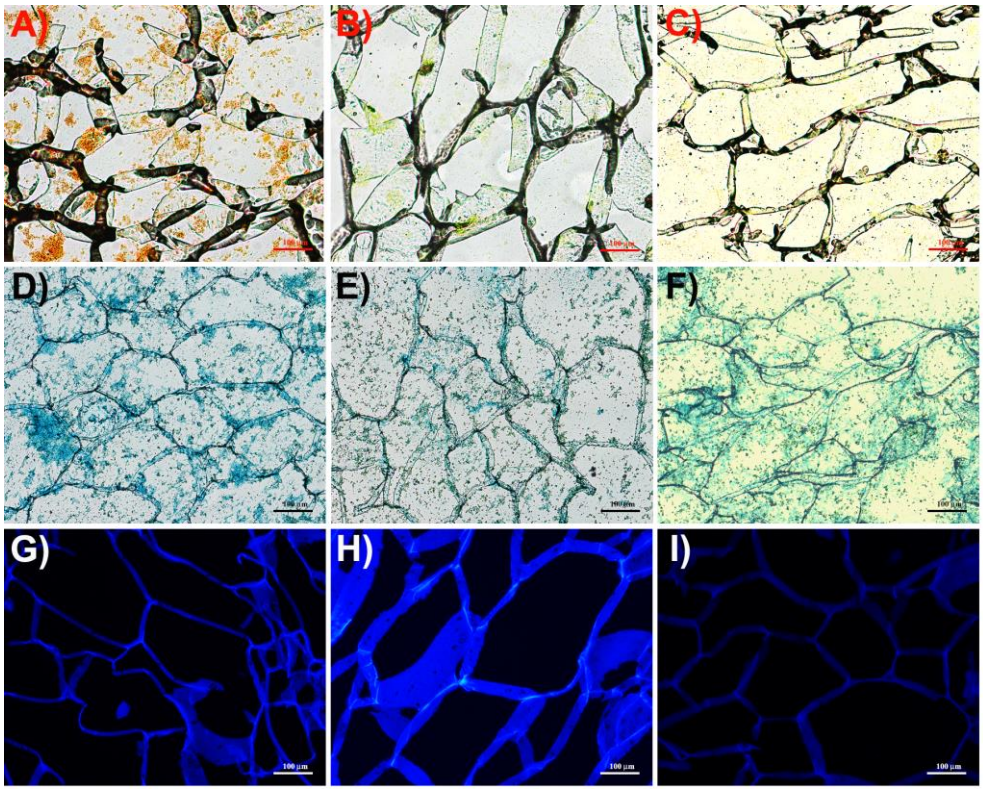

Figure 3. LM images of California sweet pepper tissues: red pepper (A, D, G), green pepper (B, E, H) and yellow pepper (C, F, I). Acidified $FeCl_3$ and $K_3Fe(CN)_6$ stain (D, E, F) and with calcofluor white stain (G, H, I). Magnification: 10x.

3.2. Carotenoid content

Figure 4A shows the mean carotenoid contents of the three types of California sweet pepper studied. Red pepper had a significantly higher ($P < 0.05$) carotenoid content than the other types. The carotenoid results for red pepper agree with those obtained by Serrano et al. (2010). The type with significantly ($P < 0.05$) lowest carotenoid content was green pepper. Deepa et al. (2007) also observed that β-carotene content increased in red pepper compared to green and yellow pepper when quantifying the antioxidant constituents in some sweet pepper genotypes during maturity. These

results were also in line with previous reports evaluating pepper carotenoids (Howard et al., 2000; Navarro et al., 2006), and the highest levels of β-carotene in pepper have been found in red pepper (Gnayfeed et al., 2001).

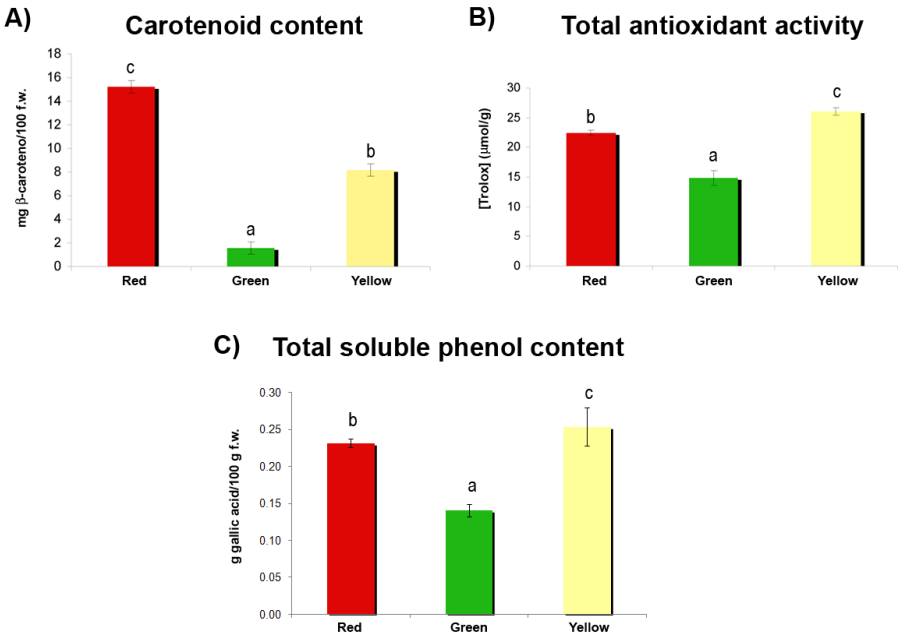

Figure 4. A) Carotenoid content, B) total antioxidant activity, and C) total soluble phenols content in red, green and yellow pepper samples. Different letters indicate significant differences ($P < 0.05$) between the samples.

3.3. Antioxidant activity

Figure 4B shows the mean total antioxidant activity values of the three types of California sweet pepper studied. Yellow pepper had significantly ($P < 0.05$) the highest antioxidant activity, followed by red pepper, and green pepper presented significantly ($P < 0.05$) the lowest antioxidant capacity. Navarro et al. (2006) when studying the antioxidant activity of green and red sweet pepper subjected to different salinity levels obtained that, regardless of salinity level, the antioxidant activity of red pepper was higher than that of green pepper. Similar results were observed by

Howard et al. (2000) when studying the influence of maturity state on the antioxidant properties of pepper cultivars. On the other hand, while red pepper had a lower antioxidant activity than yellow pepper, its carotenoid content was higher. The probable explanation is that not all the carotenoids in red pepper exhibit marked antioxidant activity – some present other beneficial effects such as vitamin activity as β-carotene and β-cryptoxanthin (De Ancos et al., 2000) – while in yellow pepper, besides carotenoids, there are other plant tissue components such as phenolic compounds, which are the main contributors of the antioxidant activity (Mertz et al., 2009).

3.4. Total soluble phenol content

Phenolic compounds have antioxidant potential and important biochemical properties (Erkan, 2012). Figure 4C shows the mean total soluble phenol contents of the three types of California sweet pepper studied. On comparing the three types, yellow pepper had a significantly higher ($P < 0.05$) total soluble phenol content, followed by the red pepper. Green pepper presented significantly ($P < 0.05$) the lowest values. This agrees with the antioxidant activity results and also with the microstructural study. As previously noted, of the three types studied it was yellow pepper that presented the highest antioxidant activity (Figure 4B). This fact could be explained as a result of its higher phenolic compound content compared to red pepper (Figure 4C) and its high carotenoid content (Figure 4A). The microstructural study also found that the quantity of phenolic compounds, both in clusters and scattered through the tissue, appeared to be higher in yellow pepper. Deepa et al. (2007) and Serrano et al. (2010) observed also an increase in total phenolic content during maturity from green to yellow/red stage in *Capsicum* cultivars.

3.5. TDF, IDF and SDF content measurement

Figure 5 shows the mean IDF (Figure 5A), SDF (Figure 5B) and TDF (Figure 5C) of the three types of California sweet pepper under study. Green pepper had significantly ($P < 0.05$) the highest IDF but no statistically significant differences ($P > 0.05$) were found between the IDF values of red and yellow peppers. This agrees with the

microstructural study finding that of the three types, green pepper tissue presented the most compact and structured cell walls and the most intact middle lamella. As regards the SDF content, there were no statistically significant differences ($P > 0.05$) between the different types of sweet pepper. For TDF, green pepper presented the highest values and yellow pepper the lowest, both to a significant degree ($P < 0.05$). As observed by LM, yellow pepper parenchyma presented the least fibril bundling and greatest dissolution of the middle lamella of the three types of tissue. Cell wall materials have a considerable nutritional importance as dietary fibre (Chesson, 1995; Southgate, 1995), so the degree of compaction and structuring of the cell wall could be directly related to the total fibre content.

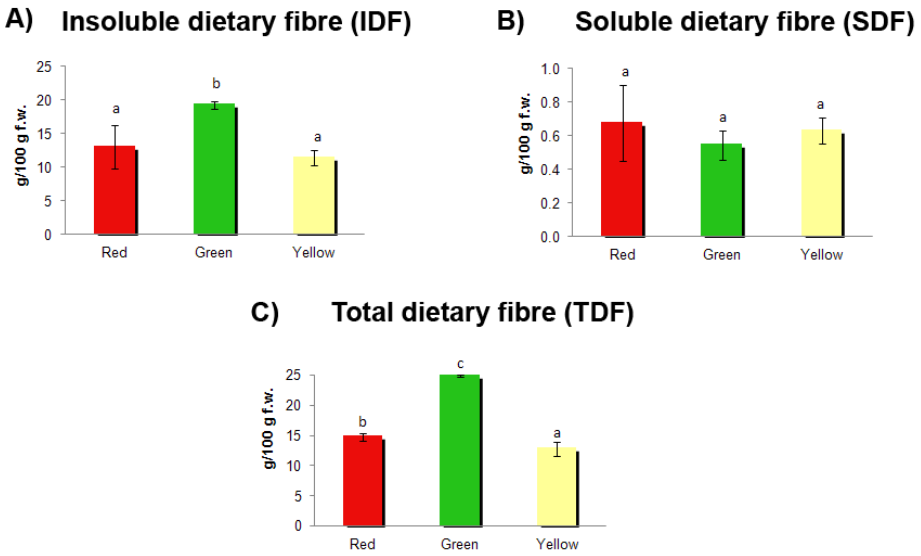

Figure 5. A) IDF, B) SDF, and C) TDF of red, green and yellow sweet pepper samples. Different letters indicate significant differences ($P < 0.05$) between the samples.
IDF, insoluble dietary fibre; SDF, soluble dietary fibre; TDF, total dietary fibre

3.6. Total soluble solids and pH

Figure 6 shows the mean TSS (Figure 6A) and pH values (Figure 6B) of the three types of California sweet pepper studied. No significant differences ($P > 0.05$) in either measurement were found between red and yellow peppers. However, green pepper registered significantly ($P < 0.05$) lower TSS and significantly higher pH values in both cases. This lower TSS in green sweet pepper could explain its lower antioxidant activity since according to previous reports in cv 'Orlando', a California type pepper, the main compounds responsible for antioxidant properties of pepper fruits are water soluble compounds (Navarro et al., 2006). This fact could also explain the higher antioxidant activity found in red and yellow sweet peppers.

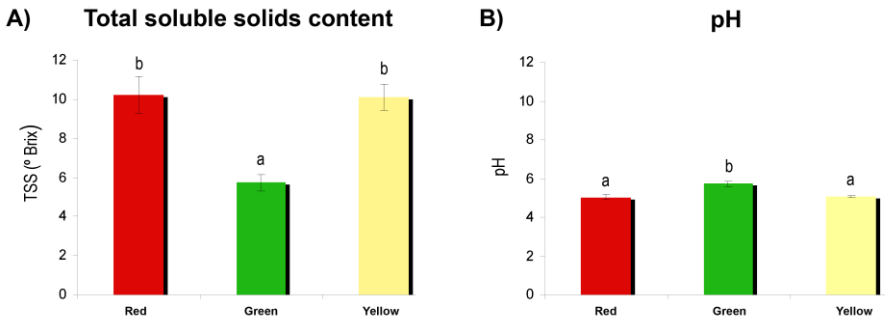

Figure 6. A) Total soluble solids content (Brix), and B) pH of red, green and yellow pepper samples. Different letters indicate significant differences ($P < 0.05$) between the samples.

3.7. Texture properties

Texture (Figure 7) is one of the key quality attributes used in the fresh and processed food industry to assess product quality and acceptability (Chen & Opara, 2013). Firmness is the pulp's resistance to pressure (Kehr, 2002) and is related to storage life, post-harvest handling and operating and processing conditions (Murayama et al., 2002; Konopacka & Plocharski, 2004; Lana et al., 2005; Farag et al., 2009; Fernández-Trujillo et al., 2009). Red and green peppers displayed greater firmness than the yellow pepper. The latter's significantly lower ($P < 0.05$) firmness

could be related to the tissue microstructure study results, as both CryoSEM and LM showed that the cell walls of yellow pepper appeared to be more labile than those of the other California sweet pepper types.

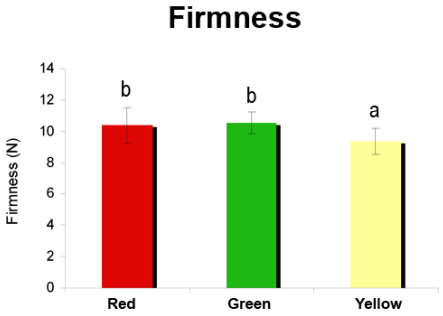

Figure 7. Firmness (N) of red, green and yellow pepper samples. Different letters indicate significant differences ($P<0.05$) between the samples.

4. Conclusions

The content of bioactive compounds of each type of sweet pepper is conditioned by their structure. For obtaining an extract rich in carotenoid compounds, red peppers could be a highly suitable plant material. Yellow pepper could be appropriate for obtaining extracts rich in phenolic compounds with a high antioxidant activity, and when extracts with high dietary fibre content were desired, green peppers could be the most suitable ones.

Mixtures of the three types of pepper could be used for obtaining a nutritionally balanced ingredient. Moreover, modifying the structural properties of the pepper tissues could enhance the extractability of some bioactive compounds such as phenolic compounds and carotenoids, so further research should be conducted on the effects of different processing methods on pepper structure.

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High hydrostatic pressure treatment as an alternative to pasteurization to maintain bioactive compound content and texture in red sweet pepper

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Abstract

Red sweet peppers (*Capsicum annuum*) are an excellent source of essential nutrients and bioactive compounds. High hydrostatic pressures (HHP) not only increase shelf-life but also maintain nutritional and organoleptic properties better in a number of food products. The aim of this work was to measure the effect of HHP and a thermal treatment, pasteurization (PA) in a water bath at 70 °C for 10 min, on some bioactive compounds (fibre, carotenoids and antioxidant activity) and on the texture (TPA; firmness and shear force) of red Lamuyo-type sweet peppers, in order to discover the relationship between treatment (HHP and PA), tissue microstructure and bioactive compound extractability. The results show that HHP at 500 MPa and PA treatments had less impact on the microstructure, bioactive compound content (fibre and antioxidant activity) and texture of red sweet peppers, than when low pressures were used. Consequently, new functional foods could be developed using red sweet pepper tissues treated with high pressures (500 MPa) and/or PA.

Keywords: bioactive compound, high hydrostatic pressure, microstructure, pasteurization, sweet pepper, texture

1. Introduction

Because food and health are closely related, consumers nowadays increasingly prefer and choose foods that not only provide the essential nutrients for life but also contain substances, such as bioactive compounds, which may have healthy effects in the long term (Drago et al., 2006). For instance, traditional foods such as some fruit and vegetables are now considered to contain important bioactive components that are beneficial to health (Santiago-Silva et al., 2011).

Sweet peppers belong to the species *Capsicum annuum*. They are an excellent source of essential nutrients such as carbohydrates, vitamins and minerals (Faustino et al., 2007). In recent years, sweet peppers have attracted the attention of researchers owing to their high content of some bioactive compounds, such as fibre, phenols, flavonoids and carotenoids, which possess antioxidant and antiinflammatory activity (Duma & Alsina, 2012). Beneficial properties are attributed to sweet peppers and their consumption appears to improve scar formation, prevent atherosclerosis and haemorrhages, stop blood cholesterol levels rising and improve stamina (Faustino et al., 2007). Sweet peppers are an important part of the daily human diet; they can be eaten fresh; however, they are usually preserved for further consumption (Gázquez, 2007).

Bioactive compounds are extra-nutritional constituents which can be found in small quantities in a variety of foods (Kris-Etherton et al., 2002). They are easily degraded by oxygen, light, temperature and pH but have protective effects in diets, as has been proved in many studies (Araya et al., 2006; Ferrari et al., 2010). They can lower the risk of cardiovascular diseases, strokes and cancer (Kris-Etherton et al., 2002). Furthermore, they appear to lessen the effects of diabetes, promote bowel movement and reduce the serum cholesterol level (Belitz et al., 2008). Bioactive compounds include, for example, carotenoids, phenols, dietary fibre and other phytochemicals. Carotenoids are important for colour and for other biological functions, such as antioxidant activity, provitamin A activity or enhancement of the immune system (Fernández-García et al., 2012). Dietary fibre can produce a sensation of fullness and therefore help in diets. Moreover it can reduce the risk of stomach cancer (Belitz et al., 2008). The insoluble fibre fraction seems to be linked to regulating the intestinal tract, while the soluble fibre is related to lowering blood cholesterol levels and to intestinal absorption (Ramulu & Udayasekhara, 2003).

It has been shown (Boileau et al., 1999) that when natural products are consumed, the assimilation of some bioactive compounds, such as carotenoids, is relatively low for the quantities ingested. Bioavailability is the fraction of a compound that is absorbed during the complete digestion process. The bioavailability of bioactive compounds like fibre, phenols and carotenoids seems to depend not only on factors related to the food matrix but also on the nutritional level and genetic profile of each individual (Maiani et al., 2009). The term "bioaccessibility" defines the fraction of nutrients that are liberated from the food matrix in the gastrointestinal tract. Some preservation treatments (osmotic dehydration, modified atmospheres, frying, microwave, freezing, and pasteurization) cause microstructural modifications in the treated foods (Llorca et al., 2003; Soliva-Fortuny et al., 2003; Quiles et al., 2004; Guardeño et al., 2011; Hernández-Carrión et al., 2011) and could influence the fraction liberated from the food matrix, and therefore also the fraction that is absorbed during digestion. Microstructural characterization of these foods is fundamental and would help to elucidate whether particular methods of treating the food might influence the ability to extract these compounds from the food matrix.

The demand for safe foods that possess sensory freshness characteristics and biological properties that go beyond the strictly nutritional have led researchers and manufacturers to develop new processing and conservation technologies. Of these new technologies, high hydrostatic pressure (HHP) is one of the most economically viable of what are known as non-thermal treatments (Devlieghere et al., 2004; Rastogi et al., 2007). The effects of HHP on the nutritional and bioactive compounds and the microstructure of the food have been studied in some foods. Hernández-Carrión et al. (2014) studied the impact of HHP on the structure and extractability of some bioactive compounds present in persimmons and concluded that this treatment favoured the structural compaction and extractability of carotenoids but appeared not to influence the fibre content. Vázquez-Gutiérrez et al. (2013) studied the changes in the structure and antioxidant properties of HHP-treated onions and found that the treatment caused structural changes and enhanced the extractability of phenols and other compounds with antioxidant effects. On studying the impact of HHP on the structure, soluble compound diffusion and texture properties of persimmons, Vázquez-Gutiérrez (2012) concluded that HHP treatments favoured the extractability of tannins and other soluble compounds and their diffusion into the intercellular

spaces and diminished the firmness and cohesiveness of the samples. It would be interesting to study the effect of HHP on the tissues of other plant products, such as sweet peppers, that are rich in bioactive compounds.

The aim of this study was to detect the effects of HHP and a traditional thermal pasteurization treatment (PA) on the bioactive compound content (fibre, carotenoids and antioxidant activity) and texture of red Lamuyo-type sweet peppers in order to ascertain the relationship between type of treatment (HHP or PA), tissue microstructure and bioactive compound extractability. This would make it possible to select the pepper tissue with the highest bioactive compound content in order to develop ingredients of interest for formulating functional foods.

2. Materials and methods

2.1. Plant material and sample preparation

The plant material used was red Lamuyo-type sweet peppers at commercial maturity stage. The red peppers, acquired from a local market in September 2013, were washed, cut into pieces measuring about 15 mm along each side and heat-sealed in 200 x 200 mm plastic bags (Doypack type, Amcor, Spain). Each bag contained approximately 100 g of sweet red pepper. One batch was not subjected to any treatment (Control). The second, third, fourth and fifth batch were treated by HHP at different pressures (100, 200, 300, and 500 MPa). The last batch was pasteurized (PA) in a water bath at 70 ºC for 10 min (come-up time to temperature = 30 min). The bags were then stored at 4 °C until they were analyzed. The microstructure, colour and texture properties were analyzed within 24 h of treatment.

2.2. High hydrostatic pressure (HHP) treatments

Bags with approximately 100 g of red sweet pepper were placed inside a hydrostatic pressure unit with a 135-L capacity (Hyperbaric type 135, Burgos, Spain), using water as the pressure medium. Different HHP treatments were studied, coded T1 (100 MPa), T2 (200 MPa), T3 (300 MPa), and T4 (500 MPa) during 15 min at 25 ºC.

2.3. Microstructural analysis

2.3.1. Light Microscopy (LM)

For the LM, the samples (2 mm^3) were fixed with a 25 g L^{-1} glutaraldehyde solution (0.025 M phosphate buffer, pH 6.8, 4 °C, 24 h), post-fixed with a 20 g L^{-1} OsO_4 solution (1.5 h), dehydrated using a graded ethanol series (300, 500 and 700 g kg^{-1}), contrasted in 20 g L^{-1} uranyl acetate, dehydrated with ethanol (960 and 1000 g kg^{-1}) and embedded in epoxy resin (Durcupan; Sigma-Aldrich, St. Louis, MO, USA) at 65.5 ºC for 72 h. The samples were cut using a Reichert Jung ultramicrotome (Leica Mycrosystems, Wetzlar, Germany). Semi-thin sections (1.5 μm) were stained with toluidine blue and examined under a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan).

2.3.2. Transmission Electron Microscopy (TEM)

The same protocol of fixation, dehydration and infiltration was followed as for LM. Ultramicrotomy was carried out in the same equipment, but in this case 0.05-μm-thick sections were obtained. These ultra-thin sections were stained with 40 g L^{-1} lead citrate and 20 g L^{-1} uranyl acetate and observed with a Philips EM 400 (Philips, Eindhoven, Holland) transmission electronic microscope at 80 kV.

2.3.3. Image analysis

The image analysis was carried out using the software ImageJ (Rasband, W.S., ImageJ v. 1.43s, National Institute of Health, Bethesda, Maryland, U.S.A.). The cell area was measured from the LM images, and the cell wall thickness from the TEM images. The area and thickness were assessed from at least six randomly acquired LM and TEM images, respectively. The cells and cells walls were manually labelled and their area (μm^2) and thickness (μm) in each image were measured.

2.4. Physicochemical analysis

2.4.1. Sweet pepper purée preparation

A 120-g portion of red sweet pepper cut into small pieces was homogenized in a food processor (Thermomix TM31, Wuppertal, Germany) using two different stirring speeds: 6500 rpm for 1 min followed by 10200 rpm for 30 s. The red sweet pepper purée was then stored in hermetically sealed glass jars at -80 °C in a deep freezer (Dairei Europe, Denmark) until its analysis, when it was thawed at room temperature before measuring the carotenoid content and antioxidant activity. The purée was prepared in triplicate.

2.4.2. Total, insoluble and soluble dietary fibre

The total dietary fibre (TDF) and insoluble dietary fibre (IDF) were determined according to AOAC official method 991.43 (AOAC, 1992) using the Fibertec E system (model TM1023, Foss Analytical AB, Höganäs, Sweden). For this purpose, 1 g of freeze-dried sample was used (72 h, -45 ºC, 1.3 10^{-3} mPa, Lioalfa-6 freeze-drier, Telstar, Terrassa, Spain). Duplicate samples underwent sequential enzymatic digestion by heat-stable α-amylase, protease and amyloglycosidase to remove the starch and protein. For TDF, the enzyme digestate was treated with ethanol to precipitate the soluble dietary fibre before filtering and the TDF residue was washed with ethanol, dried and weighed. For IDF, the enzyme digestate was filtered and the residue (IDF) was washed with warm water, dried and weighed. The TDF and IDF residue values were corrected for protein, ash, and blank. The soluble dietary fibre (SDF) was determined by the difference between TDF and IDF. The results were expressed as g/100 g of dry weight. Three different digestions were made for each treatment.

2.4.3. pH

The pH was measured in duplicate from the three separate sweet pepper purées, using a Basic 20+ pH-meter (Crison, Barcelona, Spain).

2.4.4. Carotenoid content

The total carotenoid content was measured by the method described by Hornero-Méndez & Mínguez-Mosquera (2001), with modifications. The sweet pepper purée (5 g) was extracted with 25 mL of cool acetone using a homogenizer (IKA T25 Basic Ultraturrax) and vacuum filtered until no more colour was extracted. The extract was added gradually to 50 mL of ethyl ether in a decanting funnel. With each addition of extract, enough NaCl solution (100 g L^{-1}) was added to separate the phases and transfer the pigments to the ether phase, then the aqueous phase was removed. This process was carried out in several steps to ensure maximum elimination of the aqueous phase. The ether phase was treated several times with anhydrous Na_2SO_4 (20 g L^{-1}) to remove residual water and was evaporated to dryness in a rotary evaporator (model RII; Buchi Labortechnik, Flawil, Switzerland) at a temperature below 35 ºC. Finally, the pigments were collected with acetone to a volume of 200 mL and the absorbance was measured at 450 nm using a spectrophotometer (model Helios Zeta UV Visible; Thermo Fisher Scien- tific Inc., Cambridge, UK). The calibration curve was constructed with different concentrations of β carotene (Sigma Aldrich, Madrid, Spain) in acetone (Panreac, Barcelona, Spain). The results were expressed as mg β-carotene/100 g of dry weight. Three separate carotenoid extractions were made for each treatment and the measurements were performed in triplicate.

2.4.5. Colour

The measurements were carried out with a Chroma meter CR-400 (Konica Minolta Sensing Americas, Inc. USA). The results were expressed in accordance with the CIELAB system with reference to illuminant C and a visual angle of 2°. The colorimeter was calibrated with a white standard pattern (Y = 92.9; x = 0.3137; y = 0.3198). The parameters determined were lightness, L* (L* = 0 [black] and L* = 100 [white]), a* (-a* = greenness and + a* = redness), and b* (-b* = blueness and + b* = yellowness). Total colour difference (ΔE*) was calculated as follows (Francis & Clydesdale, 1975).

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (1)$$

The values used to determine whether the total colour difference was apreciable by the human eye were the following (Bodart et al., 2008):

$\Delta E^* < 1$ colour differences are not obvious to the human eye.

$1 < \Delta E^* < 3$ colour differences are not easily distinguished by the human eye.

$\Delta E^* > 3$ colour differences are obvious for the human eye.

Measurements were performed in triplicate.

2.4.6. Antioxidant activity

The antioxidant activity was measured by a ferric reducing antioxidant power assay (FRAP). The sweet pepper purée (5 g) was homogenized in an Ultraturrax with 25 mL of 960 g kg^{-1} ethanol. The homogenate was centrifuged (27716 *g*, 20 min, 4 ºC) and filtered. The supernatant was kept. More supernatant was extracted from the pellet with 25 mL of 960 g kg^{-1} ethanol and added to the first supernatant. The total supernatant was brought up to 100 mL with 960 g kg^{-1} ethanol. Distilled water (30 µL), sample (30 µL), and FRAP reagent (900 µL) were placed in each cuvette. The cuvettes were incubated for 30 min in a water bath at 37 ºC and the absorbance was measured at 595 nm. The calibration curve was obtained using different concentrations of Trolox in 960 g kg^{-1} ethanol. The results were expressed as µmol Trolox/g of sample. Three separate extractions were made for each treatment and the measurements were performed in triplicate.

2.4.7. Texture properties

The texture properties were measured at room temperature with a TA.XTplus Texture Analyser (Stable Micro Systems, UK). Flesh firmness, shear force, and texture profile analysis (TPA) parameters were studied in the epicarp and endocarp of red sweet pepper samples. The flesh firmness was expressed as the load in newtons (N) required to break the flesh of the red sweet pepper pieces with a 2-mm diameter flat-tipped cylindrical probe at a test speed of 1 mm s^{-1}. The shear force was measured as the load in Newtons (N) needed to cut the sweet red pepper pieces (15-mm wide) with a knife blade at a test speed of 1 mm s^{-1}. A texture profile analysis was performed to measure the hardness, cohesiveness, springiness, chewiness and gumminess. The red

sweet pepper pieces (15-mm wide) were axially compressed in two consecutive cycles at a test speed of 1 mm s^{-1} with 15% compression with a 50-mm diameter flat plunger. For each treatment values were an average of the measurements from 12 pieces of red sweet pepper.

2.5. Statistical analysis

The data were subjected to variance analysis (ANOVA), using the least significant difference (LSD) test with a 95% ($P < 0.05$) confidence interval to compare the test averages (Statgraphics Plus 5.1, Manugistics, Inc., Rockville, MD, USA).

3. Results and discussion

3.1. Microstructure

The parenchyma of the red Lamuyo-type sweet peppers was composed of turgid cells, mostly round or semi-round in appearance (Figure 1A), with mean areas of 10113 ± 2311 μm^2. The cell walls, with a mean thickness of 0.82 ± 0.34 μm, turned an even blue colour when stained with toluidine blue (Figure 1B) and had a well-defined appearance in the TEM images (Figure 1D), showing their high degree of integrity.

In most of the cells the plasmalemma remained close to the cell wall (Figures 1D, 1E and 1F). The cell interior could be seen to be occupied by an enormous vacuole (Figure 1C), surrounded by the tonoplast, which remained close to the plasmalemma in most areas (Figure 1D). In general, the cell membranes showed a high level of structural integrity. Plasmodesmata could be observed, connecting the protoplasms of adjacent cells. The middle lamella could be seen to be intensely coloured, continuous and intact, keeping the cell walls united with those of the adjacent cells (Figures 1D and 1F). The intercellular spaces were mostly triangular and packed with solutes. A high carotenoid pigment content, accumulated inside the chromoplasts, could also be seen in the interior of the cells (Figures 1A and 1B). These organelles were distributed throughout the symplast, specifically between the plasmalemma and the tonoplast (Figures 1B and 1C). A clearly-defined chromoplast membrane could be seen and, within it, plastoglobuli and the carotenoid pigments clustered into tubular structures (Figure 1E).

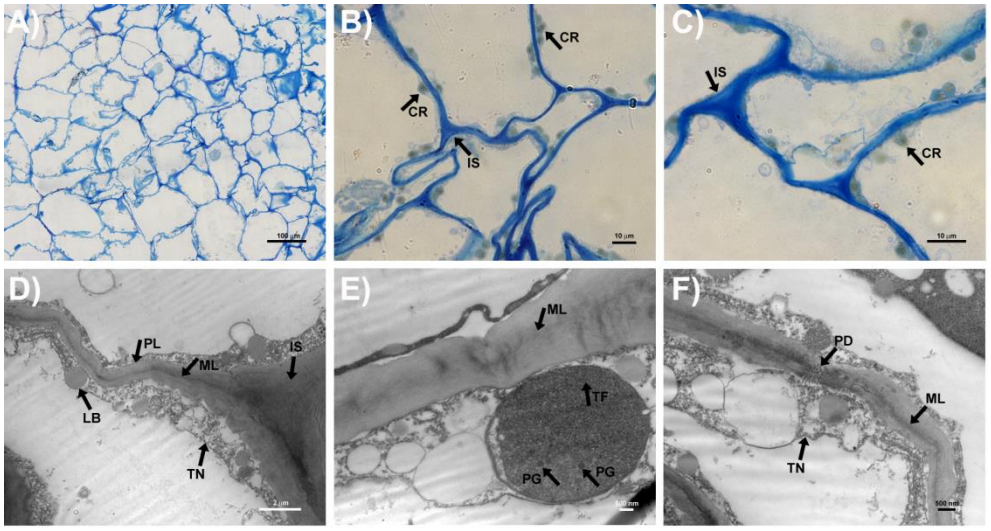

Figure 1. Light microscopy (A, B, C) and transmission electron microscopy (D, E, F) micrographs of untreated red Lamuyo sweet pepper. CR, chromoplast; IS, intercellular space; LB, lipid body; ML, middle lamella; PD, plasmodesmata; PG, plastoglobuli; PL, plasmalemma; TF, tubular formations; TN, tonoplast. Magnification: 10x (A), 60x (B), 100x (C), 1500x (D), 2000x (E), and 2500x (F).

When the red pepper was subjected to gentle HHP treatment at pressures of 100 MPa (treatment T1, Figures 2 and 3), its tissues broke down completely (Figure 2A) when compared to the untreated pepper (Figure 1A). In the pepper with the HHP T1 treatment, the parenchymal cells (mean area 7246 ± 1647 μm^2) were found to be deformed and longer in shape than those of the untreated pepper. The cell walls, which were thicker (1.57 ± 0.45 μm) than in the untreated pepper, were observed to be very lightly blue-stained or even not stained at all (Figures 2A, 2D and 2G), confirming their advanced degree of breakdown and loss of fibril bundling (Figures 3A and 3D). Areas where the cell walls had degraded completely could be seen throughout the parenchymal tissue (Figures 2D and 3D). However, these areas were occupied by clumps of matter – probably the remains of the middle lamella and cell material, possibly lignified – organized like bridges connecting the other walls to each other, maintaining the continuity and the boundaries of the cells. No middle lamella was observed in any of the walls of the pepper subjected to HHP T1 (Figures 3A and 3D), so this treatment can be said to lead to a high level of

dissolution of the middle lamella. The cell walls of the neighbouring cells were completely separated from each other (Figures 2A and 2D), greatly increasing the proportion of apoplast. In this way, the triangular intercellular spaces typical of the untreated pepper (Figure 1A) gave way to the appearance of large intercellular spaces (Figure 2A). HHP treatments with gentle pressure caused cell membrane rupture (Figure 3A) and withdrawal of the cell's content into its interior (Figures 2A and 2D). In the pepper subjected to HHP T1, the chromoplasts appeared degraded and their membranes ruptured (Figures 2D and 2G).

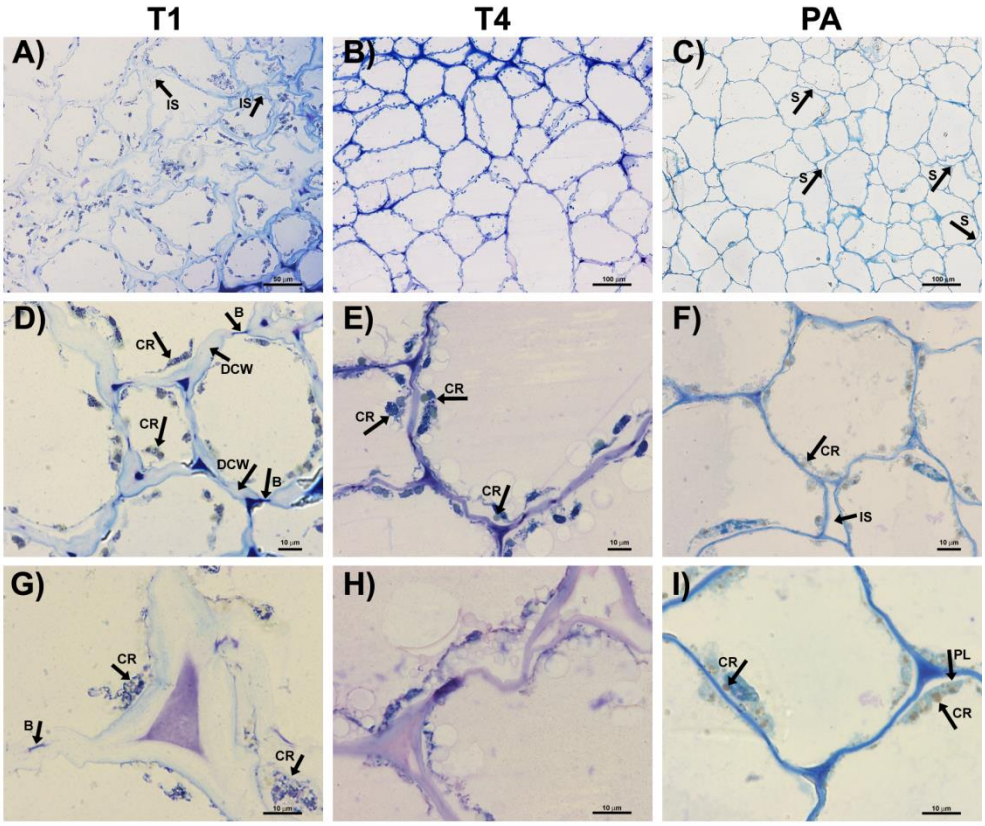

Figure 2. Light microscopy micrographs of red Lamuyo sweet pepper subjected to HHP at 100 MPa (A, D, G) and 500 MPa (B, E, H), and pasteurized (C, F, I). B, bridges of remnants of cell wall material; CR, chromoplast; DCW, degraded cell wall; IS, intercellular space; PL, plasmalemma; S, separation between cell walls. Magnification: 20x (A), 10x (B and C), 60x (D, E, F), and 100x (G, H, I).

The HHP T4 treatment (Figures 2B, 2E and 2H) led to visible structural changes in the parenchyma compared with the untreated pepper (Figure 1A), but less tissue breakdown than with the HHP T1 treatment (Figure 2A). Generally speaking, the gentle pressure applied in treatments T1 (Figures 2A, 2D and 2G) and T2 (data not shown) caused greater structural breakdown than the high pressures of treatments T4 (Figures 2B, 2E and 2H) and T3 (data not shown). The red sweet pepper parenchymal cells subjected to HHP T4, with a mean area of 9662 ± 2773 μm^2, were found to be round in shape (Figure 2B) like those of the untreated pepper (Figure 1A). Their cell walls were 1.34 ± 0.53 μm-thick and were more stained (Figures 2B, 2E and 2H) than those of the pepper that received the HHP T1 treatment (Figures 2A, 2D and 2G), though less structured than those of the untreated pepper (Figure 1E). The middle lamella could be seen in some areas (Figure 3B). HHP T4 also caused cell membrane breakdown (Figure 3E) in some areas, but without the membrane withdrawal to the centre of the cell observed in the HHP T1 pepper (Figure 2E). Chromoplasts could be seen in the interior of the cell (Figure 2E), as in the untreated pepper (Figure 1B), but the membranes of these organelles had dissolved in some areas (Figures 3B and 3E). The disintegrated chromoplasts seemed to be associated with the lipid bodies (Figures 3B and 3E), which appeared as independent structures in the untreated pepper (Figure 1D).

The thermal treatment (PA) also led to structural modifications in the pepper tissue compared with the untreated pepper (Figure 1). However, it led to less breakdown of the parenchymal tissue than the HHP treatments. The cells, with a mean area of 12127 ± 2208 μm^2, were more lightly stained (Figure 2C) than in the untreated pepper (Figure 1A). The cell walls, which were 0.75 ± 0.25 μm-thick, similar to those of the untreated pepper, presented a high degree of fibril bundling (Figure 3C) and appeared more structured than those of the HHP-treated peppers (T1 and T4). The cell walls could be seen to be ruptured in the areas of plasmodesmata (Figure 3C). Middle lamellae were present in many areas (Figure 3C), as was also the case in the pepper parenchyma subjected to HHP T4. In the pepper parenchyma that underwent PA treatment, separation between the cell walls of adjoining cells only took place in some areas giving place to irregular intercellular spaces (Figures 2C and 2F). The plasmalemma was separated (Figure 2I) and broken down in some areas (Figure 3C), as with the HHP T4 treatment. Chromoplasts were observed in the symplast areas in

the PA-treated pepper, as in the untreated and HHP T4 peppers. However, as in the HHP T4 treatment, the lipid bodies seemed to be associated with the chromoplasts that had lost their membrane (Figures 3C and 3F).

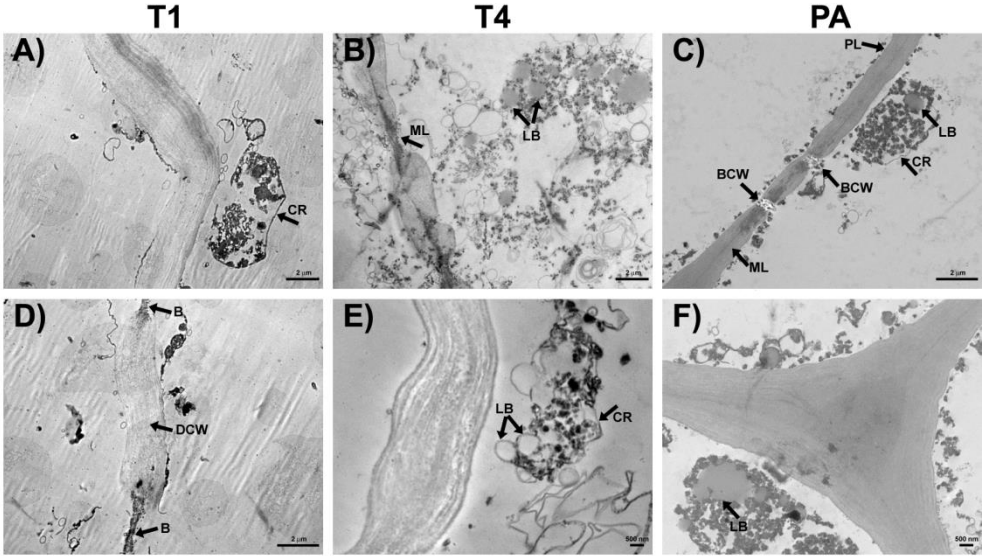

Figure 3. Transmission electron microscopy micrographs of red Lamuyo sweet pepper subjected to HHP at 100 MPa (A, D) and 500 MPa (B, E), and pasteurized (C, F). B, bridges of remnants of cell wall material; BCW, broken cell wall; CR, chromoplast; DCW, degraded cell wall; LB, lipid body; ML, middle lamella; PL, plasmalemma. Magnification: 1200x (A, B), 1500x (C, D), and 2000x (E, F).

3.2. Total, insoluble, and soluble dietary fibre

Table 1 shows the total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) content results for the red sweet peppers with the different HHP and PA preservation treatments. In general, both types of treatment led to a significant reduction in TDF content ($P < 0.05$). The reduction was less pronounced with the PA and HHP T4 treatments, and no significant differences between these two treatments were found ($P > 0.05$). Nor was any statistically significant difference ($P > 0.05$) in TDF content found between the different HHP treatments studied, although the differences between the PA-treated peppers and those to which HHP treatments T1, T2 and T3 had been applied were significant ($P < 0.05$).

Table 1. Total, insoluble and soluble dietary fibre and pH of untreated (Control), HHP-treated (T1, T2, T3, and T4) and pasteurized (PA) red Lamuyo sweet pepper.

	TDF (g/ 100 g d.w.)	IDF (g/ 100 g d.w.)	SDF (g/ 100 g d.w.)	pH
Control	20.982^{a} (0.081)	12.706^{a} (0.433)	8.276^{a} (0.514)	4.923^{a} (0.076)
T1	18.020^{b} (0.016)	12.748^{a} (0.099)	5.271^{b} (0.084)	4.393^{b} (0.127)
T2	18.349^{b} (0.096)	11.830^{ab} (0.708)	6.519^{bc} (0.804)	4.540^{c} (0.026)
T3	18.320^{b} (0.673)	12.581^{a} (0.333)	5.739^{b} (0.961)	4.567^{c} (0.021)
T4	18.726^{bc} (0.444)	11.424^{b} (0.697)	7.302^{ac} (0.518)	4.740^{d} (0.060)
PA	19.526^{c} (0.326)	12.202^{ab} (0.564)	7.325^{ac} (0.238)	4.987^{a} (0.059)

d.w., dry weight.

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

As regards the IDF content (Table 1), only the HHP T4 treatment caused a statistically significant reduction ($P < 0.05$) in this measurement, although no statistically significant differences ($P > 0.05$) in IDF content were observed between treatments HHP T4, HHP T2 and PA. Both PA and HHP T4 appeared to degrade the polysaccharides that make up the insoluble fibre. As found when studying the microstructure, although the high pressure (500 MPa) used in the HHP T4 treatment caused less tissue breakdown in general than HHP T1,T2 and T3 it did affected cellulose fibrils and 'glues' in the cell walls, as hemicellulose and lignine.

Looking now at the SDF content (Table 1), in this case HHP treatments T1, T2 and T3 did lead to a significant reduction in soluble fibre content ($P < 0.05$). Also, the differences between these HHP treatments were not significant ($P > 0.05$). The HHP T4 and PA treatments did not produce statistically significant differences ($P > 0.05$) compared to the untreated pepper. Nor were the differences between these two treatments significant ($P > 0.05$). In the microstructure study, it was found that even though the HHP T1, T2 and T3 treatments did not subject the sample to very high pressures, they did cause considerable changes in the pepper tissues. These structural changes seem to be more related to the SDF content than to the IDF content.

Kutoš et al. (2003) studied the effect of high-temperature thermal processing on canned beans and found that it solubilized certain polysaccharides (hemicelluloses and pectic substances) and reduced the TDF content, mainly owing to loss of SDF. Elleuch et al. (2011) concluded that the changes in TDF content brought about by the thermal treatment depended on the type of cell material and the conditions under which the treatment was carried out. The changes in TDF and SDF content can also be explained by the variations in tissue pH brought about by the different treatments. Rodríguez et al. (2006) established that fibre component solubilization increases as the pH rises. In Table 1 it will be seen that the treatments in which the pepper presented higher pH values (PA) had a higher SDF content than those where the pH was lower (T1).

3.3. Carotenoid content

The carotenoid content of untreated red Lamuyo-type sweet peppers (Control) subjected to the two preservation treatments – high hydrostatic pressure (HHP) and pasteurization (PA) – is shown in Table 2. These preservation treatments caused a statistically significant reduction ($P < 0.05$) in the carotenoid content of the peppers. The treatments that presented significantly lower carotenoid contents ($P < 0.05$) were HHP at 100 MPa (T1) and PA. There was no statistically significant difference between these two treatments ($P > 0.05$). The HHP treatments at 200 MPa (T2), 300 MPa (T3) and 500 MPa (T4) affected the carotenoid content least, suggesting that treatment at pressures above 100 MPa appear to preserve the carotenoid content. However, it should also be pointed out that no statistically significant differences ($P > 0.05$) in carotenoid content between treatments T4 and PA were found, suggesting that both treatments had the same effect on the carotenoid content of red Lamuyo-type sweet peppers.

Table 2. Carotenoid content and antioxidant activity of untreated (Control), HHP-treated (T1, T2, T3, and T4) and pasteurized (PA) red Lamuyo sweet pepper.

	Carotenoid content (mg β-carotene/100 g f.w.)	Antioxidant activity [Trolox] (μmol/g f.w.)
Control	7.724^{a} (0.906)	16.750^{a} (0.567)
T1	5.335^{b} (0.383)	14.533^{b} (0.586)
T2	6.768^{c} (0.086)	14.42^{b} (0.921)
T3	6.692^{c} (0.140)	15.629^{c} (1.058)
T4	6.406^{cd} (0.182)	16.099^{ac} (0.884)
PA	5.685^{bd} (0.041)	16.629^{a} (0.557)

f.w., fresh weight.

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

The effect of HHP on the bioactive compound content of red sweet peppers has not received much attention. However, studies have been made in other plant product matrices. On studying the effect of HHP in tomatoes (Butz et al., 2002), orange, lemon and carrot juice (Butz et al., 2003), gazpacho [a cold tomato soup] (Plaza et al., 2006) and carrot and broccoli (McInerney et al., 2007), these authors found no statistically significant differences ($P > 0.05$) in carotenoid content between the HHP-treated samples and the controls. Other authors (Fernández-García et al., 2001), while not finding differences in tomato carotenoid content (β-carotene or lycopene) between untreated and HHP-treated samples, did find a significant drop ($P < 0.05$) in the total carotenoid content of the samples subjected to thermal or HHP treatments in comparison to the control. They associated these differences with the modifications that take place in the tomato pulp microstructure during processing, which can induce changes in the exposure of hydrophilic structures or cellular decompartmentalization, affecting the arrangement of the internal membranes. This could cause changes in the accessibility of the carotenoids, which are located in the chromoplasts. For their part, Barba et al. (2010) studied the effect of HHP at 100, 200, 300 and 400 MPa on the total carotenoid content of a plant product-based beverage and established that the treatments at 100 and 400 MPa led to

a significant decrease in carotenoid content ($P < 0.05$). Patras et al. (2009) encountered similar results in tomato purees on applying pressures of 400 and 500 MPa for 15 min. This loss of carotenoid content could be related to carotenoid polyene chain breakdown during processing. As a result of processing, these compounds can undergo isomerization and oxidation, the main causes of carotenoid breakdown (Rodríguez-Amaya, 1997).

Persimmons seem to behave differently when subjected to HHP treatments. Several authors (Plaza et al., 2012; Hernández-Carrión et al., 2014) have obtained statistically significant ($P < 0.05$) increases in carotenoid content compared to the control by applying different HHP treatments to persimmons. It would appear, therefore, that the effects of HHP on carotenoid content are closely related to the plant material to which this technology is applied and no general conclusions can be drawn.

As regards PA, its negative effect on the levels of various bioactive compounds, including carotenoids, has been reviewed extensively. For instance, Rawson et al. (2011) found that various authors had reported reductions in the content of bioactive compounds such as anthocyanins, ascorbic acid and carotenoid, in mulberry (Aramwit et al., 2010), durian juice (Chin et al., 2010), pineapple juice (Rattanathanalerk et al., 2005) and apple and cashew juice (Zepka & Mercadante, 2009).

It is important to note that of all evaluated treatments, T1 and PA presented the highest colour differences, obvious for the human eye, 10.15 and 9.14, respectively, agree with the lowest carotenoid content obtained in these samples in comparison with untreated sweet pepper (Table 2). Furthermore, T3 and T4 showed the lowest colour differences, 1.56 and 2.52, respectively, both not appreciated by the human eye which is consistent with the highest carotenoid content present in these samples. These results suggest the existence of a close relationship between the carotenoid content and colour of the red sweet pepper samples, indicating that the lower colour difference regarding the untreated sweet pepper, the greater the carotenoid content.

3.4. Antioxidant activity

Table 2 shows the antioxidant activity of red Lamuyo-type red peppers, both untreated and after application of HHP or PA. The results show that of all the treatments tested, HHP at 100 and 200 MPa (T1 and T2) induced significantly ($P < 0.05$) the greatest drop in antioxidant activity compared to the untreated peppers. The treatments with the least effect on antioxidant activity were HHP at 500 MPa (T4) and PA, where the antioxidant activity did not differ significantly ($P > 0.05$) from that of the untreated pepper. It would appear, therefore, that both T4 and PA have a similar effect on the antioxidant activity of red Lamuyo-type sweet peppers, which would corroborate the similar effects of both treatments on the carotenoid content, as noted in the previous section.

Various studies on the effects of HHP and thermal treatments on different food products have encountered similar results to those of the present study. For instance, Clariana et al. (2011) studied the effects of HHP treatments (200, 400 and 600 MPa) on turnips. On increasing the working pressure, they found that the loss of antioxidant activity decreased, to the point where no significant differences ($P > 0.05$) compared to the control sample were observed with the 600-MPa treatment. In the same way, the authors of studies on the effects of HHP on tomato juice (Fernández-García et al., 2001) or tomatoes and carrots (Butz et al., 2002) concluded that HHP treatments at 600 MPa did not bring about significant changes ($P > 0.05$) in the antioxidant activity of the treated samples compared to those that had not been treated. Nor did they observe significant differences ($P > 0.05$) in antioxidant activity compared to the control sample when the carrots or tomatoes were treated thermally (95 ºC, 5 min) (Butz et al., 2002). Again, Butz et al. (2003) found no significant changes ($P > 0.05$) in the antioxidant activity of various HHP-treated samples (orange, carrot, apple and tomato, and orange, lemon and carrot juices) compared to the control. Sánchez-Moreno et al. (2005) studied the effects of HHP (400 MPa) and PA (70 ºC) on orange juice and found that these treatments did not significantly influence antioxidant activity ($P > 0.05$).

It should be noted that the antioxidant activity results obtained in various foods subjected to different thermal or HPP treatments differ according to the product under study. For instance, McInerney et al. (2007) studied the effects of HHP on the

antioxidant activity of different vegetables and found that they depended on the vegetable: the antioxidant activity of the broccoli was not affected significantly ($P > 0.05$), whereas that of the carrots fell significantly ($P < 0.05$) when working with pressures below 400 MPa. Keenan et al. (2010) studied the effects of HHP (450 MPa for 1, 3 and 5 min) and a thermal treatment (70 ºC, 10 min) on the antioxidant activity of a commercial smoothie elaborated with apple, apple juice, strawberry, orange and banana. They found that the HHP treatments reduced the antioxidant activity of the sample significantly ($P < 0.05$), whereas the PA treatment did not ($P > 0.05$). On applying 600 MPa for 1 min or 110 ºC for 8.6 s to a mango nectar, Liu et al. (2014) found no significant differences in the antioxidant activity of the treated samples compared to the control.

3.5. Texture properties

Table 3 shows the texture property values for the epicarp (outer skin) of red Lamuyo-type sweet peppers subjected to the preservation treatments applied in the study.

All the treatments studied (HHP treatments T1, T2, T3 and T4, and PA) led to a significant reduction ($P < 0.05$) in firmness (Table 3). The treatments that led to the least reduction in the values for this property were PA and HHP T4, in that order. Moreover, significant differences were found between all the treatments under study ($P < 0.05$), except between HHP T1 and T3.

The differences in shear force (Table 3) between the untreated samples and those subjected to PA were not significant ($P > 0.05$) but the HHP-treated peppers presented significantly lower shear force values ($P < 0.05$). T4 (500 MPa) was again the HHP treatment that produced a less sharp reduction in this property.

Table 3. Texture properties measured in the epicarp of untreated (Control), HHP-treated (T1, T2, T3, and T4) and pasteurized (PA) red Lamuyo sweet pepper.

	Firmness (N)	Shear force (N)	TPA analysis				
			Hardness (N)	Cohesiveness (%)	Springiness	Chewiness (N)	Gumminess (N)
Control	10.838^{a}	41.555^{a}	5.478^{a}	73.700^{a}	0.882^{a}	3.642^{a}	3.902^{a}
	(1.097)	(4.869)	(1.500)	(4.283)	(0.088)	(1.047)	(0.920)
T1	1.050^{b}	0.673^{b}	0.844^{b}	54.036^{b}	0.909^{a}	0.287^{b}	0.343^{b}
	(0.246)	(0.222)	(0.270)	(8.199)	(0.074)	(0.090)	(0.088)
T2	2.411^{c}	2.167^{bc}	0.639^{b}	54.869^{b}	0.913^{a}	0.335^{b}	0.343^{b}
	(0.676)	(0.870)	(0.286)	(8.233)	(0.092)	(0.135)	(0.141)
T3	1.451^{b}	5.209^{c}	0.782^{b}	60.908^{c}	0.874^{a}	0.445^{b}	0.561^{b}
	(0.519)	(1.508)	(0.337)	(4.927)	(0.086)	(0.195)	(0.211)
T4	5.598^{d}	9.197^{d}	1.407^{b}	61.700^{c}	0.857^{a}	0.535^{b}	0.780^{b}
	(0.962)	(3.111)	(0.577)	(6.028)	(0.115)	(0.230)	(0.386)
PA	8.018^{e}	43.675^{a}	2.757^{c}	73.750^{a}	0.913^{a}	1.718^{c}	1.958^{c}
	(0.762)	(6.202)	(1.521)	(6.689)	(0.088)	(0.889)	(0.883)

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

Texture profile analysis (TPA) revealed that the hardness, chewiness and gumminess values differed significantly ($P < 0.05$) between the different treatments (Table 3), but all the preservation treatments studied caused significant falls ($P < 0.05$) in the hardness, chewiness and gumminess values. PA was the treatment that caused them to fall the least, while the hardness, chewiness and gumminess values registered for the HHP-treated peppers were significantly lower ($P < 0.05$).

As regards cohesiveness, no significant differences ($P > 0.05$) were observed between the untreated and PA samples, but all the HHP treatments significantly reduced ($P < 0.05$) the values for this property, although T4 and T3 did so to a lesser extent. None of the preservation treatments studied had a significant influence on the texture property of springiness ($P > 0.05$).

The statistical study to examine the effects of each HHP treatment on the hardness, chewiness and gumminess of the red pepper epicarp (Table 4) showed statistically significant differences ($P < 0.05$) between the different treatments. The treatment with the highest hardness, chewiness and gumminess values was T4.

Table 5 shows the texture property values for the endocarp of red Lamuyo-type sweet peppers subjected to the different preservation treatments. The PA treatment did not influence the firmness of the pepper but all the HHP treatments reduced the tissue firmness values to a significant degree ($P < 0.05$). The loss was not as great with 200 MPa and 500 MPa (T2 and T4, respectively).

As regards shear force (Table 5), the PA pepper presented significantly higher values ($P < 0.05$) than the control pepper, while all the HHP treatments induced statistically significant reductions ($P < 0.05$). HHP T4 was the treatment with the lowest drop in shear force. The TPA results obtained from the measurements of the pepper endocarp (Table 5) reflected a statistically significant ($P < 0.05$) loss of hardness in the peppers subjected to HHP or PA, although less in the latter case than in the HHP peppers.

Table 4. Hardness, chewiness, and gumminess measured in the epicarp and endocarp of HHP-treated (T1, T2, T3, and T4) red Lamuyo sweet pepper.

	Epicarp			Endocarp		
	Hardness (N)	Chewiness (N)	Gumminess (N)	Hardness (N)	Chewiness (N)	Gumminess (N)
T1	0.844^{a}	0.287^{a}	0.343^{a}	0.766^{a}	0.262^{a}	0.327^{a}
	(0.270)	(0.090)	(0.088)	(0.249)	(0.104)	(0.155)
T2	0.639^{a}	0.335^{ab}	0.343^{a}	0.963^{ab}	0.405^{ab}	0.413^{ab}
	(0.286)	(0.135)	(0.141)	(0.395)	(0.143)	(0.168)
T3	0.782^{a}	0.445^{bc}	0.561^{b}	1.009^{ab}	0.558^{bc}	0.599^{bc}
	(0.337)	(0.195)	(0.211)	(0.473)	(0.231)	(0.282)
T4	1.407^{b}	0.535^{c}	0.780^{c}	1.282^{b}	0.712^{c}	0.790^{c}
	(0.577)	(0.230)	(0.386)	(0.498)	(0.329)	(0.330)

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

Table 5. Texture properties measured in the endocarp of untreated (Control), HHP-treated (T1, T2, T3, and T4) and pasteurized (PA) red Lamuyo sweet pepper.

	Firmness (N)	Shear force (N)	TPA analysis				
			Hardness (N)	Cohesiveness (%)	Springiness	Chewiness (N)	Gumminess (N)
Control	4.840^{a}	29.690^{a}	5.136^{a}	71.993^{a}	0.829^{a}	2.996^{a}	3.261^{a}
	(0.611)	(4.673)	(2.553)	(7.702)	(0.140)	(1.024)	(1.572)
T1	0.528^{b}	0.997^{b}	0.766^{b}	52.238^{b}	0.943^{b}	0.262^{b}	0.327^{b}
	(0.138)	(0.287)	(0.249)	(8.278)	(0.067)	(0.104)	(0.155)
T2	1.221^{c}	1.564^{bc}	0.963^{b}	46.462^{c}	0.909^{ab}	0.405^{b}	0.413^{b}
	(0.563)	(0.403)	(0.395)	(3.693)	(0.099)	(0.143)	(0.168)
T3	0.670^{b}	5.256^{c}	1.009^{b}	59.185^{d}	0.927^{b}	0.558^{b}	0.599^{b}
	(0.271)	(1.774)	(0.473)	(6.450)	(0.092)	(0.231)	(0.282)
T4	1.668^{c}	16.447^{d}	1.282^{b}	64.467^{e}	0.888^{ab}	0.712^{b}	0.790^{b}
	(0.355)	(5.137)	(0.498)	(6.565)	(0.124)	(0.329)	(0.330)
PA	4.452^{a}	51.644^{e}	3.775^{c}	67.625^{ae}	0.893^{ab}	2.499^{a}	2.588^{a}
	(0.572)	(7.382)	(1.639)	(6.025)	(0.110)	(1.337)	(1.105)

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

On analyzing the cohesiveness of the pepper samples (Table 5), no statistically significant differences ($P > 0.05$) were observed between the control and PA peppers, but significantly lower values ($P < 0.05$) were found in the HHP-treated samples. T4 was the HHP treatment with the least impact on cohesiveness. No significant differences were found between T4 and PA ($P > 0.0.5$).

As regards springiness (Table 5), T1 and T3 led to significant rises in this property compared to the control ($P < 0.05$). The other treatments (T1, T4 and PA) did not have a significant effect on springiness in comparison with the control ($P > 0.05$). Table 5 shows that the chewiness and gumminess measurements of the pepper endocarp did not change to a statistically significant degree ($P > 0.05$) when the sample was pasteurized (PA), but both fell significantly ($P < 0.05$) in the HHP-treated peppers.

Looking separately at the effects of the HHP treatments on hardness, measured in the pepper endocarp (Table 4), T4 was found to present significantly higher values than T1 ($P < 0.05$). Separate analysis of the HHP treatments' effect on the chewiness and gumminess measured in the pepper endocarp (Table 4) showed that the values for these properties were significantly higher ($P < 0.05$) in the high pressure treatment (500 MPa) than when low pressure (100 MPa) was applied.

Generally speaking, PA and HHP T4 can be said to be the treatments that best preserve the texture properties of red Lamuyo-type sweet peppers. When using HHP to preserve the samples, the texture was affected less by high pressures, reaching 500 MPa, than when the samples were subjected to lower pressures (100, 200, 300 MPa). As found also when studying the microstructure, increasing the working pressure reduced the damage to the sweet pepper cell tissues. Consequently, out of the HHP treatments studied, T4 was the one that best preserved the texture properties. This bears out the results of the microstructure study, in which T4 was the HHP preservation treatment that best maintained tissue integrity.

During high pressure treatment, the substrates, ions and enzymes that are located in different compartments within the cell can be released and can interact with each other, leading to enzyme and non-enzyme reactions that bring about changes in texture in the foods subjected to these treatments (Oey et al., 2008). For example, pectin is broken down by enzymes such as pectin methyl esterase (PME),

polygalacturonase (PG) and pectate lyase (PL). Thermal treatments bring about changes in the action of these enzymes, as do high pressure treatments. They can speed up or slow down chemical reactions, stimulate, delay, inactivate or stabilize pectin enzymes or dissociate enzyme inhibitors. All this induces changes in the texture properties of plant product tissues (Sila et al., 2008; Jolie et al., 2012). Red sweet peppers present high PG enzyme activity but no measurable PME activity (Ni et al., 2005; Arancibia & Motsenbocker, 2006; Castro et al., 2008).

In the present study, the damage to the texture was less noticeable with the PA and HHP T4 treatments, probably because they provided suitable conditions for inactivating enzymes such as PG. Houben et al. (2013) verified that when high temperatures (70 ºC) were applied for a relatively long time (10 min), PG was almost entirely inactivated. Crelier et al. (2001) studied PG inactivation in tomato juice and confirmed that this enzyme is sensitive to high pressure treatments, so its activity was drastically reduced by treatment with 400 MPa at 30 ºC and became almost non-existent when the pressure was raised to 500 MPa at the same temperature. Other studies (Rodrigo et al., 2006; Jolie et al., 2012; Houben et al., 2013) have found that PG activity diminishes sharply with pressures above 300 MPa at ambient temperature, falling to almost zero when working with pressures of 500 MPa or above. Rodrigo et al. (2006) found that in tomatoes, 15 min of treatment at ambient temperature with a pressure of 500 MPa totally inactivated the PG.

4. Conclusions

All the preservation treatments studied, whether PA or HHP, caused structural modifications in red Lamuyo-type sweet pepper tissues, but HHP T4 and PA were the treatments that had the least impact on the microstructure. These same treatments (HHP T4 and PA) were also the ones that least affected the bioactive compound content and the texture. High pressure treatment at 500 MPa could provide an alternative to pasteurization, the traditional thermal treatment for sweet pepper preservation, as the texture properties and bioactive compound content (fibre, carotenoids and antioxidant activity) of the red pepper tissues were found to be similar. Owing to their high bioactive compound content, these pasteurized and HHP-treated at 500 MPa sweet peppers would be also a useful ingredient for formulating new functional foods.

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Use of image analysis to evaluate the effect of high hydrostatic pressure and pasteurization as preservation treatments on the microstructure of red sweet pepper

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Abstract

The aim of this work was to evaluate the effect of HHP treatment and PAST on the microstructure of red Lamuyo-type sweet peppers using image analysis and to determine the parameters that allow characterizing the changes observed on the structure using different magnifications (100x, 200x, and 350x). The results show that all the preservation treatments evaluated, caused structural modifications on the microstructure of red sweet pepper, but HHP at 500 MPa and PAST had less impact. Fractal dimension texture, contrast, inverse difference moment and entropy are texture features that are appropriate for characterizing the effect of HHP and PAST on red pepper texture. In this context, it is important to consider the magnification at which red pepper texture is evaluated because cell damage caused by treatments is best observed at low magnification. Consequently, image analysis could be used in future studies to relate microstructure to the functionality of products subject to HHP.

Keywords: high hydrostatic pressure, image analysis, microstructure, pasteurization, red pepper.

1. Introduction

Red sweet peppers (*Capsicum annuum* L*)* are an excellent source of essential nutrients such as carbohydrates, vitamins and minerals (Faustino et al., 2007). As well as being a good source of essential nutrient, pepper is rich in fibre and other bioactive compounds such as carotenoids, which possess antioxidant and anti-inflammatory activity, phenols and flavonoids (Deepa et al., 2007; Duma & Alsina, 2012; Zhuang et al., 2012). Moreover, fresh pepper is considered to be one of the vegetables with the highest content of vitamin C within the plant kingdom (Serrano et al., 2010). Additionally, its consumption appears to prevent atherosclerosis and haemorrhages, improve scar formation and stamina, and stop blood cholesterol levels rising (Faustino et al., 2007).

The demand for safe foods that possess sensory freshness characteristics and biological properties that go beyond the strictly nutritional have led researchers and manufacturers to develop new processing and conservation technologies. Of these new technologies, high hydrostatic pressure (HHP) is one of the most economically viable of what are known as non-thermal treatments (Devlieghere et al., 2004; Rastogi et al., 2007). HHP facilitates the production of food products that have the quality of fresh foods but the convenience and profitability associated with shelf life extension (McClements et al., 2001). HPP can be applied to a range of different foods, including juices and beverages, fruits and vegetables, meat-based products, fish and precooked dishes, with meat and vegetables being the most popular applications (Norton & Sun, 2008).

On the other hand, image analysis can be particularly a useful tool for characterizing food morphology because the highly irregular structures of many food materials elude precise quantification by conventional means. This technique allows to obtain measurements from digitalized images. These measurements provide objective evaluations of the morpho-colourimetric features of samples, a method that is more quantitative and less biased than the common method of visual perception, which is prone to variation due to the personal opinions of inspectors (Russ, 2007; Sonka et al., 2008; Sun, 2008). Nowadays, many software programmes for image processing and analysis are available on the market able to analyse digital images in real time providing precise and accurate measurements of the size, shape, colour and

texture of the objects studied. Many studies have demonstrated the utility of image analysis for the study of morphometric and colourimetric characteristics in fruits and vegetables (López-García et al., 2010; Rocha et al., 2010), in legumes and durum wheat kernels (Venora et al., 2007; 2009a; 2009b) and bakery products (Abdullah et al., 2000; Grillo et al., 2014).

Image analysis can evaluate different morphometric characteristics, also known as morphometric features. Morphometric features can be divided into three groups: dimensionality, which refers to measuring various characteristics of the object such as area, perimeter, Feret diameter and maximum length, among others, using specific computer programs; form, which refers to the graphical representation of the study object as an approximate reference to an Euclidean geometric figure, including circular and elliptical form factors, eccentricity, sphericity and convexity; and texture, which refers to the information needed to describe the regularity of an object, e.g., its compactness, roughness and sinuosity (Aguilera, 2007).

Texture is one of the important characteristics used in identifying objects or regions of interest in an image, whether the image be a photomicrograph, an aerial photograph, or a satellite image (Haralick et al., 1973). Grey level co-occurrence matrix (GLCM), proposed by Haralick et al. (1973) is an image processing technique that has been widely used for measuring of texture in images. It first generates a grey level co-occurrence matrix that is defined as the distribution of co-occurring values at a given offset over a given image, then calculate a set of textual features (usually called Haralick features) from the matrix that can reflect the image texture. Different textural features can be obtained from an image using image analysis, such as: *angular second moment, contrast, correlation, inverse difference moment*, and *entropy*.

Although numerous publications can be found about the effect of different preservation treatments, such as high hydrostatic pressure and pasteurization (PAST) on the microstructure and on the size and/or shape parameters of different plant tissues (Vázquez-Gutiérrez et al., 2012; 2013; Hernández-Carrión et al., 2014a; 2014b) and about the effect of different processing techniques such as drying on the colour of sweet pepper using image analysis (Romano et al., 2012), there are no studies that quantify the effect of HHP and PAST using image texture parameters and that evaluate the effect of the magnification used on these texture parameters. In this

sense, the study of the effect of HHP and PAST on the microstructure of red sweet pepper tissue using image analysis is essential and would enable to relate the image information to structural modifications and to the extractability of some bioactive compounds, such as carotenoids, that could affect the functionality of the selected plant tissue. Also, it is important to start to develop appropriate decision algorithms, methods and magnifications that allow acceleration and optimize industrial processes that can be evaluate by image analysis, particularly in the case of important products as sweet pepper.

The aim of this work was 1) to evaluate the effect of HHP treatment and PAST on the microstructure of red Lamuyo-type sweet peppers using image analysis and 2) to determine the parameters that allow characterizing or quantitatively describe the changes observed on the structure using different magnifications.

2. Materials and methods

2.1. Plant material and sample preparation

The plant material used was red Lamuyo-type sweet peppers at commercial maturity stage. The red peppers, acquired from a local market in September 2013, were washed, cut into pieces measuring about 15 mm along each side and heat-sealed in 200 x 200 mm plastic bags (Doypack type, Amcor, Spain). Each bag contained approximately 100 g of sweet red pepper. One batch was not subjected to any treatment (CNT). The second, third, fourth and fifth batch were treated by HHP at different pressures (100, 200, 300, and 500 MPa) according to Hernández-Carrión (2014a). The last batch was pasteurized (PAST) in a water bath at 70 ºC for 10 min (come-up time to temperature = 30 min) (Hernández-Carrión et al., 2014a). The bags were then stored at 4 °C until they were analysed. The microstructure was analysed within 24 h of treatment.

2.2. High hydrostatic pressure (HHP) treatments

Bags with approximately 100 g of red sweet pepper were placed inside a hydrostatic pressure unit with a 135-L capacity (Hyperbaric type 135, Burgos, Spain), using water as the pressure medium. Different HHP treatments were studied, coded T1 (100 MPa), T2 (200 MPa), T3 (300 MPa), and T4 (500 MPa) during 15 min at 25 ºC.

2.3. Microstructural analysis

2.3.1. Light Microscopy (LM)

For the LM, the samples (2 mm^3) were fixed with a 25 g L^{-1} glutaraldehyde solution (0.025 M phosphate buffer, pH 6.8, 4 °C, 24 h), post-fixed with a 20 g L^{-1} OsO_4 solution (1.5 h), dehydrated using a graded ethanol series (300, 500 and 700 g kg^{-1}), contrasted in 20 g L^{-1} uranyl acetate, dehydrated with ethanol (960 and 1000 g kg^{-1}) and embedded in epoxy resin (Durcupan; Sigma-Aldrich, St. Louis, MO, USA) at 65.5 ºC for 72 h. The samples were cut using a Reichert Jung ultramicrotome (Leica Mycrosystems, Wetzlar, Germany). Semi-thin sections (1.5 μm) were stained with toluidine blue and examined under a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan).

2.3.2. Scanning Electron Microscopy (SEM)

Pieces (3-mm wide) from raw and treated red sweet pepper were frozen at -20 ºC and then freeze-dried at 1 Pa for 3 days (LIOALFA- 6, Telstar, Barcelona, Spain). Then, red sweet pepper samples were vacuum sealed in vials in the same freeze-drier so that they would remain stable (Llorca et al., 2001). After that, they were individually placed on SEM slides with the aid of colloidal silver and then gold-coated with (SCD005, Baltec, Germany) at 10^{-2} Pa and an ionization current of 40 mA for 120 s. The samples were observed in a scanning electron microscope (JSM-5410, Jeol, Japan) at an acceleration voltage of 15 kV. The microscope was equipped with an integrated program for digital image capture (INCA 4.09, Oxford Instruments, England). Magnifications of 100x, 200x, and 350x were used.

2.4. Image analysis

2.4.1. Morphometric analysis

Image processing was carried out according to Pedreschi et al. (2004). Images of the red sweet pepper of 1280 x 1024 pixels were captured using a light microscopy and stored as bit maps (grey-scale with brightness values between 0 and 255) (Quintanilla-Carvajal et al., 2011) by using the ImageJ software (Rasband, W.S., ImageJ v.1.43s, National Institute of Health, Bethesda, Maryland, USA). The following morphological parameters were determined for each treatment: area, perimeter, circularity, and Feret diameter. With these results, the cell size distribution was evaluated.

2.4.2. Texture image analysis

Images of the red sweet pepper of 1024 x 786 pixels were captured using an electronic microscopy and stored as bit-maps in a grey-scale with brightness values between 0 and 255 for each pixel constituting the image. A generalization of the Box Counting method was used to evaluate the fractal dimension of the images (FDT). In this work, the shifting differential box-counting method (SDBC) (Chen et al., 2001) was used to evaluate the fractal dimension of texture of SEM images using the ImageJ 1.34 software. Three different crops at the three different magnifications used (100x, 200x, and 350x) were evaluated for each treatment. The size of the crops was the same for all the evaluated magnifications (270 μm x 270 μm). The texture parameters (*angular second moment, contrast, correlation, inverse difference moment,* and *entropy*) of SEM images were evaluated using the GLCM and surface plot tools of ImageJ.

The textural feature *angular second moment*, also called energy measures the texture uniformity or orderliness of an image (Ou et al., 2014). Higher *angular second moment* values indicate more directional uniformity in the image (Yang et al., 2000). *Angular second moment* for grey-scale image is defined as (Agüera et al., 2008):

$$\text{Angular second moment} = \text{sum } (p(i, j))^2 \quad (1)$$

The textural feature *contrast* is a measure of the intensity contrast between a pixel and its neighbour over the whole image. It measures the local variation in the GLCM.

Contrast can be seen as dynamic range of grey level or sharpness of edges. The range of *contrast* lies between 0 to (size (GLCM, 1) - 1)^2. Furthermore, *contrast* is 0 for a constant image (Laddi et al., 2013).

The textural feature *correlation* is a measure of how correlated a pixel is to its neighbour over the whole image. Its range lies between -1 and +1. Also, the *correlation* is 1 or -1 for a perfectly positively or negatively correlated image. *Correlation* measures the joint probability of occurrence of pixel pairs of GLCM (Laddi et al., 2013).

The textural feature *inverse difference moment* (*IDF*) is a similar measure to the angular second moment but normalized for distance. Higher *inverse difference moment* values indicate more or less variation in image contrast (Yang et al., 2000).

The textural feature *entropy* was determined by colour to grey-scale conversion of acquired tea sample images, whereas other features were calculated by conversion of grey-scale images into GLCM, which is created by calculating how often a pixel with grey-level (grey-scale intensity) value i occurs horizontally adjacent to a pixel with the value j. Each element (i, j) in GLCM specifies the number of times that the pixel with value i occurred horizontally adjacent to a pixel with value j (Haralick et al., 1973; Haralick & Shapiro, 1992). *Entropy* is a statistical measure of randomness that can be used to characterise the texture of the input image. *Entropy* for grey-scale image is defined as:

$$\text{Entropy} = -\text{sum}\,(p(i,j)).*\log_2(p(i,j)) \quad (2)$$

where, p contains the histogram counts used for 256 bins of grey-scale image (Gonzalez et al., 2003). *Entropy* is highest when all entries in p (i, j) are of similar magnitude, and small when the entries in p (i, j) are unequal (Laddi et al., 2013).

2.5. Experimental design and statistical analysis

Morphometric parameters: Analysis of variance (one way ANOVA) was applied to study the differences between the treatments (CNT, T1, T4, and PAST) for the morphometric data; the least significant differences were calculated by Fisher's test, and the significance at $P < 0.05$ was determined.

Texture parameters: A categorical multifactorial experimental design with two factors: treatment (CNT, T1, T2, T3, T4 and PAST) and magnification (100x, 200x, and 350x) was used. Analysis of variance (ANOVA) was performed on the texture data using the Statgraphics Plus version 5.1 software package (Statistical Graphics Co., Rockville, MD, USA). The least significant differences (LSD) were calculated by Fisher's test and the significance at $P < 0.05$ was determined.

3. Results and discussion

3.1. Light microscopy (LM)

The parenchyma of the untreated red Lamuyo-type sweet peppers was composed of turgid cells round or semi-round in appearance with well-stained and well-defined cell walls (Figure 1A). The intercellular spaces were mostly triangular and packed with solutes. High carotenoid pigment content, accumulated inside the chromoplasts, could also be seen in the interior of the cells.

When the red pepper was subjected to HHP treatment at pressures of 100 MPa (treatment T1, Figure 1B), its tissues broke down completely. The cell walls were lightly blue-stained. Areas where the cell walls had degraded completely could be seen throughout the parenchymal tissue. However, these areas were occupied by clumps of matter organized like bridges connecting the other walls to each other, maintaining the continuity and the boundaries of the cells.

The HHP at 500 MPA (treatment T4, Figure 1C) led to visible structural changes in the parenchyma compared with the untreated pepper (Figure 1A), but less tissue breakdown than with the HHP T1 treatment (Figure 1B). The red sweet pepper parenchymal cells subjected to HHP T4, were found to be round in shape and their cell walls were well-stained.

The thermal treatment (PAST, Figure 1D), although led to structural modifications, caused less breakdown of the parenchymal tissue than the HHP treatments. The cells were more lightly stained than in the untreated pepper (Figure 1A). Separation between the cell walls of adjoining cells only took place in some areas giving place to irregular intercellular spaces.

So, it can be concluded that each preservation treatment (HHP and PAST) caused different tissue architecture, which suggest that the mechanisms of cell permeabilization are not the same (Tedjo et al., 2002; Vega-Gálvez et al., 2011).

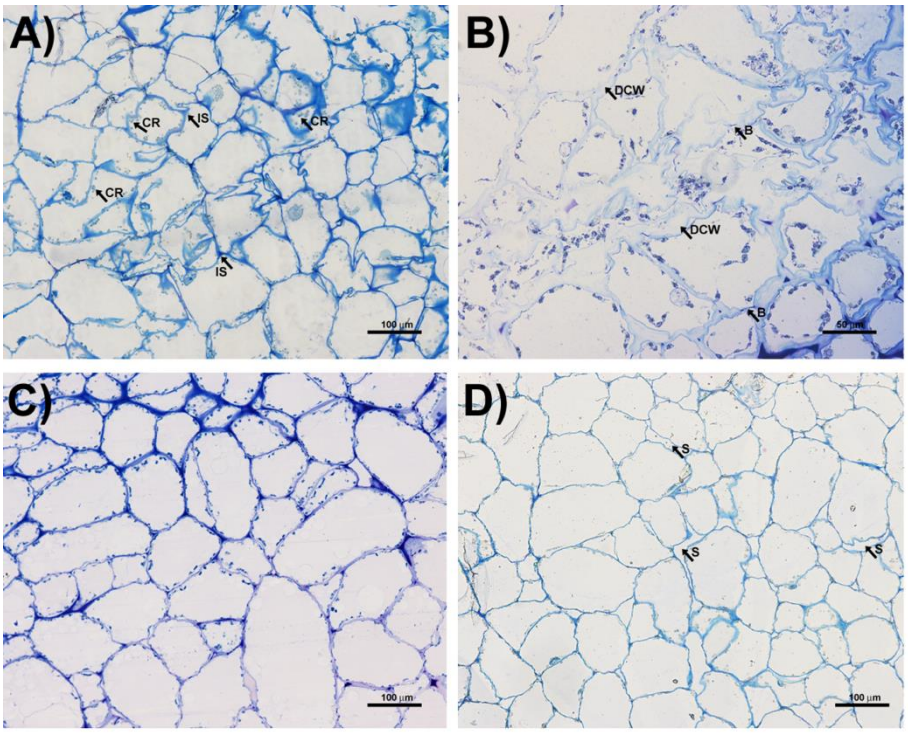

Figure 1. Light microscopy micrographs of untreated (A), HHP-treated (B, C), and pasteurized (D) red Lamuyo sweet pepper. B, bridges of remnants of cell wall material; CR, chromoplats; DCW, degraded cell wall; IS, intercellular space; S, separation between cell walls. Magnification: 20x (A), 10x (B, C, D).

3.1.1. Morphometric analysis

Table 1 shows the results obtained for the evaluated morphometric parameters (area, perimeter, circularity, and Feret diameter). Statistical analysis of the results revealed that the CNT red pepper had significantly lower circularity values ($P< 0.05$), while PAST peppers had significantly higher area, perimeter and Feret diameter ($P < 0.05$). In contrast, there were no statistically significant differences ($P > 0.05$) between the CNT, T1 and T4 red peppers with respect to area, perimeter or Feret diameter. There

were no statistically significant differences ($P > 0.05$) in circularity between cells of T1, T4 and PAST peppers. Thus, it seems that HHP and PAST promote an increase in the circularity of pepper cells. Trejo Araya et al. (2007) obtained different results when studying the effect of HHP on carrot microstructure. Those authors found that HHP decreased the circularity of cells compared with fresh carrot cells. Contrary, Penna et al. (2007) when studied the effect of high pressure treatment on the micelle microstructure of yogurt, obtained that micelles in high pressure milks were more spherical in shape and present more uniform size distribution compared to heat-treated milks.

Table 1. Morphometric parameters of the cells of untreated, HHP-treated, and pasteurized red Lamuyo sweet pepper.

	Area (μm^2)	Perimeter (μm)	Circularity	Feret Diameter (μm)
CNT	9325. 2^a	408.1^a	0.7^a	140.3^a
	(2790.6)	(70.9)	(0.1)	(20.8)
T1	10186.9^a	399.8^a	0.8^b	143.5^a
	(5577.2)	(111.1)	(0.1)	(42.3)
T4	10524.5^a	400.8^a	0.8^b	143.6^a
	(6038.7)	(119.2)	(0.1)	(38.0)
PAST	13722.6^b	469.3^b	0.8^b	164.6^b
	(2690.4)	(55.6)	(0.1)	(20.1)

Values in parentheses are the standard deviations.

In the same column, means without the same letter reveal significant differences ($P < 0.05$) according to the LSD multiple range test.

However, only PAST caused statistically significant changes ($P < 0.05$) in area, perimeter and Feret diameter in red pepper cells, contradicting the observations in the micrographs (Figure 1) where treatments T4 and (to a greater degree) T1 appeared to cause greater changes in the red pepper tissue microstructure than PAST. Therefore, it can be concluded that HHP does not result in significant changes in the evaluated morphometric parameters despite noteworthy microstructural changes in the micrographs of peppers subjected to HHP (including less staining and more degraded cell walls). This phenomenon could be explained by the fact that less aggressive treatments from a microstructural point of view (T4 and PAST) could allow water to

migrate radially and in a controlled fashion, thus favouring the maintenance of cell shape. It is also interesting to note that Hernández-Carrión et al. (2014a) found that these treatments (T4 and PAST) least affected carotenoids, antioxidant capacity, dietary fibre and red pepper texture while promoting preservation and functionality of the plant material when studying the effect of different HHP and PAST treatments on bioactive compounds and red pepper texture. These results suggest that the lower the structural damage caused by a treatment, the less impact on the functionality of the pepper. Similarly, Hernández-Carrión et al. (2014a) found that PAST caused an increase in the area of red pepper cells when studying the microstructure of Lamuyo red peppers subjected to different preservation treatments (HHP and PAST), obtaining values of 12127 μm^2 compared with CNT red pepper (9662 μm^2).

Figure 2 shows the effects of applying HHP at 100 MPa (T1) and 500 MPa (T4) as well as the effect of PAST on the cell size distribution. The figure indicates that CNT peppers show two populations of easily distinguished cell sizes, one with cells having a 125-μm Feret diameter and the other with cells having a 160-μm Feret diameter. When the pepper was subject to treatment T1, the proportion of cells with the greater diameter tended to decrease with respect to the CNT pepper, which caused widening of the curve, thus encompassing a greater range of cell diameters. Similarly, when applying treatment T4, the curve of the distribution of the cell diameters appeared to widen relative to the CNT pepper, covering a greater range of diameters and coinciding with most of the cell population of the first peak of the CNT pepper (approx. 125 μm). As shown in Figure 2, in contrast, when peppers are subjected to PAST, their cell diameter increases; in this figure, the curve corresponding to PAST is slightly shifted to the right, and its maximum value coincides with the second peak of the CNT pepper (approx. 160 μm). In this case, and as a result of the increased cell size caused by the PAST treatment, the proportion of cells with the larger diameter is decreases relative to the CNT and T4 treatment peppers. This result coincides with the result obtained by analyzing the morphometric parameters of peppers subjected to different treatments (Table 1) in which it was observed that the PAST peppers had a significantly higher Feret diameter ($P < 0.05$). It appears that the distribution of the cell diameters of red peppers varies depending on the HHP or PAST treatment. Whereas HHP tends to widen the curve, PAST tends to shift it to the right. The

widening observed upon applying the T1 and T4 treatments could be explained by microstructural changes caused by their application (Figs. 1B and 1D), which would trigger disruption of the cells and thus promote union between them, increasing their size. It is worth noting that the increase in cell diameter observed in Figure 2 was not statistically significant ($P > 0.05$) when taking into account the results shown in Table 1.

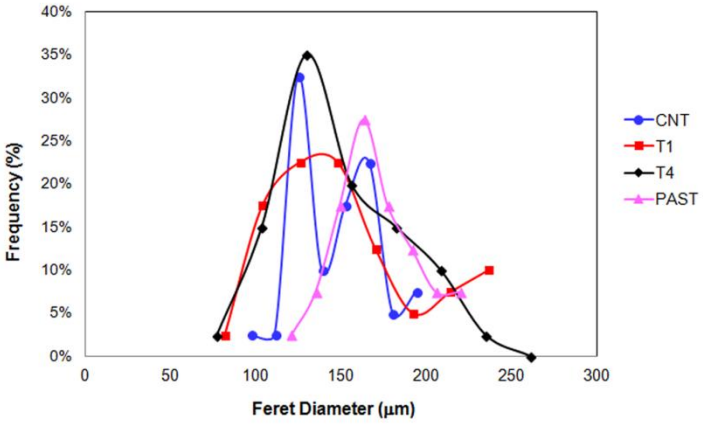

Figure 2. Particle size distribution of the cells of untreated, HHP-treated, and pasteurized red Lamuyo sweet pepper.

3.2. Scanning electron microscopy (SEM)

The parenchymal tissue of the CNT pepper (Figures 3A, 3B and 3C) was characterized by turgid cells of rounded and semi-rounded appearance intimately linked to one another with little intercellular space. Both treatments (HHP and PAST) caused visible microstructural changes. However, HHP at low and medium pressures, i.e., the T1 (Figures 3D, 3E and 3F), T2 (Figures 3G, 3H and 3I) and T3 (Figures 3J, 3K and 3L) treatments, led to more dramatic changes in the red pepper tissue. In these cases, a completely waterlogged tissue was observed in which different cells could be hardly distinguished, though they were clearly differentiated in the CNT pepper. HHP T4 treatment (Figures 3M, 3N and 3O) and PAST (Figures 3P, 3Q and 3R) caused less dramatic microstructural changes in the red peppers. Thus, in these cases, it was possible to distinguish rounded and semi-rounded cells that were slightly separated from one another, resulting in intercellular spaces filled with air.

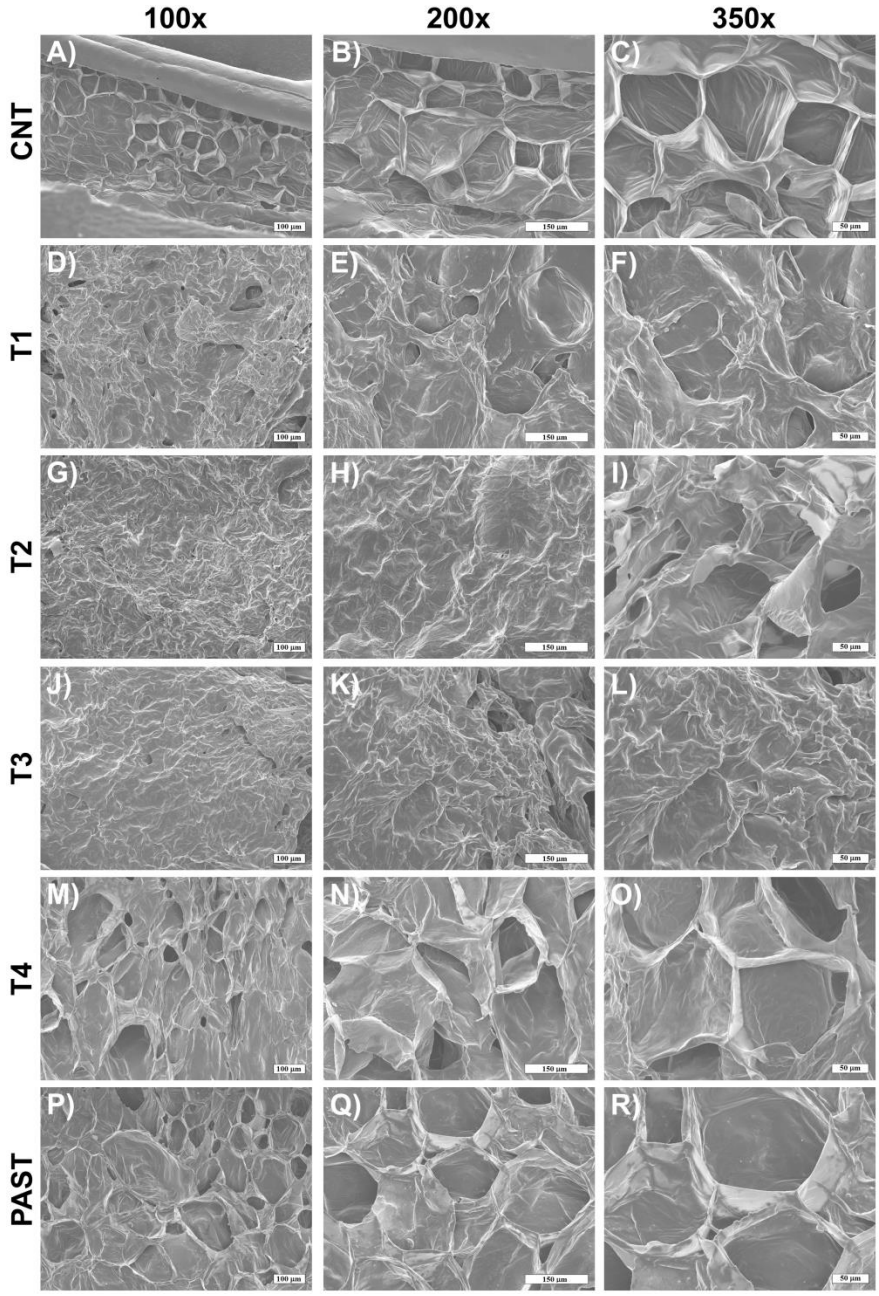

Figure 3. Scanning electron microscopy micrographs of untreated, HHP-treated, and pasteurized red Lamuyo sweet pepper. Magnification: 100x, 200x, and 350x.

These results are consistent with the results observed when studying the microstructure of samples through light microscopy where it was observed that T1 treatment at the lowest pressure caused the greatest structural changes. While the HHP T4 and PAST treatments did cause microstructural changes, they had less impact on the tissue structure of the red peppers.

Figure 4 shows cropped images (270 μm x 270 μm) that were considered for determining the image texture parameters for each of the studied treatments (CNT, T1, T2, T3, T4 and PAST) at the three magnifications used (100x, 200x and 350x) during micrograph acquisition. It is important to note that the treatments that caused greater cellular changes (T1, T2 and T3) showed a rough and irregular surface at the different magnifications studied, while the surface of the CNT pepper and the other treatments that best preserved red pepper structure (T4 and PAST) were characterized as homogenous in which holes could be distinguished that corresponded to areas occupied by the cells that make up the red pepper tissue. These results agree with the results obtained from the microstructural study (Figure 3) in which it was observed that treatments T1, T2 and T3 had a greater impact on the red pepper structure. Similarly, the T4 and PAST treatments preserved the structure best; these peppers were most similar to the CNT pepper in that some of the cells could be clearly distinguished.

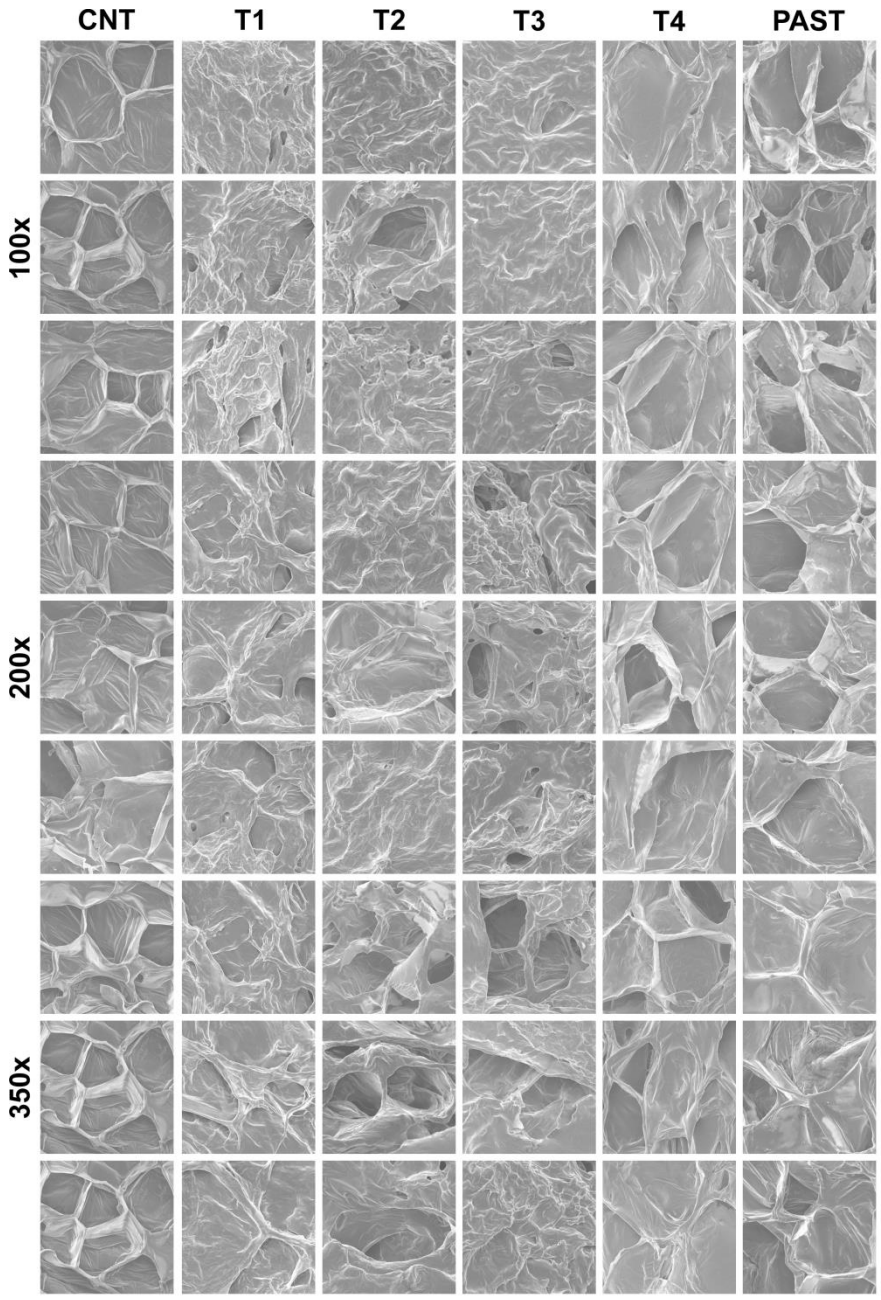

Figure 4. Scanning electron microscopy crops evaluated at 100x, 200x, and 350x of untreated, HHP-treated, and pasteurized red Lamuyo sweet pepper.

3.2.1. Texture image analysis

The statistical analysis of the results revealed a significant interaction ($P < 0.05$) between the applied treatment and the magnification used when analyzing the *texture fractal dimension (FDt)* of the Lamuyo red pepper micrographs. In this sense, it can be observed (Figure 5A) that there were no statistically significant differences ($P > 0.05$) between the micrographs of the CNT, PAST and HHP T4 peppers when using 100x magnification. However, statistically significant differences ($P < 0.05$) were found when pressures of 100 (T1), 200 (T2) and 300 (T3) MPa were applied, and an increase in pressure tended to significantly decrease the *FDt* of the Lamuyo red pepper micrographs ($P < 0.05$). This result was related to the decreased structural damage observed with increasing pressure. The treatments with higher *FDt* values (T1, T2 and T3) were also shown to have a rougher and more irregular surface, suggesting that rougher surfaces have higher *FDt* values (Quevedo et al., 2009; Arzate-Vázquez et al., 2012). Similar results were obtained by Quevedo et al. (2002) when studying the *FDt* and the surface of chocolate and pumpkin. The authors found that pumpkin had higher *FDt* values and a rougher surface compared with chocolate, which had lower *FDt* values and a more homogenous surface. In the same way, Gonzales-Barron & Butler (2008) related the higher *FDt* values to more roughness surfaces of bread crumbs images and Barrera et al. (2013) obtained in starch granules that damaged ones showed higher *FDt* values than native ones. When the 200x magnification was used, no statistically significant difference ($P > 0.05$) in *FDt* was found between the micrographs of the CNT and the T4 peppers, while the other treatments showed significantly higher ($P < 0.05$) *FDt* values. However, the increased pressure did not show the same effect as when 100x magnification was used. T2 treatment resulted in significantly higher ($P < 0.05$) *FDt* values than T1 treatment, which was in contrast to the results when using 100x magnification. Finally, when using 350x magnification, no statistically significant difference ($P > 0.05$) in the *FDt* values was found between the micrographs of the CNT, PAST and T2 and T4 treatment peppers. Treatments T1 and T3 had significantly higher ($P < 0.05$) *FDt* values, which shows that the more homogenous surfaces (CNT, T4 and PAST) tended to have lower *FDt* values. It thus appears that the magnification used to study the microstructure of the red pepper has a clear influence on the texture parameters determined from the micrographs. However, increased magnification did not exert the same effect on all of the

treatments. Thus, for the CNT, PAST, T3 and T4 peppers, the increase in magnification resulted in a significant increase ($P < 0.05$) in the *FDt* values of the micrographs. The structural damage of sweet pepper caused by treatment is best observed at smaller scales so in this case the best magnification to select would be 100x. Anyway, it would be recommendable to investigate other magnifications in future research. Similar results were obtained by Quintanilla-Carvajal et al. (2001) when studying the *FDt* in α-tocopherol agglomerated microcapsules at two different magnifications (500x and 2000x), where the *FDt* values were higher at 2000x than at 500x. The opposite behaviour was observed when treatments T1 and T2 were applied: significantly lower ($P < 0.05$) *FDt* values were obtained at 350x magnification compared with 100x and 200x. So, *FDt* seems to be a useful textural parameter to numerically describe microstructural changes. Numerous publications can be found in the literature that indicate this fact (Quevedo et al., 2002; Ersahin et al., 2006; Tang & Marangoni, 2006; Gonzales-Barron & Butler, 2008; Quevedo et al., 2009; Valous et al., 2009; Quintanilla-Carvajal et al., 2011; Barrera et al., 2013).

When analyzing the *angular second moment* (Figure 5B) of the micrographs from red peppers subjected to different preservation treatments, no statistically significant interaction ($P > 0.05$) was found between the studied parameters, and there was no statistically significant difference ($P > 0.05$) in the textural properties for any of the evaluated parameters (treatment and magnification). It thus appears that this parameter is not optimal for evaluating the effect of HHP and PAST on the image texture of red peppers. Several studies have shown the pertinence of this textural parameter to study the texture of different type of surfaces as biofilms (Yang et al., 2000), skin (Ou et al., 2014), and plastic greenhouses (Agüera et al., 2008). However, no many studies can be found that indicate the pertinence of the *angular second moment* on studying the texture of food products (Quiao et al., 2007). So, *angular second moment* seems to be a textural parameter more appropriate to study the texture of no-food products surfaces.

Regarding the textural feature *contrast*, the statistical analysis of the results revealed a significant interaction ($P < 0.05$) between the applied treatment and the magnification used. Thus, it can be observed (Figure 5C) that the micrographs of the CNT and T3 peppers had significantly lower *contrast* values ($P < 0.05$) at 100x magnification, while

the peppers subjected to T1 treatment had significantly higher *contrast* values ($P < 0.05$). The PAST, T2 and T4 treatments showed intermediate *contrast* values. When using the 200x magnification, treatments T1 and T2 resulted in significantly higher ($P < 0.05$) *contrast* values, while no significant differences ($P > 0.05$) were found for this parameter in the remaining the analysed treatments. A high *contrast* value indicates a high degree of local variation (Park & Chen, 2001; Mendoza et al., 2007), which is typical of rougher and more heterogeneous surfaces. This result would explain the higher *contrast* values observed for treatments T1 and T2 with rougher surfaces and the lower values observed for CNT peppers with a more homogenous surface. Finally, at 350x magnification, there was no statistically significant difference ($P > 0.05$) between the *contrast* values of the micrographs from peppers subjected to different treatments. This result again confirms that the magnification used to study the microstructure of red peppers has a clear influence on the textural parameters determined from micrographs, where cellular damage is clearer at smaller scales. In this case, we also observed that increased magnification resulted in a significant decrease ($P < 0.05$) in the *contrast* for all of the treatments considered in the study. Several publications can be found in the literature that indicate the pertinence of the textural feature *contrast* to numerically describe microstructural changes (Park & Chen, 2001; Mendoza et al., 2007; Zheng et al., 2007). Contrary, Laddi et al. (2013) when studying the contribution of different textural features (*entropy, contrast, energy, homogeneity*, and *correlation*) to estimate the tea quality concluded that *contrast* have no effect on tea quality. In the same way, Barrera et al. (2013) when evaluated the mechanical damage on wheat starch granules did not find significant differences of contrast parameter between damaged and native granules.

Similar to the *angular second moment* (Figure 5D), when analyzing the *correlation* between micrographs from red peppers subjected to different preservation treatments, no statistically significant interaction ($P > 0.05$) was found between the studied parameters, and no statistically significant difference ($P > 0.05$) was observed in the textural properties of any of the evaluated parameters (treatment and magnification). Just as for the *angular second moment*, its *correlation* did not appear to be an optimal parameter for evaluating the effect of HHP and PAST on the image texture of red peppers. Similar results obtained Laddi et al. (2013) when studying the contribution of different textural features (*entropy, contrast, energy, homogeneity*, and *correlation*) to estimate

the tea quality and concluded that *correlation* have no effect on tea quality. In the same way, Park & Chen (2001) obtained that correlation was not significant to differentiate unwholesome from wholesome poultry carcasses.

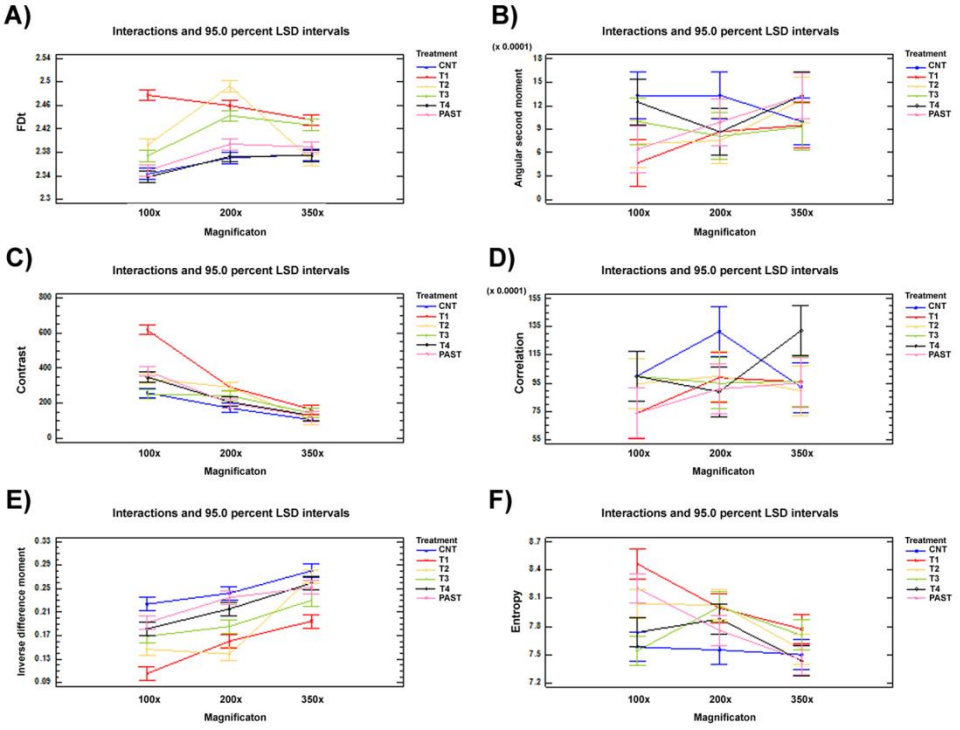

Figure 5. Interaction plots between treatment and magnification with LSD intervals for the *FDt* (A), *angular second moment* (B), *contrast* (C), *correlation* (D), *inverse difference moment* (E), and *entropy* (F) of red Lamuyo sweet pepper.

When analyzing the *inverse difference moment (IDF)* of the micrographs from red peppers subjected to different preservation treatments, the results showed a statistically significant interaction ($P < 0.05$) between the applied treatment and the magnification used. Thus, at 100x magnification (Figure 5E), there was no statistically significant difference ($P > 0.05$) in the *IDF* for PAST, T3 and T4 treatment. However, the CNT pepper micrographs had significantly higher ($P < 0.05$) *IDF* values, and the treatment T1 resulted in significantly lower ($P < 0.05$) *IDF* values. These results are logical

because it is known that *IDF* values indicate the degree of variation in image contrast, and high *IDF* values can be associated with homogeneous images (Barrera et al., 2013) such as the images obtained of the CNT red peppers. The elevated pressure tended to significantly increase ($P < 0.05$) the *IDF*, which was related to the lower structural damage observed with increased pressure. Similar results were obtained by Barrera et al. (2013) when evaluated the mechanical damage on wheat starch granules and concluded that the surface of damaged granules showed lower *IDF* values than native starch granules and suggested that the mechanical process decreased *IDF*. When using the 200x magnification, the micrographs from the CNT and PAST peppers had significantly higher *IDF* values ($P < 0.05$), while the peppers subjected to the T1 and T2 treatments had significantly lower *IDF* values ($P < 0.05$). Again, in this case, the increase in pressure tended to significantly increase the *IDF values* ($P < 0.05$) due to the decreased structural damage caused by the increased pressure. Finally, when the 350x magnification was used, treatment T1 yielded significantly lower *IDF* values ($P < 0.05$). This result was associated with the more heterogeneous surface obtained with treatment T1. It is also important to note that increasing the magnification used for making micrographs resulted in a significant increase ($P < 0.05$) of *IDF* for all of the analysed treatments, which again shows that cellular damage is best observed at smaller scales. So, *IDF* seems to be a useful textural parameter to numerically describe microstructural changes. Numerous publications can be found in the literature that indicate this fact (Yang et al., 2000; Park & Chen, 2001; Mendoza et al., 2007; Barrera et al., 2013; Laddi et al., 2013).

Finally, statistical analysis of the results revealed a statistically significant interaction ($P < 0.05$) between the applied treatment and the magnification used to analyse the *entropy* of the red pepper micrographs. Thus, it can be observed (Figure 5F) that the micrographs corresponding to the CNT and T3 and T4 treatment peppers had significantly lower *entropy* ($P < 0.05$); there was no statistically significant difference ($P > 0.05$) between these groups. In contrast, the T1 and PAST treatments resulted in significantly higher ($P < 0.05$) *entropy* values. The higher *entropy* values obtained for T1 treatment red peppers could be related to the greater heterogeneity of its structure (Yang et al., 2000) since the more complex the images, the higher the *entropy* values (Mendoza et al., 2007; Barrera et al., 2013). At 200x magnification, there was no statistically significant difference ($P > 0.05$) in the *entropy* of the micrographs

corresponding to treatments T1, T2, T3, T4 or PAST. Only the micrographs from the CNT peppers had significantly lower ($P < 0.05$) *entropy* values than the peppers treated by HHP; these micrographs were comparable to the PAST micrographs. The lower *entropy* values found in the images of the CNT and PAST peppers could be related to the greater homogeneity of their structures (Yang et al., 2000; Mendoza et al., 2007). In the same way, Barrera et al. (2013) when evaluated the mechanical damage on wheat starch granules, obtained that damage granules showed higher *entropy* values than the native granules, suggesting that the mechanical process increased the *entropy*. When the 350x magnification was used, there was no statistically significant difference ($P > 0.05$) in the *entropy* of the micrographs corresponding to the different treatments analysed, which could be expressed as cellular damage that is best observed at smaller scales. It should be noted here that the increase in magnification used in making micrographs did not result in a predictable trend in *entropy*, unlike in previous cases. That is, in some cases, the increase in magnification produced a significant decrease ($P < 0.05$) in *entropy* (T1, T2 and PAST), while in other cases, it did not cause significant changes ($P > 0.05$) in *entropy* (CNT, T3 and T4). So, *entropy* seems to be a useful textural parameter to numerically describe microstructural changes. Numerous publications can be found in the literature that indicate this fact (Yang et al., 2000; Park & Chen, 2001; Mendoza et al., 2007; Barrera et al., 2013; Laddi et al., 2013).

4. Conclusions

All the preservation treatments studied, whether PA or HHP, caused structural changes in red Lamuyo-type sweet pepper tissues, but HHP T4 and PA were the treatments that had the least impact on the microstructure. Structural modifications in the red pepper tissues caused by HHP and PAST led to variations in the area, perimeter, circularity and Feret diameter of the pepper cells, changes in the distribution of cell size and modification of the image texture parameters. Of the studied texture parameters, some were more relevant than others for characterizing the effects of HHP and PAST on the texture of the red peppers: *FDt*, *contrast*, *IDF* and *entropy*. In contrast, the magnification at which the texture was evaluated was a parameter to consider given that the cellular damage is best observed at smaller scales. Anyway, it would be recommendable to investigate other magnifications in future

research. There appears to be a relationship between the structural damage to red pepper tissues resulting from preservation treatments and the effects on the functionality of the plant material such that the lower the structural damage (T4 and PAST treatments), the lower the impact on the functionality of the pepper. These results suggest the relevance of image analysis as a quantitative and non-invasive technique that could be related in future studies to the biological functionality of products subject to HHP.

5. Acknowledgements

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New formulations of white sauces with enhanced functionality. A rheological, microstructural and sensory study

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Abstract

Red sweet peppers are good sources of antioxidant compounds, such as fibre and carotenoids. Therefore, innovative products that may replace traditional ones, such as white sauces enriched with red sweet pepper, should be developed to improve their functionality. The aim of this work was to study the rheological behaviour, microstructure, syneresis, colour, sensory characteristics and consumer acceptability of new white sauces enriched with red sweet pepper. The results of the rheological studies showed that all of the white sauces showed pseudoplastic behaviour and characteristic spectra of soft gels with G' values above G". The effect of incorporating red pepper on the rheological properties depended upon the type of starch used. Consumers scored the modified starch sauces regardless of the starch concentrations based on their highest overall liking and found them beneficial for health. Therefore, new white sauces with high nutritional value, high acceptability, good rheological properties and stability could be formulated using red pepper and modified starch.

Keywords: functionality, microstructure, red pepper, rheology, sensory perception, white sauce.

1. Introduction

Consumers now appreciate foods rich in compounds with potential long-term health effects, including bioactive compounds, such as carotenoids, fibre and flavonoids (Kapsak et al., 2011). The types of food rich in bioactive compounds are also called functional foods. The term "functional foods", which is closely related to health maintenance and preventive medical care (Sila et al., 2012), was first introduced in Japan during the 1980s (Arias-Aranda & Romerosa-Martínez, 2010). Many publications show that consuming functional foods reduces the risk of cardiovascular disease, kidney disease, obesity, macular degeneration and colon and rectal cancers. Moreover, the consumption of these phytochemicals appears to mitigate the effects of diabetes, reduce serum cholesterol levels and promote bowel movements (Morais et al., 2002; Chang & Liu, 2009; Trinidad et al., 2009).

Consequently, red sweet peppers (*Capsicum annuum)* have attracted the attention of researchers due to their high contents of bioactive compounds, such as fibre, phenols, flavonoids and carotenoids, which possess antioxidant and anti-inflammatory activities (Duma & Alsina, 2012). These beneficial properties are attributed to the sweet peppers, and their consumption appears to improve scar formation, prevent atherosclerosis and haemorrhages, prevent increases in blood cholesterol levels and improve stamina (Faustino et al., 2007).

However, convenience foods are in great demand due to recent social and cultural changes. Convenience foods are any wholly or partially prepared dish in which a significant portion of time, energy or culinary skill is assumed by the manufacturer, processor or distributor of these foods, freeing the consumer from this task. Convenience foods are prepared meals, precooked foods and various minimally processed products that might only require heating before consumption (Feliciano & Albisu, 2005). White sauces are frequently a constituent of convenience food.

Sauces are an important part of the human diet and have high added value. A standard white sauce includes milk, oil, flour or starch, and salt. Starch exerts a dominant influence on the textural properties of the sauce (Arocas et al., 2009b). The most common problem with these sauces is destabilisation after preparation and/or during storage. Two types of stability problems have been observed: those associated with the emulsion (Ostwald ripening, creaming, flocculation and coalescence) and those

caused by the interaction of two or more ingredients in the sauce. The proteins and polysaccharides act as emulsifiers and stabilizers (Mandala et al., 2004). Milk proteins are functional ingredients that can improve the nutritional and functional properties (taste, texture and shelf life) of the products containing them (Kenny et al., 2001; Gallagher et al., 2003).

Numerous studies related to the microstructural, physical and rheological properties of white sauces have been carried out. The type of corn starch affects the rheological properties of white sauces after heating and freezing (Arocas et al., 2009b), and the microstructure of white sauces produced using soy protein isolate (Quiles et al., 2012) or inulin (Guardeño et al., 2012) have been studied. The effects of the cooking time on the rheological properties and microstructure of white sauces (Arocas et al., 2010) and the effect of the type of fat and agitation speed on the stability of white sauces thawed in a microwave and conventional oven have also been examined (Hernández-Carrión et al., 2011).

In recent years, however, the consumption of convenience foods has increased, and the increasing consumer demand for functional foods designed to improve their heath create a need for the food industry to develop innovative products that may replace traditional ones. In this context, little research has been carried out on white sauces enriched with bioactive compounds, such as ground red sweet pepper, to improve their functionality and benefits.

Consequently, this study was focused on developing new white sauce formulations that were enriched with red sweet pepper. The sauces were prepared using two different types of corn starch: native and modified. The effects of using different amounts of red sweet pepper purée in the sauces were investigated. The rheological behaviour, microstructure, and some physical properties (total soluble solid content, pH, syneresis, and colour) were examined; in addition, their liking consumer acceptability was evaluated. Finally, the concept of a "white sauce enriched with red pepper" was evaluated using a "check-all-that-apply"-type form.

2. Materials and methods

2.1. Plant material and sample preparation

The plant material used to formulate the white sauces was from red Lamuyo-type sweet peppers that were ripe enough for commercial purposes. The red peppers, which were acquired from a local market in September 2013, were washed, cut into pieces measuring approximately 15 mm along each side and heat-sealed in 200 x 200 mm plastic bags (Doypack type, Amcor, Spain). Each bag contained approximately 100 g of red sweet pepper. The bags were stored at -80 °C in a deep freezer (Dairei Europe, Denmark) until use. To formulate the white sauces, the small cut pieces of red sweet pepper were homogenized in a food processor (Thermomix TM31, Wuppertal, Germany) at 6500 rpm for 1 min followed by 10200 rpm for 30 s. The red sweet pepper purée was completely thawed at room temperature and passed through a metal sieve (3-mm mesh) to standardize the particle size of the red pepper. The red pepper that remained in the sieve was discarded.

2.2. Starch

Two different types of corn starch were used to prepare the white sauces: native corn starch (NS, C Gel 03401) and modified waxy corn starch (MS medium cross-linked acetylated distarch adipate, C Tex 06214) from Cargill, Inc. (Minneapolis, Minn., USA).

2.3. White sauce

Twelve white sauces were prepared using two different types of starch (NS or MS) at two different concentrations (4 or 6 g/100 g) while varying the concentration of red pepper (0, 5 or 15 g/100 g). The sauces consisted of starch (4 or 6 g/100 g, as appropriate), sunflower oil (Coosol, Vilches, Jaen, Spain) (2.5 g/100 g); powdered skimmed milk (Central Lechera Asturiana, Siero, Spain) (3.2 g/100 g); salt (0.8 g/100 g), black pepper (0.03 g/100 g), nutmeg (0.03 g/100 g), red pepper (0, 5 or 15 g/100 g, as the case may be), and mineral water up to 100 g (Font Vella, Sant Hilari Sacalm, Girona, Spain). The sauces were prepared according to Arocas et al., (2009b); briefly,

all of the ingredients were placed in a food processor (Thermomix TM31), heated to 90 ºC (17 ºC/min) at 1100 rpm and held at 90 ºC at the same agitation speed for 6 min. The sauces were stored in Pyrex glass bottles (200 g), cooled down to 20 ºC, and stored at 4 ºC; all analyses were carried out within 24 h of preparation. To evaluate the appearance of the syneresis and the total colour differences (ΔE*) over time relative to freshly made samples, the sauces were stored at 4 ºC over 15 days.

2.4. Rheological behaviour

2.4.1. Pasting properties

The pasting properties of the sauces were studied with 6 g/100 g of starch and without red pepper or with 5 and 15 g/100 g of red pepper. A starch pasting cell (SPC) attached to a controlled stress rheometer (ARG2, TA Instruments, Crawley, England) was employed. The SPC consists of an impeller and a cylindrical cup (3.6 cm wide and 6.4 cm high). The impeller is designed to fit closely into a cylindrical cup containing the sample. The top of the mixing element shaft is gradually extended to form a non-contacting cone-shaped cover that strongly inhibits solvent evaporation. Electrical elements, which are placed concentrically relative to the cup, are used for heating the samples, and circulating water, which travels through a helical path close to the outer walls of the cup, is used for cooling. The cooling control unit, which is placed upstream of the cup, controls the flow of water.

For this test, 25 g of the system (white sauce ingredients) were placed in the cylindrical cup of the SPC. The components were strongly stirred (100 s^{-1}) for 10 s at 30 °C, and the shear rate was switched to 30 s^{-1} until the end of the test. The samples were heated from 30 to 90 °C at 15 °C/min, and the temperature was then held at 90 °C for 300 s. Subsequently, the samples were cooled to 30 °C at 15 °C/min and held at 30 °C for 300 s. The viscosity data were recorded over time; the data were collected using the TA software. The following parameters were calculated from the viscosity-temperature versus time curves (Arocas et al., 2009a): gelatinisation temperature (GT), which is the temperature at which viscosity begins to rise; peak viscosity (PV), which is the highest viscosity achieved during heating; hot paste viscosity (HPV), which is the viscosity at the end of the 90 °C isothermal period; cold paste viscosity (CPV),

which is the viscosity at the end of the 30 °C isothermal period; relative breakdown, which is calculated as (PV-HPV)/ PV. The measurements were carried out in triplicate.

2.4.2. Flow and linear viscoelastic properties

Both the flow behaviour and the linear viscoelastic properties of each white sauce were measured in triplicate. The measurements were carried out in a RS1-controlled stress rheometer (Thermo Haake, Karlsruhe, Germany) with a Phoenix P1 Circulator device (Thermo Haake) used for temperature control. A serrated plate 35 mm in diameter with 1-mm gap was employed. The RheoWin software package (version 2.93, Thermo Haake) was used to monitor the experiments. Measurements were carried out at 20 ºC and 50 ºC. The white sauces were allowed to rest on the rheometer plate for 5 min before each measurement and a fresh sample was loaded for each measurement.

Flow behaviour

A continuous ramp from 1 to 100 s^{-1} in 900 s with logarithmic distribution was applied and the apparent viscosity recorded as a function of the shear rate. The viscosity (η) versus shear rate (γ) data were fitted to the Ostwald-de Waele model (eq. 1):

$$\eta = K \cdot \dot{\gamma}^{n-1} \quad (1)$$

where η is the apparent viscosity, K is the consistency index, gamma dot is the shear rate and n is the flow index.

Linear viscoelastic properties

To determine the linear viscoelastic region (LVR), stress sweeps from 0.1 to 40 Pa were run at 1 Hz for each sample and temperature. Frequency sweeps were performed by placing a stress wave amplitude well within the LVR over the frequency range from 10 to 0.01 Hz. The storage modulus (G'), the loss modulus (G") and loss tangent (tg δ= G"/ G') were recorded as a function of the frequency (mechanical spectra) using the RheoWin Pro software.

2.5. Microstructural analysis

2.5.1. Light microscopy

Equipment and dyes

A light microscope (Nikon Eclipse 80i, Nikon Co., Ltd., Tokyo, Japan) was used to study the microstructure of the white sauces. Two different dye solutions were used: an iodine solution to stain starch and toluidine blue (1 g L^{-1}) to stain the proteins and red pepper tissue. The auto-fluorescence of the white sauces containing red pepper was also observed while using a mercury arc lamp with a FITC filter (λ_{exmax} = 482 nm, $\lambda_{em\ max}$ = 536 nm) as excitation source.

Sample viewing

A drop of white sauce was placed on a microscope slide, stained with the appropriate dye solution, covered with a cover slip and visualized at 10x, 20x, and 40x. The images were captured and stored at 1280 x 1024 pixels using the microscope software (NIS-Elements F, Version 4.0, Nikon, Tokyo, Japan).

2.5.2. Confocal laser scanning microscopy (CLSM)

Equipment and dyes

A Nikon confocal microscope C1 unit fitted on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) was used. An Ar laser line (488 nm) used to excite the Rhodamine B and Nile Red fluorescent dyes. Rhodamine B (Fluka, Sigma-Aldrich, Missouri, USA) with a $\lambda_{ex\ max}$ 488 nm and a $\lambda_{em\ max}$ 580 nm was dissolved in distilled water at 2 g L^{-1}. This dye was used to stain the proteins and carbohydrates. Nile Red (Fluka, Sigma-Aldrich, Missouri, USA) with a $\lambda_{ex\ max}$ 488 nm and a $\lambda_{em\ max}$ 515 nm was dissolved in polyethylene glycol (PEG) 200 at 0.1 g L^{-1} and was used to stain the fat. Two different oil immersion objectives lens were used: 60x/1.40NA/Oil/ Plan Apo VC Nikon and 40x/1.0/Oil DIC H/Plan Apo Nikon.

Sample viewing

A drop of white sauce was placed on a slide, and 20 μL of Rhodamine B solution and 20 μL of Nile Red solution were added. The observations were made 10 min after the dyes were added. The images were obtained and stored at a 1024 x 1024-pixel resolution using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

2.6. Physical properties

2.6.1. Total soluble solids content (TSS) and pH

TSS and pH were determined from three different white sauce samples in triplicate. The TSS was determined using a hand-held refractometer (model Pal-α, Atago, Tokyo, Japan), and the results were expressed in ºBrix. The pH was determined using a pH-meter (Basic 20+, Crison, Barcelona, Spain).

2.6.2. Syneresis

The syneresis of the white sauces was determined according to Heyman et al. (2010) with some modifications. Subsamples were introduced in centrifuge tubes and stored at 4 ºC for 15 days. After equilibrating to 20 ºC, the samples were centrifuged for 15 min at 6000 × *g*. The water released on the top was decanted and the percentage syneresis was calculated as follows:

% Syneresis = (weight of decanted liquid/ sample weight before centrifugation) × 100 (2)

2.6.3. Colour measurements

The measurements were carried out with a Chroma meter CR-400 (Konica Minolta Sensing Americas, Inc., Ramsey, N.J, USA). The results were expressed in accordance with the CIELAB system using illuminant C as a reference and a visual angle of 2°. The colorimeter was calibrated with a white standard pattern (Y = 92.9; x = 0.3137; y = 0.3198). The determined parameters included lightness, L* (L* = 0 [black] and L* = 100 [white]), a* (-a* = greenness and +a* = redness), and b* (-b* = blueness and +b* = yellowness). The total colour difference (ΔE*) relative to the freshly made sauce sample after 15 days of storage at 4 ºC was calculated as follows (Francis & Clydesdale, 1975).

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (3)$$

The values used to determine whether the total colour difference was noticeable by the human eye were the following (Bodart et al., 2008):

$\Delta E^* < 1$ colour differences are not obvious to the human eye.

$1 < \Delta E^* < 3$ colour differences are not easily distinguished by the human eye.

$\Delta E^* > 3$ colour differences are obvious for the human eye.

The measurements were performed using three different white sauces in triplicate.

2.7. Sensory analysis

A consumer test was carried out on white sauces formulated with the highest concentration of red pepper (15 g/100 g) with the two types of starch at two concentrations (4NS15RP, 6NS15RP, 4MS15RP, 6MS15RP). To evaluate the concept of an enriched white sauce consumers had to answer a check-all-that-apply (CATA) questionnaire for each sample. In addition, a liking consumer test was performed.

2.7.1. Consumers

The study was conducted in Valencia (Spain). The consumers were recruited from the employees and students of the Universitat Politècnica de València. One hundred consumers (61% were women, aged 18-62) were recruited for the study.

2.7.2. Procedure

The samples were assessed in a standardized tasting room equipped with individual booths (ISO, 1988). Each consumer received the four samples of white sauce enriched with red pepper in a sequential monadic series within a single session following a balanced complete block experimental design (William's design). The samples were served in small plastic cups randomly coded with three digit numbers. The white sauces were served at 40 ºC (consumption temperature). Mineral water was supplied for palate cleansing before each sample. The test was recorded on paper and self-completed after instructions were given by an interviewer.

2.7.3. Liking test

The consumer liking test was performed using a nine-box hedonic scale (1 = extremely dislike and 9 = extremely like). For each sample, the consumers scored their degree of liking in the following order and modalities: "overall liking", "appearance liking", "flavour liking" and "consistency liking."

2.7.4. CATA questionnaire

For each sample, the participants answered a CATA question featuring 12 attributes that had been previously selected based on an informal tasting by the researchers and their interests on the concept of a novel, functional white sauce. The following instructions were given to the participants: "which of the following characteristics better describe this sample? Please check all that you think apply. You can re-taste the sample if desired" The terms included in the questionnaire were: *red pepper provides fibre, red pepper provides antioxidants, red pepper provides calories, red pepper provides calcium, red pepper improves the taste of the white sauce, red pepper provides nutritional value, beneficial for health, prevents illnesses, prevents ageing, I would buy it, I would not buy it* and *I prefer white sauces without aggregates*. The 12 terms were presented at random to the participants.

2.8. Statistical analysis

2.8.1. Rheological and physical properties

A categorical multifactorial experimental design with three factors (type of starch (native and modified starch), concentration of starch (4 and 6 g/100 g) and concentration of red pepper (0, 5, and 15 g/100 g)) was used. An analysis of variance (ANOVA) was performed on the rheological and physical data using the XLSTAT statistical software (Version 2010.5.02, Microsoft Excel®, Barcelona, Spain). The honestly significant differences (HSD) were calculated using Tukey's test, and the significance was determined at $P < 0.05$.

2.8.2. CATA question

A Chi squared test was used to assess the differences in the perception of the white sauces with red pepper based on the CATA responses. For each sample, the frequency of use was determined for each attribute by counting the number of consumers that selected that term to describe it. Cochran's Q test (Manoukian, 1986) was carried out on the CATA data to identify the significant differences between samples for each of the characteristics. A factorial correspondence analysis (FCA) was run on the CATA frequency counts contingency table to understand the positioning of the four white sauces with red pepper as perceived by the consumers. The overall liking was superimposed in the obtained sensory space as a supplementary variable. The data analyses were performed using XLSTAT statistical software (Version 2010.5.02, Microsoft Excel®, Barcelona, Spain).

2.8.1. Consumer liking

An analysis of variance (one way ANOVA) was applied to study the differences between the formulations for the consumer liking scores; the least significant differences were calculated using Fisher's test, and the significance at $P < 0.05$ was determined. The liking data were analyzed using the Statgraphics Plus 5.1 software package (Statistical Graph Co., Rockville, MD, USA).

3. Results and discussion

3.1. Rheological behaviour

3.1.1. Pasting properties

The effects of the starch type and added red pepper on the structural changes occurring in the sauces during the cooking process were evaluated using a starch-pasting cell. In Figure 1 the viscosity and temperature profiles over time (pasting curves) for the sauces containing 6 g/100 g of starch are shown. In Table 1, the mean values of parameters GT, PV, HPV, and CPV and the relative breakdown obtained from the pasting curves are shown. The statistical results showed that the double interaction between the starch type and red pepper concentration was statistically significant ($P < 0.05$) for all of the pasting properties.

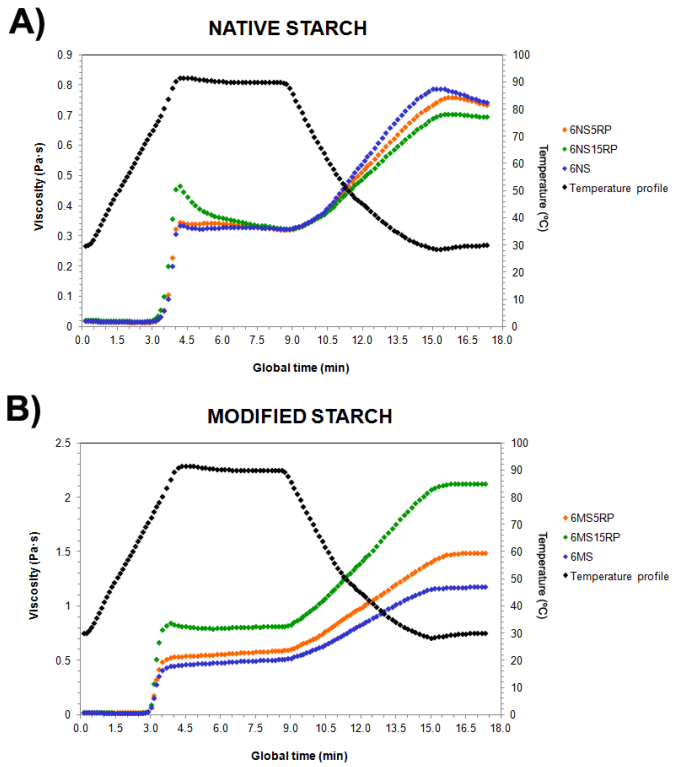

Figure 1. Viscosity-temperature profiles of white sauces elaborated with native starch (A) or modified starch (B). NS, native starch; MS, modified starch; RP, red pepper; 6, 6 g/100 g of starch; 5 and 15, 5 and 15 g/100 g of red pepper, respectively.

Adding red pepper affected the pasting parameters. In the native starch-containing sauces, increasing the red pepper concentration produced a slight but significant decrease ($P < 0.05$) in the GT; therefore, the increase in the sauce viscosity, which is associated with starch gelatinisation, occurred at a lower temperature in the presence of red pepper. Differences were also found in the values of PV. The PV value obtained at the highest red pepper concentration was significantly highest ($P < 0.05$), implying a higher starch swelling power. No significant differences ($P > 0.05$) were found in the HPV or CPV, although the CPV of the 15 g/100 g red pepper sauce was lower, implying a decrease in the final viscosity obtained at room temperature. The relative breakdown value of the sauce with 15 g/100 g of red pepper was significantly higher ($P < 0.05$) and linked with the highest PV.

Table 1. Effect of starch type, concentration of red pepper and the interaction starch type-[red pepper] in the pasting properties of the white sauces ingredients.

Type of starch	Starch content (g/100 g)	Red pepper content (g/100 g)	GT (ºC)	PV (Pa·s)	HPV (Pa·s)	CPV (Pa·s)	Relative breakdown
NS	6	0	75.85^{a} (0.07)	0.34^{d} (0.00)	0.33^{d} (0.00)	0.75^{d} (0.01)	0.05^{b} (0.01)
	6	5	74.10^{b} (0.00)	0.35^{d} (0.01)	0.33^{d} (0.00)	0.75^{d} (0.01)	0.08^{b} (0.01)
	6	15	74.10^{b} (0.00)	0.46^{c} (0.01)	0.32^{d} (0.01)	0.69^{d} (0.01)	0.31^{a} (0.00)
MS	6	0	70.75^{c} (0.07)	0.47^{c} (0.01)	0.51^{c} (0.00)	1.21^{c} (0.00)	-0.10^{d} (0.01)
	6	5	70.70^{c} (0.00)	0.54^{b} (0.01)	0.59^{b} (0.01)	1.51^{b} (0.03)	-0.10^{d} (0.00)
	6	15	70.80^{c} (0.14)	0.79^{a} (0.04)	0.80^{a} (0.02)	2.08^{a} (0.06)	-0.01^{c} (0.02)

GT, gelatinisation temperature; PV, peak viscosity; HPV, hot paste viscosity; CPV, cold paste viscosity; NS, native starch; MS, modified starch.

Values in parentheses are the standard deviations.

For each column, means with different letters are significantly different ($P < 0.05$) according to the Tukey's multiple range test.

In the sauce containing modified starch, the incorporation red pepper did not affect the GT; however increasing the red pepper content produced a significantly greater ($P < 0.05$) development of viscosity. The PV, HPV and CPV values increased significantly ($P < 0.05$) when increasing the red pepper concentration. In the presence of red pepper, the starch swelling and viscosity development were increased. No breakdown was observed, implying that the maximum viscosity developed during heating (PV) was maintained during the heating period and therefore heating stability. This result reflects the efficiency of the chemical crosslinking modification in the starch for preventing breakage in the granule. The higher viscosity development observed in the presence of the red pepper was also observed after cooling (CPV). Similar to the native starch sauces, the higher viscosity development observed in the presence of red pepper for the modified starch sauces coincides with the decrease in pH. The best processing stability and improved textural properties of the white sauces prepared with acetylated distarch adipate waxy corn starch was reported previously (Arocas et al., 2009b; 2010; 2011).

3.1.2. Flow behaviour

All of the sauces showed pseudoplastic behaviour (Figure 2), which fit the Ostwald de Waele model in the range of shear rates studied. The mean values of the consistency index (*K*), flow index (*n*), and apparent viscosity values at 10 s^{-1} (η_{10}) are shown in Table 2. Differences were found in the flow properties and effect of red pepper addition between the two types of starches. The statistical results showed that the triple interaction between starch type, starch concentration and red pepper concentration was statistically significant ($P < 0.05$) for *K* and η_{10} values at 20 ºC and for *n* and η_{10} values at 50 ºC. The three double interactions between the starch type and concentration, between the starch type and red pepper concentration, and between the starch concentration and red pepper concentration were statistically significant ($P < 0.05$) for *n* values at 20 ºC, while only the interaction between the starch type and red pepper concentration was statistically significant ($P < 0.05$) for *K* values at 50 ºC.

In the native starch at both starch concentration at 20 ºC, adding the red pepper decreased the consistency values ($P < 0.05$), and, in the 4 g/100 g sauce, increased the *n* value ($P < 0.05$), implying a decrease in pseudoplasticity. At a starch concentration of 6 g/100 g, no significant change ($P > 0.05$) was observed in the pseudoplastic index after adding the red pepper. Increasing the red pepper concentration did not have a significantly ($P > 0.05$) affect the flow properties.

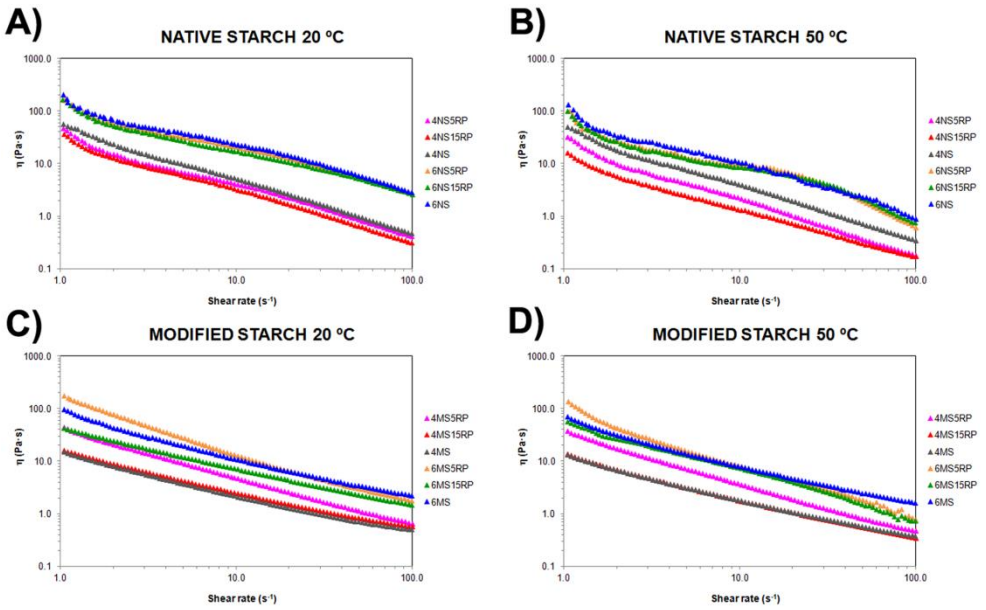

Figure 2. Flow behaviour of white sauces elaborated with native starch (A, B) or modified starch (C, D) measured at 20 ºC (A, C) and 50 ºC (B, D). NS, native starch; MS, modified starch; RP, red pepper; 4 and 6, 4 and 6 g/100 g of starch, respectively; 5 and 15, 5 and 15 g/100 g of red pepper, respectively.

The effect of incorporating red pepper in the modified starch sauces was completely different; it strongly depended on the red pepper concentration. Incorporating 5 g/100 g of red pepper increased *K* significantly ($P < 0.05$) and decreased *n* significantly ($P < 0.05$), implying an increase in pseudoplasticity. At the highest red pepper content, the effect was different. For the 4 g/100 g starch sauce with

15 g/100 g red pepper, the *K* and *n* values were very similar to the sauces without red pepper, and for the 6 g/100 g starch sauce, incorporating 15 g/100 g of red pepper decreased *K* significantly ($P < 0.05$).

The effect of incorporating the red pepper in the native starch sauces flow properties can be related to the pasting properties: the increase in red pepper concentration was associated with the highest viscosity development, the highest breakdown in viscosity, and the lowest final viscosity. In the presence of red pepper, the native starch granule became more sensitive to breakdown. With the modified starch, the pasting profile justifies the results obtained at 5 g/100 g of red pepper, but do not explain the decreased viscosity observed with 15 g/100 g of red pepper. These differences might occur because the shearing forces applied when preparing the sauces were stronger than the ones applied by the starch-pasting cell. The pasting results clearly show that increasing the red pepper concentration promote higher viscosity development. Under the shear conditions applied in the pasting procedure, no breakdown in viscosity was observed in the modified starch, implying that the starch modification imparted enough stability to the starch granule to avoid breakdown. The decreased viscosity at 15 g/100 g of red pepper observed based on the flow properties could be associated with the lower resistance of the modified starch granule under the shear conditions applied when manufacturing the sauce with other structural changes in the sauce components.

For both starches, increasing the temperature from 20 to 50 ºC decreased the *K* and η_{10} values. In general, the effect of adding red pepper on the flow properties at 50 ºC was the same as that described at 20 ºC.

Table 2. Effect of starch type, concentration of starch, concentration of red pepper and the interaction starch type-[starch]-[red pepper] in the Ostwald de Waele fit of the white sauces analyzed at 20 and 50 ºC ($0.9810 < R^2 < 0.9997$).

White sauce formulation			20 ºC			50 ºC		
Type of starch	Starch content (g/100 g)	Red pepper content (g/100 g)	K (Pa·s^n)	n	η_{10} (Pa·s)	K (Pa·s^n)	n	η_{10} (Pa·s)
NS	4	0	47.97^{d} (0.50)	0.011^{d} (0.003)	4.92^{g} (0.08)	32.95^{c} (0.93)	0.064^{cde} (0.006)	3.81^{d} (0.16)
	4	5	27.61^{def} (2.99)	0.119^{bc} (0.018)	3.63^{gh} (0.24)	18.77^{d} (0.13)	0.057^{de} (0.008)	2.14^{e} (0.02)
	4	15	26.23^{ef} (1.39)	0.048^{cd} (0.003)	2.93^{h} (0.17)	10.31^{d} (0.50)	0.112^{cd} (0.012)	1.33^{e} (0.03)
	6	0	129.71^{a} (7.93)	0.213^{a} (0.005)	21.15^{a} (1.05)	72.23^{a} (4.98)	0.114^{cd} (0.013)	9.38^{a} (0.37)
	6	5	104.31^{b} (13.44)	0.271^{a} (0.043)	19.37^{b} (0.58)	60.90^{ab} (2.12)	0.124^{bc} (0.019)	8.09^{bc} (0.07)
	6	15	88.85^{bc} (0.15)	0.269^{a} (0.001)	16.50^{c} (0.08)	52.40^{b} (2.09)	0.186^{ab} (0.018)	8.04^{bc} (0.02)
MS	4	0	14.12^{f} (1.30)	0.212^{a} (0.053)	2.29^{h} (0.07)	11.49^{d} (0.21)	0.230^{a} (0.001)	1.95^{e} (0.04)
	4	5	39.93^{de} (0.79)	0.093^{cd} (0.005)	4.94^{g} (0.04)	33.39^{c} (0.51)	0.044^{e} (0.014)	3.70^{d} (0.06)
	4	15	14.00^{f} (0.37)	0.268^{a} (0.004)	2.59^{h} (0.05)	12.06^{d} (0.33)	0.196^{a} (0.023)	1.89^{e} (0.05)
	6	0	77.85^{c} (5.12)	0.198^{ab} (0.006)	12.28^{e} (0.65)	53.70^{b} (1.29)	0.191^{a} (0.022)	8.34^{ab} (0.62)
	6	5	139.47^{a} (5.81)	0.017^{d} (0.009)	14.48^{d} (0.30)	71.68^{a} (8.76)	0.054^{de} (0.028)	8.08^{bc} (0.48)
	6	15	39.85^{de} (0.91)	0.273^{a} (0.007)	7.47^{f} (0.05)	56.77^{b} (0.95)	0.099^{cde} (0.006)	7.12^{c} (0.22)

NS. native starch; MS, modified starch.

Values in parentheses are the standard deviations.

For each column, means with different letters are significantly different ($P < 0.05$) according to the Tukey's multiple range test.

3.1.3. Viscoelastic properties

The mechanical spectra at 20 and 50 ºC of the different white sauces analyzed during this study are compared in Figure 3. In addition, the mean values of the storage modulus (G'), loss modulus (G") and loss tangent (tg δ) at 1 Hz, as well as the results of the statistical analyses, are shown in Table 3. Statistical results showed that the triple interaction between the starch type, starch concentration and red pepper concentration was statistically significant ($P < 0.05$) for the G' and G" values at 20 ºC and for the G' and tg δ values at 50 ºC. The three double interactions between the starch type and starch concentration, between the starch type and red pepper concentration, and between the starch concentration and red pepper concentration were statistically significant ($P < 0.05$) for tg δ values at 20 ºC, while the interactions between the starch type and starch concentration was only statistically significant ($P < 0.05$) for the G" values at 50 ºC.

All of the sauces (both NS and MS) showed the characteristic spectra of soft gels with G' values above G" and a slight frequency dependence for both moduli within the studied frequency range, similar to previous results reported in conventional white sauces (Arocas et al., 2011).

For both starch types, increased starch concentrations were associated with a significant increase ($P < 0.05$) in G' and G" and a decrease in tan delta (values closer to 0), revealing an increase in the system structuration associated with higher starch concentrations.

The viscoelastic properties varied between the sauces made using different starches. The MS sauces showed higher G' and G" values than the NS sauces; however, the tan delta values were closer to 0 in the NS sauces (higher predominance of the elastic component relative to the viscous). These results reveal the differences between the inner sauce structures associated with the starch structures, which will be described in detail within the microstructure section of this article.

Similar to previous results, the effect of adding red pepper addition depended on the starch type. In the NS sauce, increasing the red pepper concentration did not affect the linear viscoelastic functions significantly ($P > 0.05$). In the MS sauce containing 4 g/100 g of starch, increasing the red pepper concentration increased G' and G'

slightly', but the differences were not significant. Small yet significant differences were found in the values of tan d; the viscoelasticity increased at 5 g/100 g of red pepper and decreased at 15 g/100 g of red pepper. In the 6 g/100 g MS sauce, increasing the red pepper content decreased G' and G" and increased tan δ (decrease in viscoelasticity) slightly yet significantly.

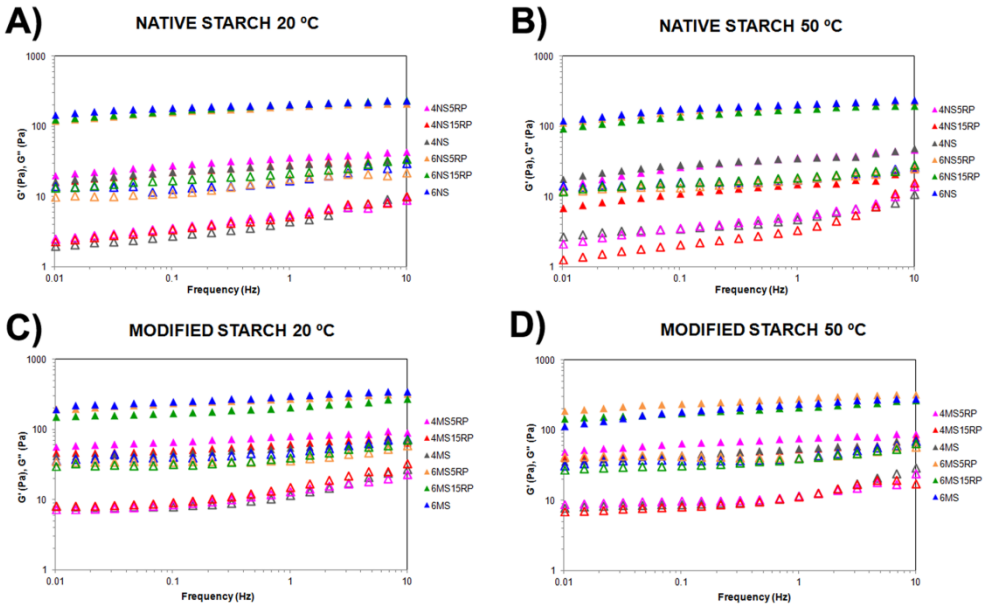

Figure 3. Viscoelastic properties of white sauces elaborated with native starch (A, B) or modified starch (C, D) measured at 20 ºC (A, C) and 50 ºC (B, D). G', full symbols; G", empty symbols; NS, native starch; MS, modified starch; RP, red pepper; 4 and 6, 4 and 6 g/100 g of starch, respectively; 5 and 15, 5 and 15 g/100 g of red pepper, respectively.

Table 3. Effect of starch type, concentration of starch, concentration of red pepper and the interaction starch type-[starch]-[red pepper] in the viscoelastic properties of the white sauces analyzed at 20 and 50 ºC.

White sauce formulation			20 ºC			50 ºC		
Type of starch	Starch content (g/100 g)	Red pepper content (g/100 g)	G' (Pa)	G'' (Pa)	tg δ (G''/G')	G' (Pa)	G'' (Pa)	tg δ (G''/G')
NS	4	0	28.1^{d} (3.8)	4.38^{f} (0.26)	0.16^{def} (0.01)	34.7^{fg} (5.8)	4.70^{de} (0.02)	0.14^{e} (0.01)
	4	5	35.8^{d} (2.5)	5.55^{f} (0.03)	0.16^{ef} (0.01)	35.4^{fg} (1.5)	5.24^{de} (0.21)	0.15^{de} (0.00)
	4	15	29.1^{d} (0.3)	5.20^{f} (0.26)	0.18^{cd} (0.01)	14.9^{g} (1.0)	3.34^{e} (0.27)	0.22^{a} (0.00)
	6	0	206.5^{b} (21.1)	16.64^{cd} (1.69)	0.08^{g} (0.00)	205.3^{c} (7.6)	17.40^{bc} (0.91)	0.08^{f} (0.00)
	6	5	192.8^{b} (3.0)	17.53^{cd} (0.15)	0.09^{g} (0.00)	198.7^{c} (5.5)	17.45^{bc} (0.22)	0.09^{f} (0.00)
	6	15	205.5^{b} (11.3)	21.18^{c} (0.40)	0.10^{g} (0.00)	174.4^{d} (4.2)	18.73^{b} (0.64)	0.11^{f} (0.00)
MS	4	0	54.5^{cd} (2.8)	11.56^{e} (0.66)	0.21^{b} (0.00)	54.9^{f} (1.6)	11.62^{cd} (0.41)	0.21^{ab} (0.01)
	4	5	80.7^{c} (2.2)	12.98^{de} (0.40)	0.16^{de} (0.00)	77.2^{e} (0.9)	11.59^{cd} (0.11)	0.15^{de} (0.00)
	4	15	62.1^{cd} (3.6)	15.01^{de} (1.16)	0.24^{a} (0.00)	53.0^{f} (3.0)	11.40^{cd} (0.41)	0.22^{ab} (0.00)
	6	0	300.5^{a} (4.5)	46.02^{a} (0.74)	0.15^{ef} (0.00)	236.0^{b} (3.1)	40.98^{a} (4.09)	0.17^{cd} (0.02)
	6	5	269.4^{a} (25.5)	36.35^{b} (2.43)	0.14^{f} (0.00)	283.0^{a} (8.9)	40.60^{a} (3.66)	0.14^{e} (0.01)
	6	15	207.5^{b} (11.8)	39.21^{b} (2.71)	0.19^{bc} (0.00)	211.3^{c} (11.0)	39.63^{a} (2.57)	0.19^{bc} (0.00)

NS, native starch; MS, modified starch.

Values in parentheses are the standard deviations.

For each column, means with different letters are significantly different ($P < 0.05$) according to the Tukey's multiple range test.

To understand the differences induced by the two different starches, the major events that occur during the different processes including sauce-making, cooling and heating must be analyzed.

When the sauces are heated during preparation, the crystallinity of the starch granules decreases, and the absorb water as starch molecules, particularly amylose, are released. After cooling to 4 °C, the starch molecules, particularly amylose and amylopectin, may partially crystallize (retrogradate). During heating, the starch molecules may become susceptible to retrogradation once more. Based on these events, starch retrogradation, particularly amylose retrogradation, is primarily responsible for the stability of starch during processing (Miles et al., 1985).

No changes were found in the viscoelastic properties of any of the sauces after increasing the temperature from 20 to 50 ºC. The thermal stability of the viscoelastic functions from 20 to 50 ºC was reported previously in fresh white sauces by Arocas et al. (2009a). During this study, we concluded that the presence of red pepper in the sauces did not affect the stability of the viscoelastic functions from 20 to 50 ºC.

3.2. Microstructural analysis

Figure 4 shows the micrographs obtained by light microscopy (LM) and confocal laser scanning microscopy (CLSM) of the sauces elaborated with different types of corn starch (native and modified) at different concentrations (4 and 6 g/100 g). The sauces formulated with native starch and stained with iodine (Figures 4A and 4B) presented a continuous, homogeneous and fluid blue phase. This phase was more consistent, thicker and more intensely blue when the starch concentration was higher (6 g/100 g). The dispersed phase was primarily composed of fat globules. The continuous phase was composed of disintegrated starch granules, particularly amylose, and milk components. When the sauce was stained with toluidine blue (Figures 4E and 4F), an intensely blue network could be observed; this network most likely formed from the milk proteins and the interactions between the milk proteins and the amylose from the starch. This network was thicker in the 6NS sauce than in the 4NS sauce, suggesting that the starch polymers form stable interactions with the milk proteins. Some isolated, gelatinized starch granules that have not been disintegrated and fat globules

could be observed as dispersed phases distributed throughout the sauce (Figures 4E and 4F). The NS sauces showed a complex matrix composed primarily of bright red protein and dark red polysaccharides when subjected to CLSM (Figures 4I and 4J). Most of the polysaccharides are the amylose and amylopectin that have been leached from the granules disintegrated during cooking. The fat globules were green, homogenously dispersed and associated with the protein phase. A similar fat distribution was observed by Auty et al. (2001) and Guardeño et al. (2011) in processed cheese and white sauces, respectively. The CLSM images of the native starch sauces confirmed that the continuous phase of the sauce made using a higher starch concentration (6NS) was thicker than that obtained in the 4NS sauce.

The sauces produced with modified starch (Figures 4C, 4D, 4G, and 4H) contained a phase composed of gelatinized starch granules that interacted with each other to form a tapestry. This tapestry of granules was thicker when the sauces were elaborated using a higher starch concentration (6MS). The dispersed phase and the holes left between the starch granules, fat globules and milk components could be observed, maintaining continuity and forming a network. The fat globules were associated with the protein phase of the sauce (Figures 4K and 4L), and in some areas, these fat globules appeared agglomerated. The sauces produced with modified starch (Figures 4K and 4L) seemed to exhibit greater aggregation among the fat globules than those with the native starch (Figures 4I and 4J). These fat globules tended to cluster in the holes left between the starch granules, promoting aggregation. The sauces with modified starch showed additional swollen starch granules compared to the native starch sauces and a protein matrix stabilizing the fat globules. This structure could be related to the cross-linked chemical modifications present in this type of starch, which reinforce the granular structure and might explain the higher granule swelling power of the MS before rupture relative to the NS sauces.

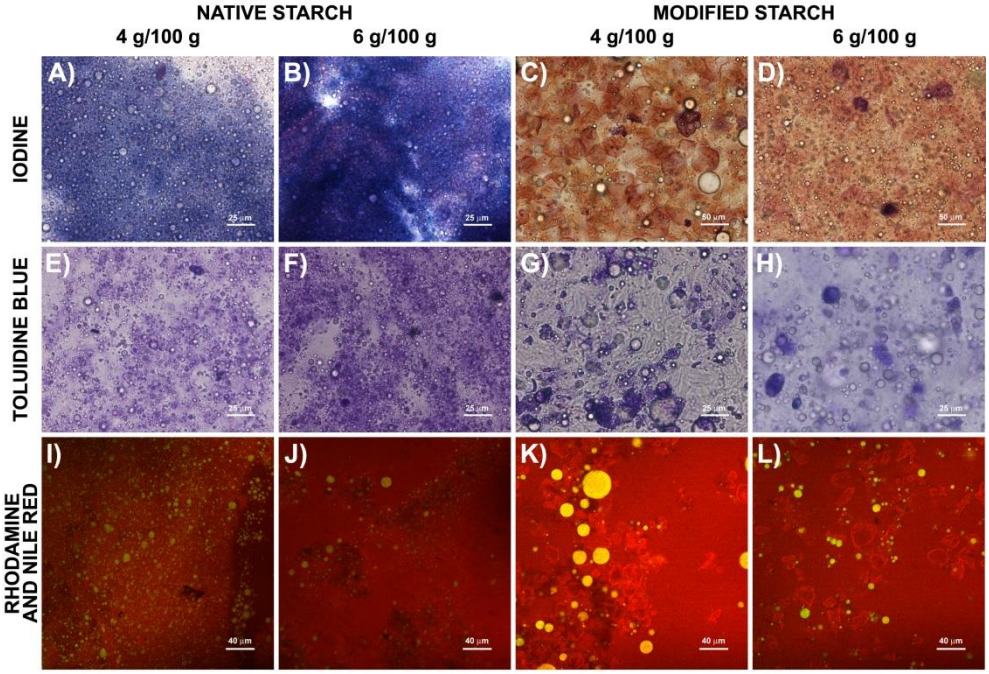

Figure 4. Light microscopy (A, B, C, D, E, F, G, H) and Confocal Laser Scanning Microscopy (I, J, K, L). White sauces without red pepper elaborated with native and modified starch. (A, B, C, D) staining with iodine solution; (E, F, G, H) staining with toluidine blue; (I, J, K, L) staining with Rhodamine B and Nile Red. Green: carotenoids and fat globules. Red: proteins and starch. Magnification: 40x (A, B, E, F, G, H, I, J, K, L), 20x (C, D).

When 5 g/100 g of red pepper were added to the sauces elaborated with native starch, the continuous phase, which had become homogeneous, became less fluid and more compact in appearance (Figures 5A and 5B) than those without red pepper did. When sauce containing 6 g/100 g of starch (6NS5RP), compaction of the sauce seemed to be higher. In sauces made with red pepper and native starch in general, a continuous phase stained blue by iodine (Figures 5A, 5B, 5I, and 5J) and violet by toluidine blue (Figures 5E, 5F, 5M, and 5N) could be observed. These phases were homogenous, but more aggregated and less fluid than those obtained in sauces made without red pepper. The dispersed phase consisted of fat globules, which seemed to occupy the space between the holes left by the components of the disintegrated starch granules.

When sauce was produced using 15 g/100 g of red pepper, the agglomerated milk protein distributed throughout the sauce seemed to interact with the starch components. Slices of the red pepper tissue were distributed throughout the sauce and seemed to overlap with the other two phases. No interactions were observed between plant tissue and the components of the continuous and dispersed phase (Figures 5E, 5F, 5M, and 5N). When a higher concentration of red pepper (15 g/100 g) was incorporated, the compaction of the continuous phase was evident. All of the components of the sauce seemed to compete for water, generating little fluid and a compact appearance sauce. The red pepper tissue appeared intact, and the cell walls (Figures 5E and 5F) and carotenoids responsible for the red colour of the pepper (Figures 5E, 5F, 5M, and 5N) could be observed. The red pepper sauces formulated with modified starch exhibited a different structure compared to the sauces produced with native starch. However, the sauces produce with and without red pepper were not appreciably different. When the sauces contained 5 g/100 g of red pepper, a tapestry consisting of gelatinized starch granules could be observed (Figures 5C, 5D, 5G, and 5H). This tapestry was thicker after the concentration of starch was increased. Fat globules and milk protein agglomerates could be observed in the holes left by the starch granules. The red pepper tissue did not interact with the other components of the sauce significantly. In sauces produced with 5 g/100 g and 15 g/100 g of red pepper, the red pepper tissue appeared intact (Figures 5G, 5H, 5O, and 5P). According to the rheological data, the sauce produced using 15 g/100 g of red pepper was more fluid compared to that without and with 5 g/100 g of red pepper. The decreased viscosity could be attributed to some interactions between the fibre from the red pepper and the glucose chains from the starch granules. Consequently, the fibre would occupy the holes left by the amylopectin chains when the granules are swollen.

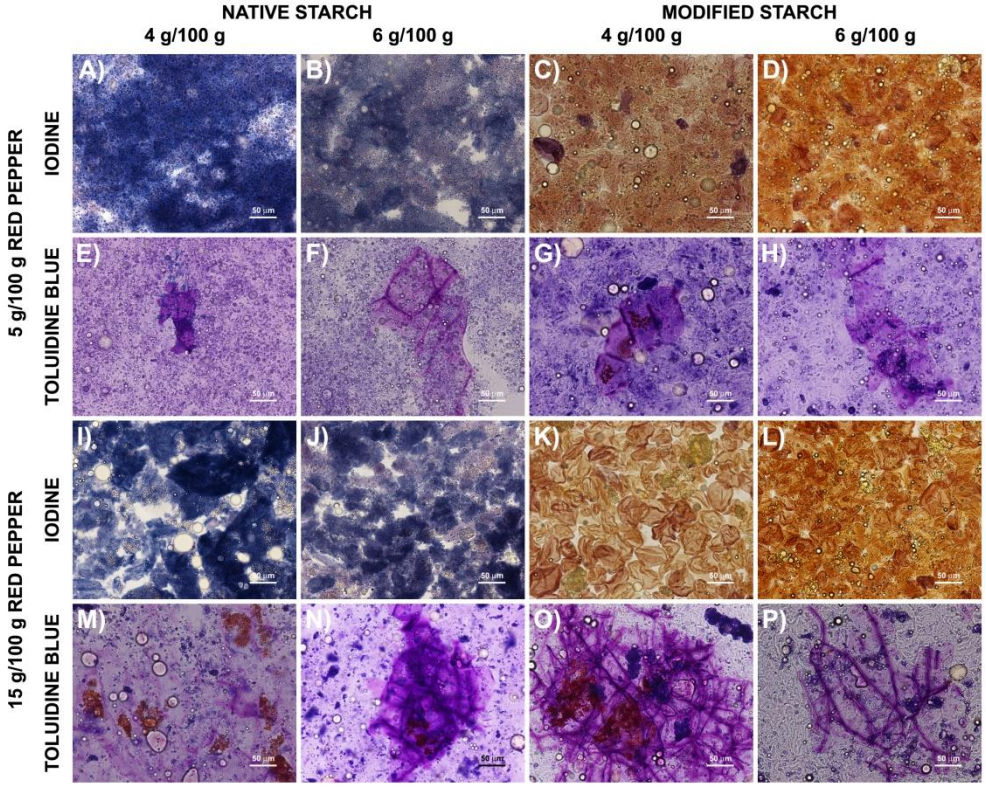

Figure 5. Light Microscopy. White sauces enriched with red pepper elaborated with native and modified starch. (A, B, C, D, I, J, K, L) staining with iodine solution; (E, F, G, H, M, N, O, P) staining with toluidine blue. Magnification: 20x.

Figure 6 shows the CLSM image of the functional sauces. All of the studied sauces exhibited a significant intrinsic auto-fluorescence (Figures 6A, 6B, 6C, 6D, 6I, 6J, 6K, and 6L) due to the presence of carotenoids. Similar results were obtained by Vázquez-Gutiérrez et al. (2011) and Hernández-Carrión et al. (2014) while studying the microstructure of persimmons and milk-based persimmon beverages; a high auto-fluorescence was observed due to the high carotenoid content of persimmons. In the pieces of plant tissue, the cell walls could be observed. The red pepper tissue survived the preparation process and appeared intact. In the NS sauces, the granules disintegrated during preparation (Figures 6E, 6F, 6M, and 6N), while in those elaborated with modified starch, the granules gelatinized and interacted with each

other (Figures 6G, 6H, 6O, and 6P). In the NS sauces, the fat globules were homogenously distributed between the components of the disintegrated starch granules. In the MS sauces, the fat globules were distributed between the gelatinized starch granules, and seemed to present a higher degree of coalescence compared to the NS sauces.

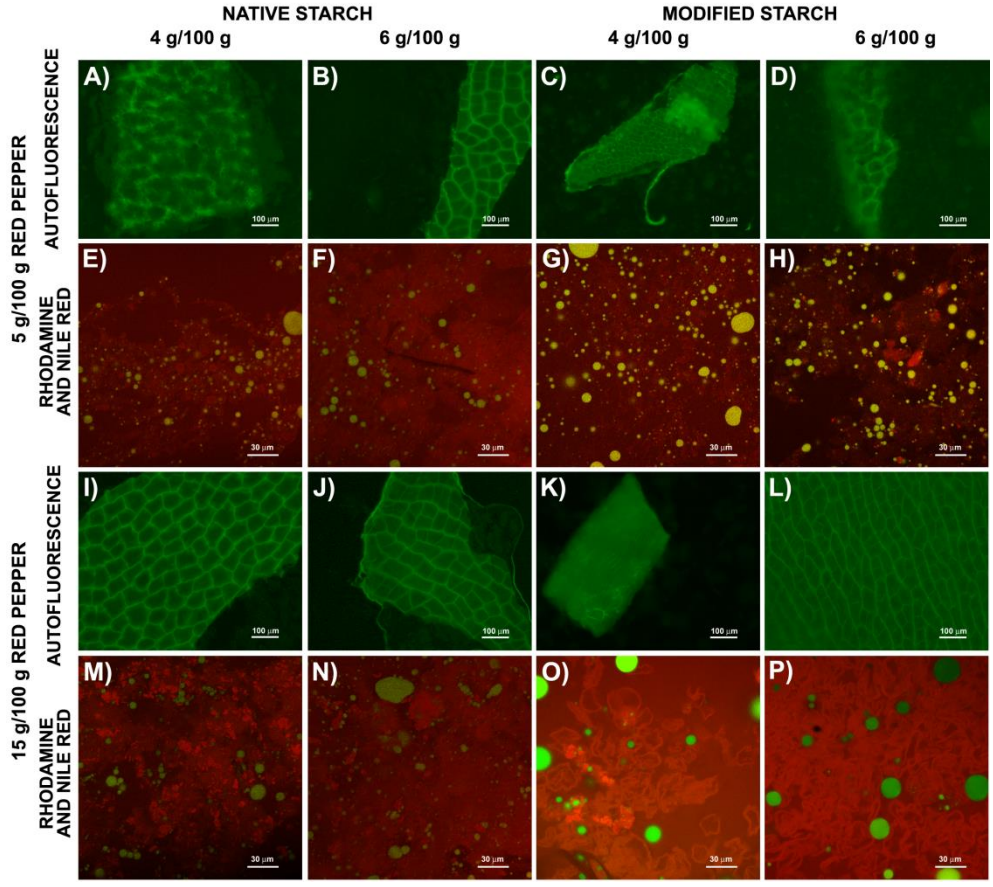

Figure 6. Light microscopy (A, B, C, D, I, J, K, L) and Confocal Laser Scanning Microscopy (E, F, G, H, M, N, O, P). White sauces enriched with red pepper elaborated with native and modified starch. (A, B, C, D, I, J, K, L) auto-fluorescence; (E, F, G, H, M, N, O, P) staining with Rhodamine B and Nile Red. Green: carotenoids and fat globules. Red: proteins and starch. Magnification: 10x (A, B, C, D, I, J, K, L), 60x (E, F, G, H, M, N, O, P).

3.3. Physical properties

3.3.1. Total soluble solids content (TSS) and pH

Table 4 shows the TSS and pH values of the different white sauces produced with and without red pepper. The statistical results revealed the triple interaction between starch type, starch concentration and red pepper concentration was statistically significant ($P < 0.05$) for the TSS values, while only the interaction between the starch type and red pepper concentration was statistically significant ($P < 0.05$) for the pH values. In sauces formulated with 4 g/100 g of modified starch (MS), the TSS content increased significantly ($P < 0.05$) when increasing the concentration of red pepper. Furthermore, the TSS content of sauces produced with higher starch content was significantly higher ($P < 0.05$) compared to the sauces formulated with 4 g/100 g of starch.

The pH values of the sauces elaborated without and with 5 g/100 g of red pepper were significantly higher ($P < 0.05$) than those obtained for the sauces prepared with 15 g/100 g of red pepper at two different concentrations of NS and MS. Therefore, adding 15 g/100 g of red pepper ($pH = 5.01 \pm 0.03$) significantly decreased ($P < 0.05$) the pH of the sauce. This decreased pH might be related to the acids in the red pepper, such as ascorbic acid.

3.3.2. Syneresis

Water mobility is critical when determining the quality of starch-containing systems. A common phenomenon observed in sauces containing starch was the release of water or syneresis, which is primarily related to amylose retrogradation (Arocas et al., 2009b). In white sauces, syneresis is a negative factor in terms of sensory quality and consumer acceptability. The statistical results showed that the triple interaction between the starch type, starch concentration and red pepper concentration was statistically significant ($P < 0.05$) for syneresis. After 15 days of storage at 4 ºC, syneresis was only observed in the NS sauces (Table 4), which was attributed to the high retrogradation suffered by this native starch after cold storage.

Table 4. Effect of starch type, concentration of starch, concentration of red pepper and the interaction starch type-[starch]-[red pepper] in the physical properties of the white sauces.

Type of starch	Starch content (g/100g)	Red pepper content (g/100g)	TSS	pH	% Syneresis	ΔE*
NS	4	0	8.03^{f} (0.23)	6.70^{a} (0.01)	3.71^{b} (1.09)	2.72^{cd} (0.40)
	4	5	8.37^{ef} (0.06)	6.57^{a} (0.05)	27.21^{a} (6.87)	1.73^{cd} (0.42)
	4	15	8.63^{def} (0.06)	6.20^{c} (0.20)	1.77^{b} (0.69)	3.46^{c} (1.16)
	6	0	10.27^{bcd} (0.31)	6.70^{a} (0.05)	0.00^{b} (0.00)	1.95^{cd} (0.40)
	6	5	10.43^{bc} (0.25)	6.51^{ab} (0.01)	3.49^{b} (0.55)	2.25^{cd} (0.32)
	6	15	10.07^{cde} (0.06)	6.29^{c} (0.04)	4.38^{b} (0.87)	9.04^{a} (0.14)
MS	4	0	5.50^{g} (0.30)	6.52^{ab} (0.03)	0.00^{b} (0.00)	1.28^{d} (0.28)
	4	5	9.40^{cdef} (0.30)	6.56^{a} (0.05)	0.00^{b} (0.00)	2.73^{cd} (0.35)
	4	15	10.60^{bc} (1.31)	6.34^{bc} (0.04)	0.00^{b} (0.00)	3.25^{c} (0.30)
	6	0	12.43^{a} (0.40)	6.61^{a} (0.01)	0.00^{b} (0.00)	1.37^{d} (0.40)
	6	5	13.20^{a} (0.35)	6.52^{ab} (0.10)	0.00^{b} (0.00)	2.83^{cd} (0.31)
	6	15	11.93^{ab} (1.42)	6.28^{c} (0.05)	0.00^{b} (0.00)	6.39^{b} (1.51)

TSS, total soluble solids; NS, native starch; MS, modified starch.

Values in parentheses are the standard deviations.

For each column, means with different letters are significantly different ($P < 0.05$) according to the Tukey's multiple range test.

The absence of syneresis after 15 days of storage at 4 ºC in sauces produced with MS (Table 4) is attributed to the low amylose content of the starch and to its chemical substitution, which prevents retrogradation. Moreover, cross-linking and substituting groups within the starch exerted an extra protective effect against retrogradation because these modifications prevented the leaching of starch polymers from the granules (Guardeño et al., 2012). Mason (2009) stated that acetylation provided stability at high temperatures, although less so than hydroxypropyl substitution because the hydroxypropyl groups appeared to create more steric hindrance. These results are consistent with those observed by Quiles et al. (2012) and Guardeño et al. (2012; 2013) in their studies of white sauces prepared with different types of starch, which concluded that syneresis did not occur when using modified starches.

3.3.3. Colour measurements

Finally, Table 4 shows the total colour differences (ΔE*) between the different white sauces after 15 days of storage at 4 ºC compared to the freshly made samples. The statistical data showed that the triple interaction between the starch type, starch concentration and red pepper concentration was statistically significant ($P < 0.05$) during the colour measurements. The ΔE* values ranged from 1.28 to 9.04; the 6NS15RP sauce exhibited the highest ΔE* value ($P < 0.05$), and the 4MS sauce exhibited the lowest ΔE* value ($P < 0.05$). The differences in ΔE* between sauces produced without and with 5 g/100 g of red pepper were not significant ($P > 0.05$), and their values were below 3, which was not considered visible to the human eye (Francis & Clydesdale, 1975). These results were consistent to those observed by Guardeño et al. (2012; 2013) when studying ΔE* in different white sauces on different days during storage; in these cases, the ΔE* values were below 3. The higher the red pepper content, the higher ΔE* values are, indicating that red pepper makes sauce less stable during storage. Sauces produced with 15 g/100 g of red pepper exhibited ΔE* values above 3, which was considered obvious to the human eye (Francis & Clydesdale, 1975).

These ΔE* values were significantly higher ($P < 0.05$) when the sauces were produced with starch concentrations above 4 g/100 g. Moreover, the white sauces produced with MS had lower ΔE* values than the NS sauces, meaning that the MS sauces were more stable toward storage than NS sauces.

3.4. Sensory analysis

3.4.1. CATA question

Although most of the CATA terms were not significantly different between samples (Table 5), a correspondence analysis was performed to analyze the trends in the consumer responses (Ares et al., 2014). Figure 7 shows the symmetric plot generated for the new concept of functional white sauce with red pepper with an additional variable "overall liking" superimposed. The first two factors of the attribute map accounted for 95.56% of the variance of the original dataset (90.42% and 5.14%, respectively) indicating that the X axis accounts for the major differences in consumer perception. Most of the attributes were well represented in the perceptual space defined by the first two factors of the FCA. The first factor (X axis) was related to the opposing perceptions (*I would buy/I would not buy*). *I prefer white sauces without aggregates* located closer to *I would not buy it*, on the right of the axis, seemed to be the major reason for this differentiation indicating that consumers preferred traditional white sauce. *Red pepper provides calories,* (probably associated with the sauces in general), was also mentioned as a driver for rejecting sauces. On the other hand, close to *I would buy it*, on the left of the axis, were located most of the claimed functional attributes of the white sauces, such as *red pepper improves the taste and beneficial for health.* The most positively perceived attributes were primarily correlated to the left part of the first factor of the FCA. Those characteristics are primarily associated with MS sauces.

Table 5. Frequency of selection of CATA terms and Cochran's Q test for significant differences between four white sauces with enhanced functionality.

CATA Terms	4NS15RP	6NS15RP	4MS15RP	6MS15RP	Q
Provides fibre	29	33	36	34	0.205
Provides antioxidants	54	55	61	63	0.115
Provides calories	13	19	15	14	0.224
Provides calcium	3	5	5	5	0.651
Improves the taste	51	45	61	59	**0.012**
Provides nutritional value	62	52	68	60	**0.029**
Beneficial for health	45	39	48	47	0.104
Prevents illnesses	23	19	26	23	0.055
Prevents ageing	22	23	19	25	0.353
I would buy it	44	27	59	63	**<0.0001**
I would not buy it	35	49	19	18	**<0.0001**
I prefer white sauces without aggregates	27	34	29	22	**0.036**

NS, native starch; MS, modified starch; RP, red pepper.

Highlighted terms correspond to those for which significant differences between samples were identified according to Cochran's Q test ($P < 0.05$).

The Chi squared test ($P = 0.022$) indicates statistically significant differences between the descriptions of the samples which means that the samples were not all equal perceived. Cochran's Q test was used to identify the significant differences between samples for each of the terms included on the CATA question. The results (Table 5) showed that no statistically significant differences ($P > 0.05$) were found between the samples for most of the analyzed attributes. However it worth to note that all attributes had a large number of mentions indicating that all samples were considered positively formulated since the consumer trusted in the messages. The white sauces prepared with MS (4MS15RP and 6MS15RP) were significantly perceived ($P < 0.05$) by the consumers as *provides more nutritional value* and significantly ($P < 0.05$) *improves the taste* compared to the NS sauces. Figure 7 shows that the white sauces were clearly separated into three groups according to their functionality perception by consumers. The white sauces prepared with MS (4MS15RP and 6MS15RP) were related to *red pepper provides antioxidants*, *beneficial for health, prevents illnesses*, *red pepper improves the taste, red pepper provides nutritional value* and *I would buy it* by the consumers. The white sauces

formulated with higher NS content (6NS15RP) were related to the following attributes: *prevents ageing, red pepper provides calories, I would not buy it* and *I prefer white sauces without aggregates*. According to Table 5, MS sauces were significantly ($P < 0.05$) associated with *I would buy it* by consumers, while the NS sauces were significantly ($P < 0.05$) related to *I would not buy it* and *I prefer white sauces without aggregates*. These results are consistent with the rheological results, where the NS sauces were more viscous and less creamy than that prepared with MS. Concurrently, this area of the perceptual space was associated with the overall liking (superimposed as supplementary variable) and to the CATA parameter: *I would buy it*. Therefore, the new taste, the different for the flavour of traditional white sauces, and a high antioxidant content would drive the liking and the purchase intent in these categories, fitting the intended concept of a functional product very well.

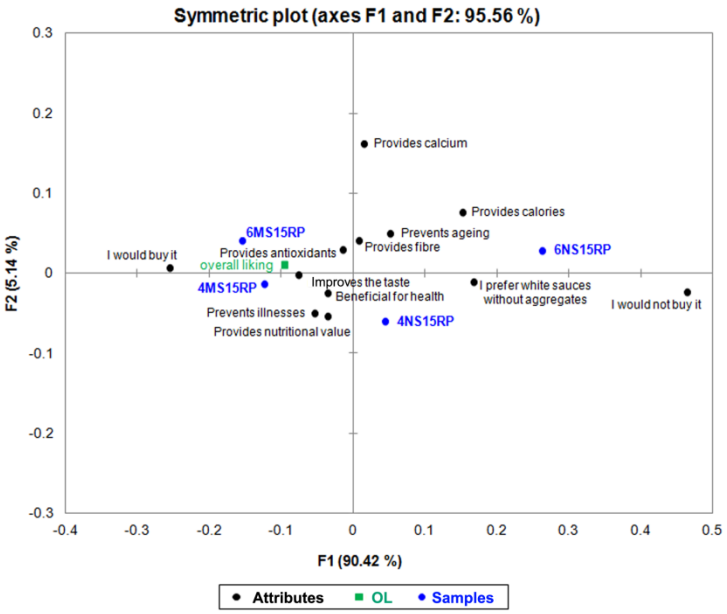

Figure 7. Symmetric plot. Representation of the terms from the CATA question with the additional variable "overall liking" superimposed and the four white sauces enriched with red pepper, in the first two dimensions of the Factorial Correspondence Analysis (FCA) of the CATA counts. NS, native starch; MS, modified starch; RP, red pepper; 4 and 6, 4 and 6 g/100 g of starch, respectively; 15, 15 g/100 g of red pepper.

3.4.2. Liking test

An analysis of variance (ANOVA) was conducted to determine the consumer acceptability of the white sauces enriched with the highest red pepper content. The mean scores for the overall liking and the liking of appearance, flavour and consistency are shown in Table 6. The overall liking (OL) scores were between 6.7 and 4.8 for the four white sauces. The liking of the specific modalities (appearance, flavour, texture) agreed with the OL. The white sauces prepared with MS (4MS15RP and 6MS15RP) attained the significantly highest ($P < 0.05$) scoring for the overall liking, appearance, flavour and consistency liking, with no significant differences ($P > 0.05$), indicating that these sauces were the most acceptable for consumers. The white sauces prepared with NS and the highest concentration of starch (6NS15RP) had the significantly lowest ($P < 0.05$) overall liking, appearance, flavour and consistency scores, indicating that this white sauce was the least acceptable for consumers based on these modalities. The white sauces prepared with NS and the lowest concentration of starch (4NS15RP) obtained intermediate scores between the MS sauces and the 6NS15RP sauce for overall liking, appearance, flavour and consistency liking, appearance and consistency liking. The 6NS15RP sauce was outperformed by the 4NS15RP sauce most likely because the latter was more consistent due to its higher starch content. The consumers rejected white sauces they related to statements such as *red pepper provides calories,* and *I prefer white sauces without aggregates* (6NS15RP). Considering the frequency of the selection of the first two attributes (Table 5), the consumers checked *red pepper provides calories* and *prevents ageing* for most of the samples; therefore, these attributes did not drive their dislike. Most likely, *I prefer white sauces without aggregates* and the poor consistency are the attributes that drove the dislike of the 6NS15RP sauce.

Table 6. Scores for overall liking, appearance liking, flavour liking, and texture liking for white sauces with enhanced functionality.

	Overall liking	Appearance liking	Flavour liking	Texture/consistency liking
4NS15RP	5.5[a] (1.9)	5.0[a] (2.1)	5.9[a] (2.2)	4.4[a] (2.2)
6NS15RP	4.8[b] (2.1)	3.7[b] (2.0)	5.4[b] (2.0)	3.8[b] (2.3)
4MS15RP	6.6[c] (1.6)	6.6[c] (1.9)	6.5[c] (1.9)	6.5[c] (2.0)
6MS15RP	6.7[c] (1.7)	6.7[c] (1.7)	6.8[c] (1.8)	6.7[c] (2.1)

NS, native starch; MS, modified starch; RP, red pepper.

Values in parentheses are the standard deviation.

For each column, means with different letters are significantly different ($P < 0.05$) according to the Fisher's test.

4. Conclusions

A novel, functional white sauce added with red pepper purée was proposed. The studied sauces exhibited considerable intrinsic auto-fluorescence due to the presence of carotenoids from the red pepper that seemed to be preserved during the preparation process. The sauces prepared with modified starch that were creamier and more consistent were the most liked; consumers found them beneficial for health because red pepper provides antioxidants and nutritional value and improves the sauces' taste. The sauces with 6 g/100 g of native starch that were lighter in texture were scored the lowest in terms of overall liking.

These results suggest that novel, functional creamy white sauces with high nutritional value, high acceptability, good rheological properties and stability could be formulated using red pepper. Adding red pepper to traditional products, such as white sauces, is an augmentation that provides antioxidants and fibre, and consumer are confident on this new concept. This study opens the door to other augmentations with plant tissues that would help to achieve a healthier diet. Therefore, the normally "reviled" sauces might become high-added-value products.

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Resumen de resultados

El caqui 'Rojo Brillante' es una variedad de **caqui** astringente con alto contenido en compuestos bioactivos tales como taninos solubles, carotenoides, ácidos fenólicos y fibra dietética. A través de diversas técnicas microscópicas (microscopía óptica, LM; microscopía electrónica de transmisión, TEM y microscopía electrónica de barrido a bajas temperaturas, CryoSEM) y de la cuantificación de ciertos compuestos bioactivos y propiedades fisicoquímicas (carotenoides, contenido en sólidos solubles totales, actividad antioxidante, fibra diétetica, color y textura) se ha logrado investigar el efecto de un tratamiento de altas presiones hidrostáticas (APH) seleccionado a partir de investigaciones previas (200 MPa/6 min/25 ºC) y de pasteurización (70 ºC/15 min) sobre la estructura de dicho fruto así como la extractabilidad de ciertos compuestos bioactivos. Los resultados mostraron que ambos tratamientos causaron cambios estructurales en el tejido parenquimático del **caqui**. El **caqui** sometido a APH presentó una estructura más compacta, con poco aire en su interior y espacios intercelulares llenos de material celular, indicativo de un elevado movimiento de solutos a través del tejido. Por otro lado, el tejido de **caqui** pasteurizado presentó células más pequeñas, paredes celulares más deformadas y espacios intercelulares mayoritariamente vacíos. Estos cambios microestructurales podrían ser los responsables de las modificaciones observadas en la extractabilidad de compuestos bioactivos del **caqui**. Ambos tratamientos favorecieron la precipitación de taninos y la formación de células tánicas, lo que podría relacionarse con la pérdida de astringencia del fruto. El procesamiento por APH mejoró la extractabilidad de compuestos carotenoides y mantuvo las propiedades antioxidantes del fruto. Por tanto, el **caqui** tratado por APH podría ser empleado en la formulación de nuevos alimentos funcionales debido a su alto valor nutritivo. Asimismo, el tratamiento por APH podría ser una alternativa ventajosa al tratamiento de pasteurización convencional.

A partir de estos resultados se formularon bebidas lácteas haciendo uso de **caqui**: no tratado, sometido a APH y pasteurizado; y tres matrices lácteas diferentes: leche entera, semi-desnatada y desnatada. Se estudió la microestructura y comportamiento reológico de estas nuevas bebidas lácteas. El estudio de la microestructura mediante la técnica de microscopía láser confocal de barrido (CLSM) puso de manifiesto la integridad estructural de los carotenoides. Esta evidencia junto con la cuantificación del contenido en carotenoides, que había mostrado que el tratamiento por APH favorece su extractabilidad aumentando su difusión a la matriz láctea, indicaron que

estas nuevas bebidas lácteas mantienen las propiedades funcionales del **caqui**. El tratamiento por APH permitió formular bebidas lácteas con un alto contenido en carotenoides haciendo uso de menores cantidades de **caqui** que cuando se usó **caqui** pasteurizado o no tratado. Asimismo, las bebidas elaboradas con **caqui** sometido a APH mostraron unas adecuadas propiedades reológicas ya que no gelificaron como las elaboradas con **caqui** no tratado, ni sedimentaron como las formuladas con **caqui** pasteurizado.

Por último, se estudiaron las características sensoriales y aceptabilidad de las bebidas lácteas elaboradas con **caqui**. Se estudiaron seis bebidas lácteas con **caqui** diferentes variando el tipo de leche (entera y desnatada) y el tratamiento del **caqui** (no tratado, APH y pasteurizado). Los resultados mostraron que los consumidores percibieron las bebidas lácteas con **caqui** como una bebida altamente antioxidante. La muestra de consumidores entrevistados constituyó un grupo en general interesados por la salud, que también presentaron una actitud positiva y perceptiva ante el concepto de que consumir ciertos alimentos puede tener un efecto positivo en la salud. Las bebidas lácteas elaboradas con **caqui** tratado por APH independientemente del tipo de leche utilizada y las elaboradas con **caqui** no tratado y leche entera fueron las favoritas de los consumidores. Los beneficios nutricionales que supone la ingesta de **caqui**, las ventajas a nivel tecnológico y organoléptico que supone la aplicación de APH para la prolongación de la vida útil de los alimentos, unidos a la elevada aceptabilidad por parte de los consumidores de las bebidas lácteas elaboradas con **caqui** tratado por APH hacen de este tratamiento una opción idónea para la formulación de nuevos alimentos lácteos. Por tanto, el tratamiento por APH hace posible la formulación de bebidas lácteas con **caqui** con alto valor nutricional, variable contenido graso y elevada aceptabilidad independientemente de la estacionalidad del fruto.

En cuanto al **pimiento**, el primer objetivo se centró en la localización y cuantificación del contenido en algunos compuestos bioactivos (carotenoides, contenido en fenoles solubles, capacidad antioxidante y fibra dietética) y propiedades fisicoquímicas (sólidos solubles totales, pH y textura) de tres tipos diferentes de **pimiento**: rojo, verde y amarillo. Para la localización de los compuestos bioactivos se utilizaron diferentes técnicas microscópicas como microscopía electrónica de barrido a bajas temperaturas (CryoSEM) y microscopía óptica (LM). Los resultados mostraron que el contenido en

compuestos bioactivos de cada tipo de **pimiento** estuvo condicionado por su estructura. El grado de compactación y estructuración de la pared celular estuvo indirectamente relacionado con el transporte de solutos a nivel celular y directamente relacionado con el contenido en fibra dietética. Las paredes celulares más lábiles y el mayor transporte de solutos se observó en el **pimiento amarillo** mientras que el **pimiento verde** presentó las paredes celulares más compactas y con un alto grado de integridad estructural. El tejido parenquimático de **pimiento rojo** presentó paredes celulares más compactas que el **pimiento amarillo** y menos que el **verde**, con un elevado contenido en pigmentos carotenoides dispuestos principalmente en forma de racimos a través de todo el tejido celular. Los tres tipos de **pimiento** presentaron formación y acumulación de agregados fenólicos y una activa circulación de solutos. Para obtener un extracto rico en compuestos carotenoides, el **pimiento rojo** sería el material vegetal más adecuado. Por otro lado, el **pimiento amarillo** sería apropiado para obtener extractos ricos en compuestos fenólicos con elevada actividad antioxidante, mientras que el **pimiento verde** sería adecuado si lo que pretendemos es obtener extractos con elevado contenido en fibra dietética.

A partir de este punto, se plantea la posibilidad de si modificando la estructura del tejido de **pimiento** mediante la aplicación de diferentes tratamientos de conservación se podría aumentar la extractabilidad de ciertos compuestos bioactivos tales como compuestos fenólicos y carotenoides. Es por ello que el siguiente objetivo se centró en la investigación del efecto de diferentes tratamientos de APH (100, 200, 300 y 500 MPa durante 15 min a 25 ºC) y de un tratamiento de pasteurización convencional (70 ºC/10 min) sobre la microestructura, contenido en compuestos bioactivos y textura de **pimiento rojo** con el objetivo de descubrir la relación entre el tratamiento de conservación, la microestructura del **pimiento** y la extractabilidad de compuestos bioactivos. La elección de **pimiento rojo** se basó en el elevado contenido en compuestos carotenoides que éste presenta. Los resultados mostraron que todos los tratamientos de conservación estudiados, APH y pasteurización, causaron modificaciones microestructurales en el tejido de **pimiento rojo**. Sin embargo, los tratamientos de APH a 500 MPa y la pasteurización fueron los que menos impacto tuvieron. Estos mismos tratamientos fueron a su vez los que menos afectaron al contenido en compuestos bioactivos (fibra y actividad antioxidante) y la textura del **pimiento rojo**. El tratamiento de APH a 500 MPa podría ser, por tanto, una

alternativa a la pasteurización, tratamiento tradicional de conservación de **pimiento rojo**, ya que tanto las propiedades texturales como el contenido en compuestos bioactivos (fibra, carotenoides y actividad antioxidante) fue similar en ambos casos. En consecuencia, podrían desarrollarse nuevos alimentos funcionales mediante el uso de tejido de **pimiento rojo** sometido a APH a 500 MPa y/o pasteurización.

A continuación se evaluó el efecto de los tratamientos de conservación anteriormente descritos, APH y pasteurización, sobre la microestructura de **pimiento rojo** mediante el uso de herramientas de análisis de imagen y se determinaron aquellos parámetros que permiten caracterizar los cambios observados en la estructura utilizando para ello diferentes aumentos (100x, 200x y 350x). Tal y como se ha descrito anteriormente, todos los tratamientos estudiados provocaron modificaciones en la microestructura del **pimiento rojo**, pero fueron los tratamientos de APH a 500 MPa y la pasteurización los que provocaron un menor impacto. Las modificaciones microestructurales causadas por la aplicación de APH y pasteurización provocaron variaciones en el área, perímetro, circularidad y diámetro de Feret de las células de **pimiento rojo**. Asimismo, provocaron cambios en la distribución del tamaño de célula y modificaciones en los parámetros de textura de la imagen. De los parámetros de textura estudiados, la dimensión fractal de textura, el contraste, el momento de diferencia inversa y la entropía fueron los más relevantes para caracterizar el efecto de las APH y la pasteurización sobre la textura de **pimiento rojo**. Por otro lado, el aumento al cual se evalúa la textura de la imagen es un parámetro a considerar ya que el daño celular causado por los tratamientos de conservación se observa mejor a escalas bajas. Dado que existe una relación entre el daño estructural provocado sobre un tejido vegetal como consecuencia del tratamiento de conservación y su funcionalidad, estos resultados sugieren la pertinencia del uso del análisis de imagen como técnica no invasiva y cuantitativa para evaluar la funcionalidad biológica de productos sometidos a APH.

Por último, ya que el **pimiento rojo** supone una fuente importante de compuestos antioxidantes como fibra y carotenoides, el trabajo se centró en el desarrollo de productos innovadores, como salsas bechamel enriquecidas con **pimiento rojo**. Para ello, se emplearon dos tipos de almidón de maíz (nativo y modificado) a dos concentraciones diferentes (4 y 6 g/100 g) y diferentes cantidades de **pimiento rojo**

(0, 5 y 15 g/100 g). Se estudió el comportamiento reológico, la microestructura, sinéresis, características sensoriales y aceptabilidad por parte de los consumidores de estas salsas. Los resultados reológicos mostraron que todas las salsas bechamel estudiadas presentaron un comportamiento pseudoplástico y un espectro mecánico típico de geles débiles con valores de G' superiores a G". El efecto de la incorporación de **pimiento** sobre las propiedades reológicas dependió del tipo de almidón utilizado. La microestructura de las salsas elaboradas con almidón nativo (AN) reveló la presencia de una compleja matriz compuesta por proteína, y amilosa y amilopectina lixiviadas de los gránulos de almidón desintegrados durante la elaboración de la salsa. Los glóbulos de grasa aparecieron homogéneamente dispersados y asociados con la fase proteica. Las salsas elaboradas con almidón modificado (AM) mostraron gránulos de almidón más hinchados y una fase proteica estabilizando dichos glóbulos. Las salsas presentaron una considerable auto-fluorescencia intrínseca debido al contenido de carotenoides del **pimiento rojo**. Tras 15 días de almacenamiento a 4 ºC, la presencia de sinéresis únicamente fue observada en las salsas elaboradas con AN debido a la elevada retrogradación sufrida por este tipo de almidón tras el almacenamiento en refrigeración. Las salsas preparadas con AM, más cremosas y consistentes, fueron las que más gustaron a los consumidores; éstos las encontraron beneficiosas para la salud ya que el **pimiento rojo** proporciona antioxidantes y valor nutricional y mejora el sabor de la salsa. Las salsas elaboradas con 6 g/100 g de AN, más ligeras en términos de textura, fueron las peor valoradas en términos de aceptabilidad global. Los resultados sugieren que podrían formularse nuevas salsas bechamel, funcionales, cremosas, con alto valor nutricional, elevada aceptabilidad, buenas propiedades reológicas y estabilidad utilizando **pimiento rojo** y almidón modificado.

Conclusions

The main conclusions drawn from this thesis are:

- High hydrostatic pressure (HHP) and pasteurization treatments cause structural changes in the parenchymal tissue and modifications in the bioactive compounds content of **persimmon**. Both treatments lead to a fall in the total soluble tannin content that could be related to the loss of astringency of **persimmon**. Nevertheless, HHP processing improves the extraction of carotenoids and keeps the antioxidant properties of the fruit.

- HHP treatment can be an alternative to pasteurization in order to preserve and extend the shelf life of **persimmon** and allows the **persimmon** to be consumed throughout the year despite its seasonality. Moreover, a high nutritional value ingredient is obtained to be used when formulating new functional foods as milk-based beverages, so it is possible to widen the forms of consumption of **persimmon**.

- The milk-based beverages prepared with the HHP-treated **persimmon** have high carotenoid content using smaller quantities of **persimmon**, and present the best rheological properties because unlike the untreated and pasteurized **persimmon** milk-based beverages, they do not form a gel-like structure or separate out.

- Consumers perceive **persimmon** beverages as high antioxidant foods. The beverages with HHP-treated **persimmon**, regardless the type of milk, and the ones enriched with untreated **persimmon** and whole milk are the consumers' favourites.

- The degree of compaction and structuring of the cell wall of the **sweet pepper** is found to be indirectly related to solute transport at the cellular level and directly related to total dietary fibre content. The content of bioactive compounds of each type of **sweet pepper** is conditioned by their structure. **Red peppers** can be suitable for obtaining extracts rich in carotenoid compounds. **Yellow pepper** can be appropriate for obtaining extracts rich in phenolic compounds with a high antioxidant activity and **green peppers** for extracts with high dietary fibre content.

- HHP and pasteurization treatments cause structural modifications in **red sweet pepper** tissues, but HHP at 500 MPa and pasteurization are the treatments that have the least impact on the microstructure, bioactive compound content and texture. So, HHP treatment at 500 MPa can be an alternative to pasteurization, for **sweet pepper** preservation. Owing to their high bioactive compound content, new functional foods can be developed using **red sweet pepper** tissues treated with HHP at high pressures or pasteurization.

- Structural modifications in the **red sweet pepper** tissues caused by HHP and pasteurization lead to variations in the morphometric and texture image parameters. Fractal dimension texture, contrast, inverse difference moment and entropy are texture parameters that are appropriate for characterizing the effect of HHP and pasteurization on **red sweet pepper** texture. The magnification at which the texture is evaluated is a parameter to consider given that the cellular damage is best observed at low magnifications.

- All the white sauces show pseudoplastic behaviour and characteristic spectra of soft gels with G' values above G". The effect of incorporating **red sweet pepper** on the rheological properties depends upon the type of starch used.

- The white sauces enriched with **red sweet pepper** exhibit considerable intrinsic auto-fluorescence due to the presence of carotenoids from the **red sweet pepper** that seem to be preserved during the preparation process.

- The sauces prepared with modified starch, which are creamier and more consistent, are the most liked. Consumers find them beneficial for health because **red sweet pepper** provides antioxidants and nutritional value and improves the sauces' taste. Therefore, novel, functional creamy white sauces with high nutritional value, high acceptability, good rheological properties and stability can be formulated using **red sweet pepper**.