Potencial Tecnológico y Digestibilidad *in vitro* de Diferentes Extractos Ricos en Fibra Procedentes de Coproductos del Caqui (*Diospyros kaki*). Aplicación a Alimentos

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Que la Tesis Doctoral Titulada "**Potencial tecnológico y digestibilidad in vitro de diferentes extractos ricos en fibra procedentes de coproductos del caqui (*Diospyros kaki*). Aplicación a Alimentos**" de la que es autora la graduada en Ciencia y Tecnología de Alimentos, Dña. Raquel Lucas González, ha sido realizada bajo nuestra dirección y autorizamos a que sea presentada para optar a la obtención del grado de Doctor por la Universidad Miguel Hernández.

Y para que conste a los efectos oportunos se firma el presente certificado en Orihuela a 20 de marzo de 2021.

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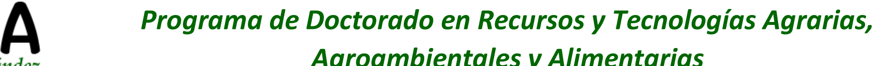

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"Hasta que nos comprometemos
hay vacilación,
la posibilidad de retroceder,
inefectividad.

En lo concerniente a todos los actos
de iniciativa (y creación)
hay una verdad elemental
cuya ignorancia mata incontables ideas
y espléndidos planes: Que en el momento
en que nos comprometemos definitivamente,
la Providencia da el paso también.
Todo tipo de cosas ocurren para ayudarnos
que de otra manera nunca hubieran ocurrido.

Una corriente de eventos surgidos de la decisión
genera a nuestro favor
toda clase de incidentes y encuentros imprevistos
y asistencia material
que ningún hombre podría haber soñado jamás
que vendría en su ayuda.

Aquello que puedes hacer
o sueñas que puedes hacer,
comiénzalo.

La audacia tiene genio, poder y magia"

Johann Wolfgang von Goethe

" *Nuestras convicciones más arraigadas,*
más indubitables, son las más sospechosas.
Ellas constituyen nuestros límites,
nuestros confines, nuestra prisión"

José Ortega y Gasset

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Prólogo

Esta Tesis Doctoral se presenta bajo la modalidad de ***Tesis por compendio de 8 publicaciones*** (5Q1, 2Q2 y 1Q3).

Publicación 1

Evaluation of particle size influence on proximate composition, physicochemical, techno-functional and physio-functional properties of flours obtained from persimmon (*Diospyros kaki Thunb.*) coproducts

Autores: Raquel Lucas-González, Manuel Viuda-Martos, José Ángel Pérez-Álvarez, Juana Fernández-López

Revista	Plants Foods for Human Nutrition, 72, 67-73
DOI	10.1007/s11130-016-0592-z
Editorial	Springer
eISSN	15773-9104
Categoría de JCR	Food Science & Technology
Cuartil	Q2
Rango	39/132
Factor de Impacto (2017)	2,465
Año de publicación	2017

Publicación 2

Effect of particle size on phytochemical composition and antioxidant properties of two persimmon flours from *Diospyros kaki* Thunb. vars. 'Rojo Brillante' and 'Triumph' co-products

Autores: Raquel Lucas-González, Juana Fernández-López, José Ángel Pérez-Álvarez, Manuel Viuda-Martos

Revista	Journal of the Science of Food & Agriculture, 98, 504-510
DOI	10.1002/jsfa.8487
Editorial	Willey
eISSN	1097-0010
Categoría de JCR	Agriculture (Multidisciplinary)
Cuartil	Q1
Rango	9/57
Factor de Impacto (2018)	2,422
Año de publicación	2018

Publicación 3

In vitro digestion models suitable for foods: Opportunities for new fields of application and challenges

Autores: Raquel Lucas-González, Manuel Viuda-Martos, José Ángel Pérez-Álvarez, Juana Fernández-López

Revista	Food Research International, 107, 423-436
DOI	10.1016/j.foodres.2018.02.055
Editorial	Elsevier
eISSN	0963-9969
Categoría de JCR	Food Science & Technology
Cuartil	Q1
Rango	27/197
Factor de Impacto (2018)	3,579
Año de publicación	2018

Publicación 4

Changes in bioaccessibility, polyphenol profile and antioxidant potential of flours obtained from persimmon fruit (_Diospyros kaki_) co-products during _in vitro_ gastrointestinal digestion

Autores: Raquel Lucas-González, Manuel Viuda-Martos, José Ángel Pérez-Álvarez, Juana Fernández-López

Revista	Food Chemistry, 1, 252-258
DOI	10.1016/j.foodchem.2018.02.128
Editorial	Elsevier
eISSN	0963-9969
Categoría de JCR	Food Science & Technology
Cuartil	Q1
Rango	7/197
Factor de Impacto (2018)	5,399
Año de publicación	2018

Publicación 5

Persimmon (*Diospyros kaki* Thunb.) coproducts as a new ingredient in pork liver pâté: influence on quality properties

Autores: Raquel Lucas-González, Marika Pellegrini, Manuel Viuda-Martos, José Ángel Pérez-Álvarez, Juana Fernández-López

Revista	International Journal of Food Science & Technology, 54, 1365-2621
DOI	10.1111/ijfs.14047
Editorial	Institute of Food Science & Technology
eISSN	2193-4126
Categoría de JCR	Food Science & Technology
Cuartil	Q2
Rango	47/139
Factor de Impacto (2019)	2,773
Año de publicación	2019

Publicación 6

Persimmon flours as functional ingredients in spaghetti: chemical, physico-chemical and cooking quality

Autores: Raquel Lucas-González, Manuel Viuda-Martos, José Ángel Pérez-Álvarez, Clemencia Chaves-López, Blerina Shkembi, Salvatore Moscaritolo, Juana Fernández-López, Giampiero Sacchetti

Revista	Journal of Food Measurement & Characterization, 14, 1634-1644
DOI	10.1007/s11694-020-00411-6
Editorial	Springer
eISSN	0963-9969
Categoría de JCR	Food Science & Technology
Cuartil	Q3
Rango	89/139
Factor de Impacto (2019)	1,648
Año de publicación	2020

Publicación 7

Evaluation of polyphenol bioaccessibility and kinetic of starch digestion of spaghetti with persimmon (*Dyospyros kaki*) flours coproducts during *in vitro* gastrointestinal digestion

Autores: Raquel Lucas-González, José Ángel Pérez-Álvarez, Salvatore Moscaritolo, Juana Fernández-López, Giampiero Sacchetti, Manuel Viuda-Martos

Revista	Food Chemistry, 338, 128142
DOI	10.1016/j.foodchem.2020.128142
Editorial	Elsevier
eISSN	0963-9969
Categoría de JCR	Food Science & Technology
Cuartil	Q1
Rango	6/139
Factor de Impacto (2019)	6,306
Año de publicación	2021

Publicación 8

Pork liver pâté enriched with persimmon coproducts: Effect of *in vitro* gastrointestinal digestion on its fatty acid and polyphenol profile stability

Autores: Raquel Lucas-González, José Ángel Pérez-Álvarez, Manuel Viuda-Martos, Juana Fernández-López

Revista	Nutrients, 13(4), 1332
DOI	10.3390/nu13041332
Editorial	MDPI
eISSN	2072-6643
Categoría de JCR	Nutrition & Dietetics
Cuartil	Q1
Rango	17/89
Factor de Impacto (2019)	4,546
Año de publicación	2021

Estructura de la Tesis

La presente Tesis doctoral es un compendio de publicaciones científicas, tanto de investigación como de revisión, por lo tanto, su estructura sigue la Normativa de estudios de doctorado de la Universidad Miguel Hernández de Elche para la presentación de "Tesis por compendio de publicaciones". Además, con esta Tesis se pretende obtener la mención "Doctor Internacional" para tal fin se ha seguido el Real Decreto 99/2011.

La Tesis está dividida en 7 capítulos. Previo a los capítulos se aporta un resumen de la Tesis **"Resumen/Abstract"** tanto en castellano como en inglés.

Capítulo 1. Introducción.

Este capítulo consta de cuatro apartados, en los que se recoge información acerca de la producción, valor nutricional y propiedades saludables relativas al caqui, así como su industrialización. Se profundiza en la valorización de los coproductos de la industria alimentaria, así como en las pautas a seguir para el desarrollo e innovación de alimentos. El capítulo termina exponiendo los diferentes métodos de digestión *in vitro* y sus usos para evaluar la funcionalidad de los alimentos.

Capítulo 2. Objetivos

En este capítulo se detalla el objetivo general y los objetivos específicos de la presente Tesis Doctoral.

Capítulo 3. Materiales y Métodos

En este capítulo se especifican todos los materiales, procesos, protocolos, análisis y métodos aplicados durante la elaboración de la presente Tesis Doctoral. Está dividido en 6 apartados: tres de ellos concernientes a la obtención, caracterización y evaluación de la funcionalidad de las harinas de caqui; otros dos relativos al desarrollo, caracterización y evaluación de la funcionalidad de las matrices alimentarias; y el último apartado, donde se resumen los análisis estadísticos.

Capítulo 4. Resultados y Discusión

En este capítulo se muestran todos los resultados obtenidos en las publicaciones de investigación que componen la presente Tesis Doctoral, así como la discusión de los resultados más relevante. El capítulo se divide en 5 apartados, tres relativos a la obtención caracterización y evaluación de la funcionalidad de las harinas de caqui y dos concernientes a la calidad y funcionalidad de las matrices alimentarias desarrolladas.

Capítulo 5. Conclusiones

En este capítulo se recogen las conclusiones obtenidas de todos los trabajos científicos que conforman la presente Tesis Doctoral. Dichas conclusiones se presentan en castellano y en inglés.

Capítulo 6. Referencias

En este capítulo se recopila toda la bibliografía consultada para la elaboración de la presente Tesis Doctoral.

Capítulo 7. Publicaciones

En este capítulo se recopilan todas las publicaciones científicas que forman parte de esta Tesis Doctoral, presentadas en el idioma original de publicación. Está dividido en 8 apartados, uno por cada publicación presentada.

Las *dos primeras publicaciones* hacen referencia a la caracterización de las harinas de caqui. La primera se centra en su composición química, propiedades físico-químicas, tecnofuncionales y fisiofuncionales, y la segunda publicación se centra en los compuestos bioactivos de las harinas y su capacidad antioxidante:

1. **Lucas-González R,** Viuda-Martos M, Pérez-Álvarez JÁ, Fernández-López J. Evaluation of particle size influence on proximate composition, physicochemical, techno-functional and physio-functional properties of flours obtained from persimmon (*Diospyros kaki* trumb.) coproducts. **Plant Foods Hum Nutr. 2017**, 72:67–73. https://doi.org/10.1007/s11130-016-0592-z

2. **Lucas-González R**, Fernández-López J, Pérez-Álvarez JÁ, Viuda-Martos M. Effect of particle size on phytochemical composition and antioxidant properties of two persimmon flours from *Diospyros kaki* Thunb. vars. 'Rojo Brillante' and 'Triumph' co-products. **J Sci Food Agric. 2018**; 98:504-510. https://doi.org/10.1002/jsfa.8487

La *tercera publicación* es un trabajo de revisión donde se analiza el estado del arte de los métodos de digestión gastrointestinal *in vitro* que servirán de guía, tanto para la introducción de la presente Tesis Doctoral, como para la realización de los posteriores trabajos de investigación que incluyan digestiones de ingredientes y/o alimentos:

3. **Lucas-González R**, Viuda-Martos M, Pérez-Alvarez JA, Fernández-López J. *In vitro* digestion models suitable for foods: Opportunities for new fields of application and challenges. **Food Res Int. 2018**. 107:423-436. https://doi.org/10.1016/j.foodres.2018.02.055

La *cuarta publicación* se centra en estudiar la funcionalidad de las harinas de caqui después de haber sido sometidas al proceso de digestión *in vitro*:

4. **Lucas-González R**, Viuda-Martos M, Pérez Álvarez JA, Fernández-López J. Changes in bioaccessibility, polyphenol profile and antioxidant potential of flours obtained from

persimmon fruit (*Diospyros kaki*) co-products during *in vitro* gastrointestinal digestion. **Food Chem. 2018**, 1:252-258. https://doi.org/10.1016/j.foodchem.2018.02.128

Las dos siguientes publicaciones (*quinta y sexta publicación*) incluyen la elaboración y caracterización de los dos alimentos desarrollados enriquecidos con harinas de caqui:

5. **Lucas-González R**, Pellegrini M, Viuda-Martos M, Pérez-Álvarez JÁ, Fernández-López J. Persimmon (*Diospyros kaki* Thunb.) coproducts as a new ingredient in pork liver pâtè: influence on quality properties. **Int J Food Sci Technol**. **2019**, 54:1232-1239. https://doi.org/10.1111/ijfs.14047

6. **Lucas-González R**, Viuda-Martos, M, Pérez-Álvarez JÁ, Chaves-López C, Shkembi B, Moscaritolo S, Fernández-López J, Sacchetti G. Persimmon flours as functional ingredients in spaghetti: chemical, physico-chemical and cooking quality. **J Food Meas Charact**. **2020**, 14:1634–1644. https://doi.org/10.1007/s11694-020-00411-6

Finalmente, los dos últimos artículos (*séptima y octava publicación*) hacen referencia a la digestión *in vitro* de cada uno de los dos alimentos desarrollados:

7. **Lucas-González R**, Pérez-Álvarez JA, Moscaritolo S, Fernández-López J, Sacchetti G, Viuda-Martos M. Evaluation of polyphenol bioaccessibility and kinetic of starch digestion of spaghetti with persimmon (*Dyospyros kaki*) flours coproducts during *in vitro* gastrointestinal digestion. **Food Chem. 2021**, 338:128142. https://doi.org/10.1016/j.foodchem.2020.128142

8. **Lucas-González R**, Pérez-Álvarez J.A, Viuda-Martos M, Fernández-López J. Pork liver pâté enriched with persimmon coproducts: Effect of *in vitro* gastrointestinal digestion on its fatty acid and polyphenol profile stability. **Nutrients 2021,** 13:1332. **https://doi.org/10.3390/nu13041332**

Resumen

La producción de alimentos de manera sostenible junto con la elaboración de alimentos saludables que promuevan la salud, son temas actuales de gran calado social. Los coproductos de la industria agroalimentaria (pieles, pulpa, semillas, hojas, etc), los cuales tienen altas cantidades de nutrientes y/o compuestos bioactivos, pueden ser procesados y usados de nuevo en la industria alimentaria para la elaboración de nuevos alimentos.

La presente Tesis ha tenido como objeto la valorización de los coproductos (piel y pulpa) generados tras la producción de zumo de caqui de dos cultivares, "Rojo Brillante" y "Triumph". Tras someter a los coproductos a varias operaciones unitarias se obtuvieron ingredientes intermedios, denominados harina de caqui, con diferentes tamaños de partícula. Estas harinas destacaron por sus capacidades de hidratación: capacidad de retención de agua e hinchamiento, y por su habilidad para retener bilis. Tras analizar qué tamaño de partícula de las harinas presentaba mejor composición química, contenido de compuestos bioactivos, propiedades tecnofuncionales, fisiofuncionales y antioxidantes, se seleccionaron dos harinas de caqui, ambas con el menor tamaño de partícula estudiado (< 210 µm), una procedente de cada cultivar, que se denominaron harina Rojo Brillante y harina Triumph. Ambas harinas se sometieron a los procesos de digestión gastrointestinal *in vitro*, donde se dilucidó la bioaccesibilidad de sus compuestos (poli)fenólicos y su capacidad protectora frente a los radicales libres durante los procesos digestivos.

Las dos harinas de caqui seleccionadas se usaron para enriquecer, con dos concentraciones diferentes (3 y 6%), dos matrices alimentarias diferentes, una cárnica, paté de hígado de cerdo, y otra amilácea, espaguetis de sémola de trigo duro. En total se trabajó con 10 formulaciones, 8 alimentos enriquecidos con harina de caqui, y dos sin enriquecer, que se usaron como controles, para estudiar y comparar los efectos del enriquecimiento sobre la composición química, las propiedades fisicoquímicas, la calidad de la matriz y el comportamiento del alimento tras ser sometido a la digestión gastrointestinal *in vitro*.

Ambas harinas pueden ser consideradas fuente de fibra dietética, especialmente insoluble, de monosacáridos y de ácido gálico. Los efectos más destacados de las harinas de caqui sobre las matrices desarrolladas son: su capacidad colorante, en ambas matrices se intensificó de manera significativa la tonalidad roja (a*); su capacidad para reducir el tiempo de cocción en los espaguetis; su capacidad como agentes reductores de nitritos, en los patés. Tras la digestión *in vitro* se comprobó que los espaguetis con menor porcentaje de harina de caqui (3%) reducen la hidrólisis del almidón, y en los patés (ambas concentraciones) reducen la oxidación lipídica. Además, las harinas de caqui aportan en ambas matrices alimentarias, (poli)fenoles que podrán ser usados por la microbiota intestinal al resistir los procesos digestivos (*in vitro*).

En conclusión, las harinas de caqui (Rojo Brillante y Triumph) pueden usarse como ingredientes intermedios en la industria alimentaria para enriquecer diferentes matrices alimentarias. La incorporación de dichas harinas a un 3% es la más recomendada. La inclusión de las harinas de caqui en la industria alimentaria conlleva la valorización de los coproductos de la industria del caqui, la reducción de residuos y la aparición en el mercado de nuevos alimentos.

Abstract

The sustainable production of food and the elaboration of foods that provide health benefits (functional foods) are current issues that have attracting more and more interest in our society. Agri-food industry coproducts (peels, pulp, seeds, leaves, etc.), which have high amounts of nutrients and bioactive compounds, can be processed, and used again in the food industry to produce new foods.

The aim of the current Doctoral Thesis was the valorization of coproducts (peel and pulp) from persimmon (cv. "Rojo Brillante" and "Triumph") juice industry. After undergoing coproducts to several unit operations, intermediate ingredients were obtained, called persimmon flours (which several particle sizes). Their hydration ability (water holding ability and swelling ability) and their bile holding ability have been highlighted as the most relevant technofunctional properties. Flours with the smallest particle size (<210 µm) (from both cultivars, "Rojo Brillante" and "Triumph"), showed better chemical composition, higher amount of bioactive compounds, and better technofunctional, physhyofunctional, and antioxidants properties than the others, and so they were selected for the following studies. Both flours were undergone to *in vitro* digestion process where the bioaccessibility of their (poly)phenolic compounds and protective capacity against free radicals during the digestive process was assessed.

These persimmon flours were used to enrich (at two concentrations, 3 and 6%) foods: a meat product (pork liver pâté) and a cereal-based food (durum wheat semolina spaghetti). Ten food formulations were evaluated: eight of them were persimmon flours enriched (4 pâtés and 4 pasta) and two without persimmon flours, which were used as controls to study and to compare the effect of the persimmon flour addition on their chemical composition, physicochemical and quality properties. Their behavior after *in vitro* gastrointestinal digestion was also evaluated.

Both flours can be considered a source of dietetic fiber (especially insoluble), monosaccharides and gallic acid. One of the common effects of persimmon flours on both foods is their colorant ability; redness was significantly intensified in both food matrices. Also, it is important to highlight their ability to reduce the optimum cooking time in spaghetti, and to reduce the nitrite residual level in pâtés. After *in vitro* digestion, it was found that the starch hydrolysis in spaghetti (added with 3% persimmon flours) was decreased, and lipid oxidation in pâtés (both concentrations) was reduced. Furthermore, (poly)phenols were contributed by persimmon flours

in both type of foods (meat product and cereal-based food), which could be used by intestinal microbiota to resist the digestive process (*in vitro*).

Persimmon flours (Rojo Brillante and Triumph) can be used as intermediate ingredients by food industry to enrich different food matrices. The incorporation of these flours at 3% is the most recommended. The use of persimmon flours in the food industry allows not only persimmon coproducts valorisation, but also contribute to waste reduction. Finally, several foods (with interesting nutritional and functional properties) can become available at the market.

Capítulo 1.-
INTRODUCCIÓN

1.1.- EL CAQUI *(Diospyros kaki* Thunb.)

1.1.1.- Historia y producción

El caqui (*Diospyros kaki*) es una fruta de colores amarillos, rojos o naranjas originaria del este de Asia, donde se han encontrado fósiles de hojas de caqui salvajes de cerca de 2,5 millones de años (Wang y col., 2013). Su nombre significa la comida de los Dioses (Dio, en griego significa Dios y spyros comida). Muchos autores concentran su origen en China (Wang y col., 2013; Zheng y col., 2013), debido a los innumerables textos que recogen la presencia del árbol de caqui en este país, tales como "The book of songs", la presencia de árboles antiguos y la presencia de hallazgos arqueológicos. Posteriormente el caqui se extendió a Japón y Corea, en los siglos VII y XIV, respectivamente; a partir del siglo XVII se introdujo en Europa y a otras partes del mundo con climas templados como Israel, Brasil y Nueva Zelanda (Sugiura, 1997; Ullio, 2003). No obstante, Asia sigue liderando la producción mundial (Figura 1), siendo China, con diferencia, el mayor productor, el cual concentra, entorno al 88 % de la producción total mundial (FAOSTAT, 2020).

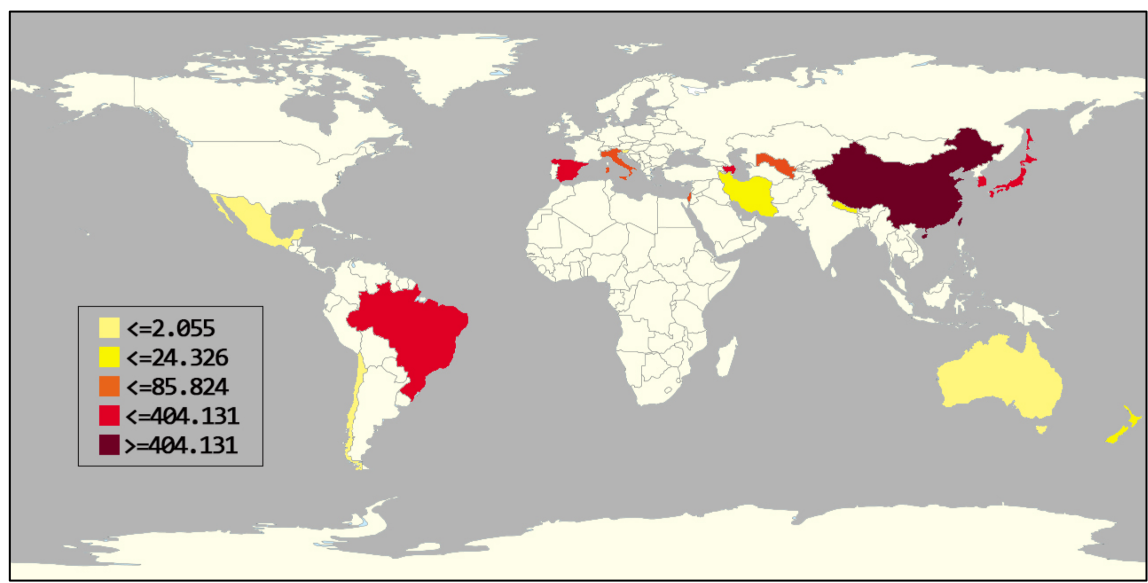


Figura 1.- Producción mundial (toneladas) de caqui en 2017. Elaboración propia. Fuente: FAOSTAT (2020).

Con respecto a Europa, durante varias décadas (60-80) Italia era el único productor con impacto en la comercialización (Llácer, 2002), hasta que en 1991 España comienza a cultivar caqui para su comercialización, pasando de encontrarse en novena posición en dicho año, a segunda posición en el 2017 (FAOSTAT, 2020).

Botánicamente el caqui es un fruto tipo baya perteneciente a la familia Ebanaceae, género *Diospyros*, el cual es el más importante, tanto numérica como económicamente dentro de la familia Ebanaceae (Mallavadhani, 1998). Se conocen más de 900 variedades de caqui en todo el

mundo (Wang y col. 1997), muchas de ellas autóctonas de determinados países, tales como las cultivares "Rojo Brillante" autóctona de España, "Kaki tipo", procedente de Italia, "Triumph" encontrada en Israel o "Rama Forte" originaria de Brasil (Badenes y col., 2003) (Figura 2).

Figura 2.- Imagen de los cultivares de caqui "Rojo Brillante" y "Triumph".

Una peculiaridad del caqui es que presenta una variedad fenotípica especial en la que se pueden dividir los frutos entre astringentes y no astringentes (Yonemori y col, 2003), que responde al contenido de taninos solubles presentes en la fruta tras su recolección (Novillo y col., 2016). Los taninos son compuestos (poli)fenólicos con capacidad de unirse a las proteínas presentes en la saliva, lo que implica que estas precipiten, provocando la sensación de sequedad en la boca. En ambos grupos la astringencia de los frutos está condicionada por el tipo de polinización, por lo tanto, el caqui se puede clasificar en cuatro grupos (Khokhlov y Plugatar, 2016; Besada y Salvador, 2018):

1. **Polinización constante no astringente (PCNA)**: frutos no astringentes independientemente de la presencia de semillas. Estos pueden comerse maduros cuando el fruto está turgente como una manzana.
2. **Polinización variable no astringente (PVNA):** frutos no astringentes si contienen semillas en la cosecha. Las frutas no son comestibles cuando están firmes si no han sido polinizadas.

3. **Polinización constante astringente (PCA):** fruto astringente cuando se encuentra firme. Solamente se puede comer cuando el fruto está sobremadurado, 10-30 días después de la cosecha.
4. **Polinización variable astringente (PVA):** frutos sin semilla, astringentes cuando están firmes.

Es por ello, que a excepción de las variedades PCNA, el resto de las variedades deben sobremadurar en el árbol para perder la astringencia y ser consumidas (Besada y Salvador, 2018). Una de las consecuencias de esta sobremaduración es la pérdida de firmeza del fruto, lo que conlleva que sea consumido con cuchara y que su comercialización quede reducida a mercados locales. Para permitir la comercialización de las variedades astringentes y fomentar el consumo del caqui existen diferentes métodos de desastringencia, cuyo objetivo es eliminar taninos, y con ello la astringencia sin perder la firmeza, de esta manera se aumenta la vida comercial de la fruta y se hace más atractiva para el consumidor. Los tratamientos más actuales se realizan en cámaras donde la fruta es expuesta a altas concentraciones de CO_2 o vapores de etanol, consiguiendo así condiciones de anaerobiosis (Besada y Salvador, 2018).

1.1.1.1.- El caqui en España

El caqui fue introducido en España en el siglo XIX como árbol ornamental y por la calidad de su madera (Llácer, 2002). Su producción y comercialización no tenían ningún impacto en la década de los setenta, debido, sobre todo a que las variedades cultivadas en España son astringentes ("Rojo Brillante", "Tomatero", "Triumph"), lo que conlleva que la fruta estuviera sobremadurada para poder ser consumida. El auge de las técnicas de post-recolección junto con la aparición del cultivar "Rojo Brillante", el cual es autóctono de España, impulsó la producción y comercialización de caqui. Tal es el caso, que en 20 años la producción del caqui pasó a ser 66 veces mayor (1995-2015) (FAOSTAT, 2020) (Figura 3). La comunidad Valenciana ha sido el gran impulsor de este crecimiento, siendo diversos los factores que han propiciado dicho aumento productivo, tales como: innovación tecnológica, innovación organizativa y comercial (Consejo Regulador de la Denominación de Origen Ribera del Xúquer) e innovación empresarial (Vendrell, 2017). Todo ello ha contribuido a convertir a la Comunidad Valenciana, concretamente a Valencia, en el mayor productor de caqui de España.

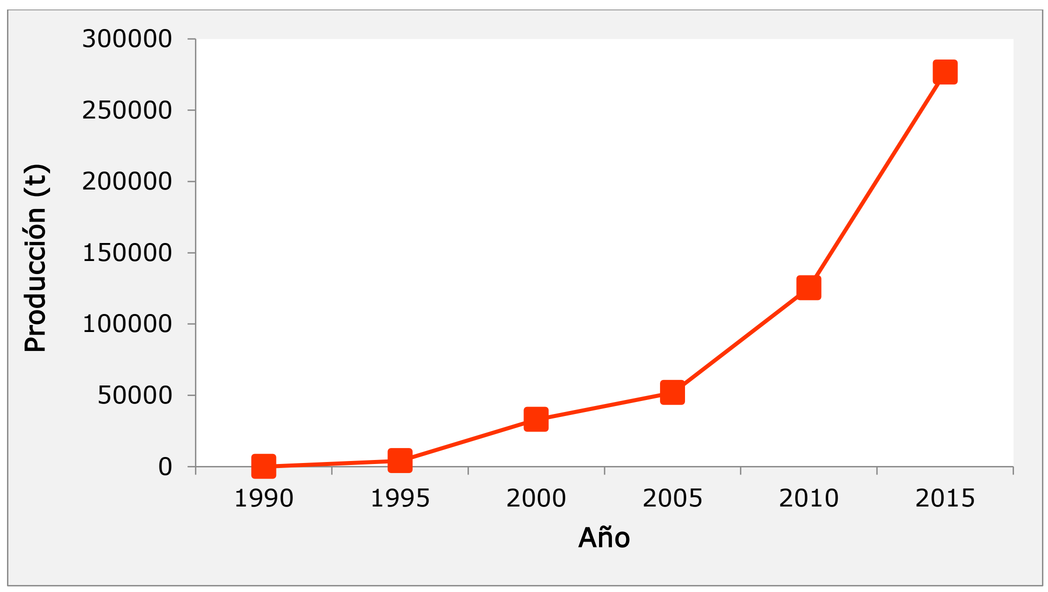


Figura 3.- Evolución de la producción del caqui en España a lo largo del tiempo. Elaboración propia. Fuente: FAOSTAT (2020).

1.1.2.- Composición química

El caqui destaca por ser una fruta rica en azúcares, pectina y compuestos bioactivos. Después del agua, su componente mayoritario es el azúcar, seguido de la fibra dietética (Tabla 1). Al igual que ocurre con la mayoría de las frutas, su composición depende de la variedad, el estado de maduración y los tratamientos post-recolección (Chen y col., 2016; Ancillotti y col., 2019). Su contenido en azúcares simples varía de 9,5 a 28 g por 100 g de fruta fresca (Khokhlov y Plugatar, 2016; Chen y col., 2016; Veberic y col., 2010), considerándose un valor medio de 15,3 g/100g fruta fresca, según las tablas de composición de alimentos española (BEDCA, 2020), lo que la convierte en una fruta muy dulce, pues presenta mayores contenidos de azúcares simples que otras frutas altamente consumidas, tales como manzanas, peras, melocotones o naranjas (Giordani y col., 2011). Los monosacáridos glucosa y fructosa son sus azúcares mayoritarios, seguidos del disacárido sacarosa. Con respecto a sus compuestos bioactivos (Tabla 2), destaca su contenido en vitamina C, que varía entre 35 y 218 mg/100 g (Giordani y col., 2011) y en fitoquímicos como los carotenoides y los (poli)fenoles.

Tabla 1.- Composición química del caqui, manzana, melocotón, naranja, pera y piña.

	Caqui	Manzana	Melocotón	Naranja	Pera	Piña
Parte comestible (%)	84,0	84,0	88,0	73,0	87,0	57,0
Energía (Kcal)	279,0	209,0	163,0	160,0	187,0	204,0
Agua (g)	80,7	85,7	89,0	88,6	86,7	86,8
Proteínas (g)	0,7	0,3	0,6	0,8	0,4	0,5
Grasas (g)	0,2	Traza	Traza	Traza	Traza	Traza
Carbohidratos (g)	15,3	12,0	9,0	8,6	10,6	11,5
Fibra (g)	2,5	2,0	1,4	2,0	2,3	1,2
Vitamina A* (µg)	163,0	4,0	17,0	46,0	1,0	10,0
Vitamina D (µg)	0,0	0,0	0,0	0,0	0,0	0,0
Vitamina E** (mg)	0,6	0,5	0,5	0,2	Traza	0,1
Folato, total (µg)	11,0	1,0	3,0	37,0	11,0	11,0
Niacina*** (mg)	0,3	0,2	1,0	0,3	0,2	0,3
Riboflavina (mg)	0,06	0,04	0,05	0,03	0,03	0,02
Tiamina (mg)	0,03	0,03	0,03	0,1	0,03	0,07
Vitamina B12 (µg)	0,0	0,0	0,0	0,0	0,0	0,0
Vitamina B6 (mg)	0,10	0,04	0,02	0,06	0,02	0,09
Vitamina C (mg)	7,0	3,0	8,0	50,0	3,0	20,0
Calcio (mg)	21,0	6,0	8,0	36,0	12,0	12,0
Hierro (mg)	0,4	0,4	0,4	0,3	0,2	0,5
Potasio (mg)	171,0	99,0	260,0	200,0	130,0	250,0
Magnesio (mg)	9,0	5,0	9,0	12,0	7,0	14,0
Sodio (mg)	2,0	2,0	3,0	3,0	2,0	2,0
Fósforo (mg)	20,0	9,0	22,0	28,0	17,5	11,0
Selenio (µg)	0,6	Traza	1,0	1,0	Traza	Traza
Zinc (mg)	Traza	0,10	0,06	0,18	0,14	0,15

Fuente: Base de datos española de composición de alimento (BEDCA, 2020).

*Equivalentes de retinol de actividades de retinos y carotenoides

**Equivalentes de alfa tocoferol de actividades de vitámeros E

***Equivalentes de niacina, totales.

Tabla 2.- Compuestos bioactivos mayoritarios presentes en el caqui

Familia	Compuestos	Referencias
Vitaminas	Vitamina C	Giordani y col. (2011)
Carotenoides	β-caroteno, β-cryptoxanthin, α-caroteno, zeaxantina, luteína, violaxantina, neoxantina, licopeno	de Ancos y col. (2000); Veberic y col. (2010); Zhao y col. (2011); Plaza y col. (2012)
(Poli)fenoles		
Ácidos fenólicos	Ácido gálico, *p*-cumárico, ácido vanílico	Veberic y col. (2010); Kou y col. (2015); Sentandreu y col. (2015); Suzuki y col. (2015)
Flavanoles	Catequina, epicatequina, epigalocatequina, galocatequina,	
Flavonoles	Kaempferol, quercetina, rutina	

Los carotenoides son los fitoquímicos mayoritarios del caqui, y los responsables del color de su piel y pulpa (Thomas y Chen, 1998). Estos tienen un relevante papel en la dieta humana, tanto por su actividad pro-vitamina A como por su capacidad antioxidante. En el caqui los carotenoides mayoritarios son la β-criptoxantina y el β-caroteno, ambos con actividad pro-vitamina A (de Ancos y col., 2000) (Figura 4). El caqui destaca también por ser rico en compuestos (poli)fenólicos (Figura 5), tales como flavonoides, ácidos fenólicos y taninos. Sin embargo, su contenido en taninos disminuye drásticamente con la maduración del fruto, lo que permite que pueda ser consumido, y es más alto en frutos astringentes que no astringentes (Novillo y col., 2016). En cuanto a su contenido en minerales destaca su contenido en calcio, hierro, magnesio y potasio (Mir-Marqués y col., 2015; Khokhlov y Plugatar, 2016).

β-caroteno

β-cryptoxantina

α-caroteno

Zeaxantina

Luteína

Licopeno

Figura 4.- Estructura química de los carotenoides presentes en el caqui.

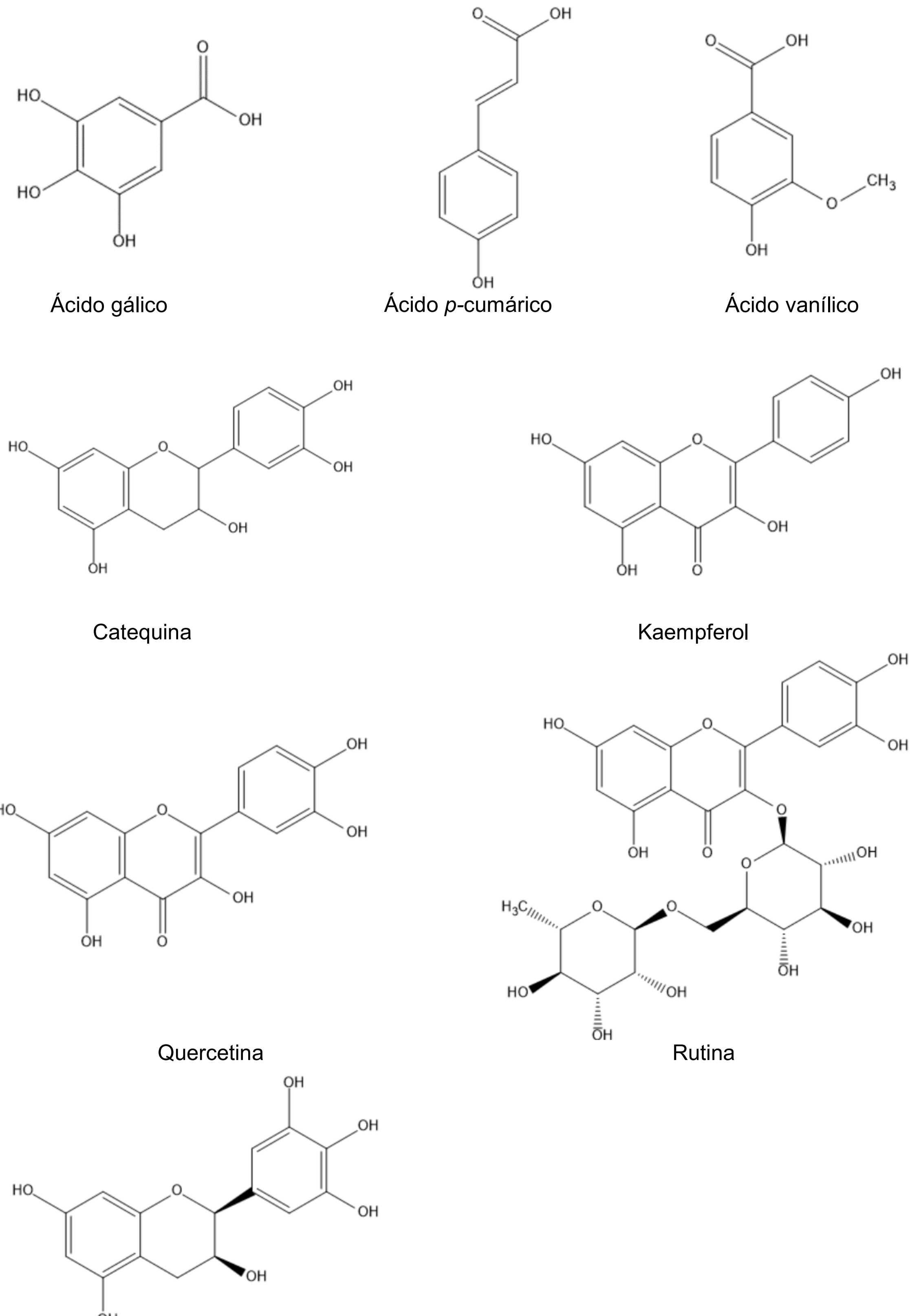


Figura 5.- Estructura química de los (poli)fenoles mayoritarios presentes en el caqui.

1.1.3.- Efectos saludables

Además de por su alto valor nutricional, el caqui es un fruto muy apreciado por la medicina tradicional asiática, habiéndose usado en Japón como antídoto contra la picadura de serpientes (extracto de taninos) (Martz, 1992) o en Corea para frenar la diarrea (Song y Kim, 2011). Es preciso destacar que, además del fruto, también a las hojas del caqui se les atribuyen diversos efectos saludables derivados de su alto contenido en compuestos bioactivos (Xie y col., 2015). Recientemente, diversos estudios *in vitro* e *in vivo* han puesto de manifiesto que el consumo de caqui puede prevenir o mejorar diferentes enfermedades no transmisibles, que actualmente son una pandemia, tales como:

- **Enfermedades cardiovasculares**, las cuales engloban los trastornos del corazón y los vasos sanguíneos, concretamente la cardiopatía isquémica y el accidente cerebrovascular, las cuales, son en la actualidad la primera y segunda causa de muerte en el mundo respectivamente (OMS, 2020). Los ensayos con ratas o ratones a los que se les suministraba caqui o extractos ricos durante 4-6 semanas han concluido que su consumo tiene efectos hipocolesterolémicos, hipotrigliceridémicos y antiaterogénicos, además de reducir la oxidación de las lipoproteínas de baja densidad (LDL) y la lipogénesis hepática, y mejorar el perfil lipídico sanguíneo (Gorinstein y col., 1998; Gorinstein y col., 2000; Matsumoto y col., 2010; Gorinstein y col., 2011; Son y col., 2013; Fushimi y col., 2015; Ahn y col., 2017). Un reciente estudio aleatorio y de doble ciego con 14 sujetos concluyó que el consumo de galletas elaboradas con un extracto de caqui rico en fibra y taninos disminuye el colesterol en sangre (Gato y col., 2013). Los mecanismos de acción, por los cuales el caqui o sus extractos presentan un rol protector frente a las patologías cardiacas se deriva de su actividad antioxidante, que conlleva un efecto protector frente a la oxidación de las lipoproteínas, y su capacidad de retención de bilis, (Matsumoto y col., 2010; Gorinstein y col., 2011; Takekawa y Matsumoto, 2012; Ahn y col., 2017; Hamauzu y Suwannachot, 2019), que provoca que el cuerpo tenga que sintetizar ácidos biliares de nuevo a partir del colesterol (Houten y col., 2006; Staels y col., 2010).
- **Diabetes mellitus**, enfermedad que se produce cuando el páncreas no produce insulina (tipo I) o cuando los órganos diana de la insulina no responden a ésta (tipo II); en ambos casos la consecuencia son niveles de glucosa elevados en sangre. Actualmente, se ha convertido en la novena causa de muerte a nivel mundial (OMS, 2020) y es también un factor de riesgo cardiovascular. Los estudios en animales a los que se les suministraba caqui o preparados con extractos de caqui sugieren que su consumo regula los niveles de glucosa en sangre (Izuchi y col., 2011) o los disminuye en ratas y conejos diabéticos (Lee y col., 2006; Sindu y col. 2019). Además, los (poli)fenoles presentes en el caqui tienen la capacidad de disminuir el estrés oxidativo derivado de la hiperglucemia (Yokozawa y col., 2007). El efecto antidiabético del

caqui se puede deber, tanto a la capacidad que presentan sus compuestos (poli)fenólicos de inhibir a la α-amilasa y a la amiloglucosidasa (ambas enzimas responsables de la degradación del almidón a glucosa), según un estudio *in vivo* con extractos de hoja de caqui (Kawakami y col., 2010), como también a su capacidad de retener bilis, pues diversos estudios apuntan que la activación del gen colesterol 7α-hydroxilasa, gen inicial de la ruta metabólica de la síntesis de ácidos biliares, mantiene la homeostasis de la glucosa (Li y col., 2006).

– **Otros**: algunos autores apuntan otros beneficios al consumo de caqui, tales como: efectos antiobesidad, dada su capacidad de inhibir la lipasa pancreática (Kim y col., 2016), lo que conllevaría la disminución de la absorción de triglicéridos; capacidad para prevenir y tratar las lesiones neurológicas (Forouzanfar y col., 2016); o incluso como herramienta para el tratamiento de las enfermedades antiinflamatorias del intestino y para prevenir el cáncer colorectal (Direito y col., 2017), basándose en un estudio con ratas que atribuía tales efectos al potencial antiinflamatorio de los (poli)fenoles del caqui.

1.1.4.- Industrialización del caqui

Además del consumo del caqui en fresco, este se utiliza para la producción de multitud de alimentos (Figura 6). En China, Japón y Corea, es frecuente usar el caqui para producir piezas deshidratadas, vino, vinagre, e incluso fideos chinos (Akter y col., 2010; Ubeda y col., 2011; Han y col., 2012; Lu y col., 2020), pues su alta estacionalidad (octubre a enero) hace que la fruta se encuentre poco tiempo disponible para su consumo. En la mayoría de los países productores del caqui se producen diversos alimentos cuyo ingrediente principal es el caqui. Italia produce mermeladas, compotas, conservas en aceite de oliva de caqui, extractos ricos en caqui y piezas deshidratadas. Otros innovadores usos son los de la empresa “My goodness” de Nueva Zelanda, que usa el caqui como base para producir helados sin lácteos.

En España, a raíz del aumento de la producción del caqui, se han empezado a elaborar productos a partir de él, sobre todo piezas deshidratadas, mermeladas, purés, néctares y zumos (Carbonell y col., 2012; Jiménez-Sánchez y col., 2015). La producción de estos alimentos genera coproductos, sobre todo piel, pulpa y semillas. Además, durante los tratamientos de desastringencia, también se producen pérdidas de frutos, debido a sobreexposiciones por CO_2 o daños por frío. Todos estos coproductos, sino son debidamente tratados, generan residuos que conllevan un impacto medioambiental, a la par que una pérdida económica.

Figura 6.- Alimentos elaborados con caqui.

1.2.- VALORIZACIÓN DE COPRODUCTOS DE LA INDUSTRIA AGROALIMENTARIA

La valorización de los coproductos de la industria agroalimentaria se percibe como una potente estrategia para reducir los desechos y aumentar la economía de las industrias agroalimentarias. Además, esta valorización de coproductos se engloba dentro de la estrategia europea "*more food, less waste*" a la que también está adherida España con un programa para la

reducción de las pérdidas y el desperdicio alimentario y la valorización de los alimentos desechados, con el objetivo de mejorar la eficiencia de la cadena agroalimentaria (MAPA, 2018). De entre todas las etapas de actuación previstas en dicha estrategia, una de ellas se centra específicamente en "la industria agroalimentaria", para evitar pérdidas asociadas al procesamiento y proceso de producción y recursos que han perdido valor alimentario.

Dentro de este marco, son muchas las estrategias que se pueden seguir para reducir residuos y valorizar los coproductos agroalimentarios, tales como generación de compost y biocombustible, la extracción de compuestos bioactivos para usos farmacológicos o cosméticos, hasta el uso de los coproductos como agentes bioadsorbentes de metales pesados para la purificación del agua (Inoue y col., 2017). Otra alternativa es la de producir ingredientes intermedios, ricos en fibra, y compuestos bioactivos, a partir de los coproductos de la industria agroalimentaria, principalmente de la pulpa y la piel, para el desarrollo de alimentos innovadores y funcionales, alimentos que pueden aportar un beneficio para la salud más allá de la nutrición básica (International Food Information Council Foundation, 2020). Como se expondrá en el siguiente apartado, existe una fuerte demanda por parte de los consumidores y las entidades públicas (Organización Mundial de la salud, OMS, Organización de las Naciones Unidas para la Alimentación y la Agricultura, FAO), de desarrollar alimentos saludables y funcionales. Es por todo esto que la comunidad científica busca nuevas fuentes de ingredientes con potencial funcional. Los coproductos de la industria agroalimentaria están siendo estudiados como candidatos a convertirse en nuevos ingredientes, dado su alto valor nutricional y sus diversas propiedades tecno-funcionales tales como conservantes, agentes antioxidantes, colorantes, emulsionantes, texturizantes, y/o espumantes (Faustino y col., 2019).

Son muchos los ejemplos de coproductos de la industria agroalimentaria que han sido estudiados por su potencial como ingredientes intermedios y/o funcionales para el desarrollo de alimentos, tales como los coproductos procedentes de cítricos (Lario y col., 2004a; Viuda-Martos y col., 2011a; López-Marcos y col., 2015), de la chufa (Sánchez-Zapata y col., 2009, 2012a) del dátil (Martín-Sánchez y col., 2014a,), de la manzana (Cerda-Tapia y col., 2015), del tomate (Viuda-Martos y col., 2014), de la granada (Viuda-Martos y col., 2011b, 2012, 2013; Hasnaoui y col., 2014; Gullon y col., 2016), del higo (Viuda-Martos y col., 2015a,b), de la chía (Fernández-López y col., 2018), de frutas tropicales (López-Vargas y col., 2014; Tril y col., 2014; Selani y col., 2016), de la zanahoria (Ma y col., 2016) del cacao (Martínez y col., 2012), de las nueces (Chang y col., 2016) de la industria del vino (García-Lomillo y González-Sanjosé, 2016), entre otros; o que han sido utilizados en la formulación de nuevos alimentos, como barritas de cereales elaboradas con coproductos de dátil, pera y manzana (Bchir y col., 2017), pastas de trigo duro con pequeños porcentaje de coproductos de tomate y orujo de oliva (Padalino y col., 2017; Simonato y col., 2019), yogures con coproductos de cítricos (Lario y col., 2004b; García-Pérez y col., 2005, 2006)

productos cárnicos, a los que se les ha incorporado coproductos de cítricos, chufa, dátil, quinoa, chía o avellana (Aleson-Carbonell y col., 2003, 2004, 2005; Fernández-Ginés y col., 2003, 2004; Fernández-López y col., 2004, 2007, 2008, 2019, 2020a; Viuda-Martos y col., 2009, 2010a; Sánchez-Zapata y col., 2010, 2012b; Sayas-Barberá y col., 2012; Martín-Sánchez y col., 2013, 2014b; Longato y col., 2019) o productos de panadería, elaborados con coproductos del brócoli, de la molienda de la cebada, del aceite de oliva, de la banana o de la patata (Verardo y col., 2011; Eshak, 2016; Jeddou y col., 2017; Lafarga y col., 2018, 2019; Di Nunzio y col., 2020).

1.3.- DESARROLLO E INNOVACIÓN DE ALIMENTOS

La industria alimentaria está constantemente diseñando nuevos alimentos y reformulando los alimentos tradicionales para cumplir con las demandas del consumidor, las presiones de la competencia y las recomendaciones gubernamentales. Para llevar a cabo estos fines, si además de desarrollar un alimento se quiere innovar, los conocimientos científico-técnicos no son suficientes y se deben trabajar otras competencias tales como curiosidad, creatividad, empatía, visión sistemática, capacidad de aprendizaje, resiliencia, y capacidad de observación. No obstante, la innovación de nuevos productos en España ha disminuido en los últimos años (Valencoso, 2019). Los expertos en este ámbito apuntan a que la falta de introducción de los productos desarrollados en las grandes cadenas de distribución, limitan el acceso del consumidor a ellas y por ende el éxito del producto, provocando que los fabricantes, que son los que más innovan, frenen la inversión en I+D+i (Cabanas, 2015; Valencoso, 2019).

Es por todo esto, que el desarrollo de alimentos requiere tener en cuenta diversos factores, que abarcan tanto aspectos técnicos, como conocimientos de mercadotecnia. Para tener éxito al desarrollar un alimento y que este perdure en el mercado, se deben tener en cuente los siguientes puntos:

- Conocer los ingredientes a usar:
  - Su composición química: proteínas, carbohidratos, grasas, etc.
  - Sus propiedades fisicoquímicas: pH, actividad de agua, color, densidad, textura, comportamiento reológico, etc, y
  - Sus propiedades tecnológicas y tecnofuncionales: capacidad de retención de agua, de aceite, de hinchamiento, actividad emulsionante, gelificante y espumante, etc.
- Formular una matriz estable en la que todos los ingredientes estén integrados.
- Aceptación organoléptica
- Aceptación socio-cultural
- Estabilidad microbiológica
- Análisis coste-beneficio
- Evaluación del potencial saludable

- Canales de distribución

Tener en cuenta todos estos factores en el desarrollo de nuevos alimentos, determinará el éxito del producto final, pues si algunos de estos factores no se tienen en cuenta muy probamente el producto no será aceptado por el consumidor final y desaparecerá del mercado. Por ejemplo, si nos centramos sólo en aumentar el valor nutritivo del alimento sin tener en cuenta el sabor, nadie lo querrá consumir, o si usamos ingredientes que son culturalmente rechazados por la población a la que se destina el producto, estos lo rechazarán sin tan siquiera querer probarlo.

Por otro lado, las tendencias actuales en innovación de alimentos según Techfood magazine (2019) y Whole Foods Market (2019) son las siguientes:

- Desarrollo de “productos cárnicos” a base de vegetales.
- Desarrollar carne de laboratorio.
- Productos prebióticos, probióticos y fermentados.
- Reducir o eliminar el azúcar añadido de los alimentos.
- Snack saludables y refrigerados
- Upcycling. Convertir los desechos en productos de valor.
- Uso de harinas de frutas y verduras.
- Alimentos procedentes de África occidental, tales como la moringa, el tamarindo, el jengibre, la citronela o el fonio.
- Cremas untables y mantequillas.
- Productos saludables
- Sustitutivos del azúcar a base de frutas y verduras
- Mezclas de carne y verdura
- Bebidas alternativas al alcohol

Como se puede apreciar, la tendencia en el desarrollo de alimentos saludables sigue en auge, pues la población es cada vez más consciente del binomio salud-alimentación.

Además, desde hace más de una década se persigue un movimiento holístico en el campo de la nutrición frente a la filosofía reduccionista, puesto que el potencial saludable de un alimento depende tanto de su valor nutricional como de su matriz (Fardet y Rock, 2015). En este contexto se entiende al movimiento holístico aquel que contempla que el todo es más que la suma de sus partes, entendiendo que un alimento es más que un conjunto de nutrientes. En contraposición la tendencia reduccionista, la cual ha imperado hasta la actualidad en la ciencia de la nutrición, se centra en los compuestos o cantidades de los alimentos, sin tener en cuenta, las sinergias, las diferentes respuestas interindividuales, etc (Kwon, 2019). Un ejemplo palpable de este movimiento en España es el “realfooding” impulsado por el dietista-nutricionista Carlos Ríos, que ha convertido las tradicionales pautas dietéticas de la dieta mediterránea y las actuales, como

evitar los alimentos ultraprocesados, en un estilo de vida con multitud de adeptos, que se autodenominan "realfooder" o aquellos que comen comida "real".

Por otro lado, la Organización de las Naciones Unidas lanzó en abril de 2016 "*El decenio de acción sobre la nutrición*", con el objetivo de reducir todas las formas de malnutrición, desnutrición y obesidad, puesto que el panorama actual es preocupante, con 821 millones de personas subalimentadas, más de 1.900 millones de adultos con sobrepeso u obesidad y 2.000 millones de personas con carencia de micronutrientes, lo que conlleva unos costos para la economía mundial que alcanzan los 3,5 billones de dólares al año (FAO y col., 2018).

Teniendo en cuenta todo esto, es necesario el desarrollo de alimentos con alto valor nutritivo, sostenibles, con potencial saludable y que cumplan las expectativas de los consumidores y se adapten a su ritmo de vida.

Sin embargo, pese a que en el mercado existen muchos alimentos bajo el paraguas de alimentos "funcionales" o "saludables", la mayoría de ellos carecen de respaldo científico para realizar estas alegaciones. En este sentido, únicamente Japón presenta una legislación adecuada para la comercialización de alimentos funcionales, a los que se exige que tengan respaldo con ensayos en humanos.

1.4 LA DIGESTIÓN "*in vitro*" COMO HERRAMIENTA EN LOS ESTUDIOS DE FUNCIONALIDAD ALIMENTARIA

La digestión humana es un complejo proceso fisicoquímico, regulado hormonalmente, que tiene como objetivo reducir el tamaño de los alimentos a nutrientes asimilables por las células del organismo. Durante el proceso de digestión se producen, de forma simultánea, transformaciones mecánicas, que reducen el tamaño de partícula de los alimentos mediante la masticación y los movimientos peristálticos, y transformaciones enzimáticas, que rompen las macromoléculas para liberar las unidades monoméricas. En ella intervienen diversos órganos y compartimentos: la boca, el esófago, el estómago, el intestino delgado y grueso, donde se produce la rotura de los alimentos y la absorción de estos (principalmente en el intestino delgado), el hígado y el páncreas, que segregan enzimas digestivas y sales biliares que permiten la hidrólisis de los alimentos. Recientemente la microbiota intestinal ha ganado relevancia en términos de digestión y el paradigma actual ha cambiado, dando especial importancia a las transformaciones metabólicas llevadas a cabo por los microorganismos, pues el microbioma intestinal contiene alrededor de 100 veces más genes que nuestro propio genoma, lo que conlleva que se pueden llevar a cabo más transformaciones mediadas por enzimas (Gill y col., 2006).

Conocer el comportamiento de los alimentos tras la digestión gastrointestinal permite aumentar el conocimiento científico en términos de nutrición y desarrollo de alimentos con un mejor balance de nutrientes, matrices estables y compuestos bioactivos con funcionalidad en el

organismo. Los ensayos en humanos son los que arrojan la mayor información, sin embargo, son costosos y entrañan limitaciones éticas y técnicas, sobre todo cuando se quiere estudiar la evolución de un determinado nutriente a lo largo del conducto gastrointestinal o se requiere evaluar la bioaccesibilidad de un compuesto tóxico como, por ejemplo, las micotoxinas. En consecuencia, los ensayos de digestión *in vitro*, los cuales tienen como objetivo reproducir las condiciones físicas y químicas de la digestión humana mediante el control de la temperatura, el pH, el tiempo de exposición, el movimiento y la concentración de electrolitos y enzimas, presentan ventajas sobre los modelos humanos, pues son rápidos, baratos, y no presentan restricciones éticas (Minekus y col., 2014). Estos ensayos se pueden llevar a cabo de modo estático o dinámico (Guerra y col., 2012). Las diferencias entre ambos modelos recaen principalmente en la adición de enzimas y el control del pH. En los modelos dinámicos el alimento o ingrediente investigado se mueve a diferentes compartimentos, a los que se les va añadiendo de forma automática las diferentes soluciones gastrointestinales (Guerra y col., 2012). Por otro lado, en los modelos estáticos, estas operaciones se realizan de forma manual. Existen diversas metodologías para reproducir en el laboratorio la digestión gastrointestinal humana, las cuales difieren entre sí, sobre todo, en el número y tipo de fases que se simulan (oral, gástrica, intestinal y colónica), en la composición de los fluidos digestivos empleados y en la forma de movimiento empleada para simular los movimientos peristálticos del organismo (Hur y col., 2011). Dada la gran diversidad de protocolos llevados a cabo por la comunidad científica, en el 2014 se harmonizó el método de digestión *in vitro* estático (Minekus y col., 2014), con el fin de que los resultados puedan ser reproducibles y comparables entre científicos. A través de los ensayos de digestión *in vitro* estáticos se puede estudiar:

(i) **la digestibilidad de los alimentos**, la cual hace referencia a la facilidad con la que un alimento o macronutriente es transformado en moléculas asimilables para las células. En la literatura científica existes múltiples estudios sobre la digestibilidad del almidón (Donlao y Ogawa, 2017; Cattaneo y col., 2019; Freitas y col., 2019; Selma-Gracia y col. 2020; Rocchetti y col., 2020) y la digestibilidad de las proteínas (Elkonin y col., 2013; Zahir y col., 2018; Zhang y col., 2020a; Aderinola y col., 2020),

(ii) **la bioaccesibilidad**, la cual es definida como la cantidad de un nutriente o compuesto químico ingerido que está disponible para ser absorbido (Etcheverry y col., 2012). En este contexto los compuestos fitoquímicos, tales como (poli)fenoles y carotenoides (Schweiggert y col., 2012; Gullon y col., 2015a,b; Kamiloglu y col., 2016; Pellegrini y col., 2017, 2018; Hilary y col., 2020), así como vitaminas y oligoelementos han sido ampliamente estudiados (Pupin y col., 2018; Gonçalves y col., 2020; Silva y col., 2020),

(iii) **el efecto de la matriz del alimento** sobre diversos aspectos como la bioaccesibilidad, las sinergias, la estabilidad de los compuestos, etc. (Stanisavljević y col., 2015; Pineda-Vadillo y col., 2017; Pešić y col., 2019),

(iv) **la viabilidad de la encapsulación** de ciertos compuestos bioactivos (Liang y col, 2013; Zou y col., 2014),

(v) **la supervivencia de probióticos al proceso digestivo** (Lo curto y col., 2011; Betoret y col., 2019).

Es por todo esto, que los ensayos de digestión *in vitro*, pueden ser usados para estudiar la funcionalidad de nuevos ingredientes y alimentos. Además, dado que son rápidos y baratos, se pueden usar para escoger entre diferentes formulaciones.

No obstante, como todo ensayo *in vitro,* presenta muchas limitaciones, pues no se reproducen todos los eventos que ocurren durante la digestión humana *in vivo*, como el control hormonal y nervioso, los mecanismos de retroalimentación, la actividad de las células mucosas, los movimientos peristálticos o la implicación del sistema inmune ubicuo (Guerra y col., 2012). Sin embargo, pese a que los modelos de digestión *in vitro* estático simplifican los complejos mecanismos de la digestión *in vivo*, se han visto correlaciones positivas entre los modelos de digestión *in vitro* y la digestión *in vivo* cuando se estudia, por ejemplo, la digestibilidad del almidón y de las proteínas, y la bioaccesibilidad de carotenoides y de (poli)fenoles totales (Bohn y col., 2018). Por todo ello, la digestión *in vitro* se considera una herramienta útil, sin restricciones éticas y con alta correlación con la realidad para el estudio y la comprensión de los alimentos una vez son digeridos (Bohn y col., 2018).

ZUMO DE CAQUI
Capítulo 2.
OBJETIVOS
<210 µm

2.1. OBJETIVO GENERAL

El objetivo principal de esta Tesis ha sido valorizar los coproductos de la industrialización del caqui, de dos cultivares diferentes, "Rojo Brillante" y "Triumph", como posibles ingredientes intermedios ("harinas de caqui") para el desarrollo de alimentos potencialmente saludables.

2.2. OBJETIVOS ESPECÍFICOS

2.2.1.- Obtención y caracterización de harinas de caqui

(i) Desarrollar y optimizar el proceso para la obtención de ingredientes intermedios ("harina de caqui") a partir de los coproductos de caqui de los cultivares "Rojo Brillante" y "Triumph"

(ii) Determinar la influencia del tamaño de partícula de las harinas de caqui en su composición química, propiedades fisicoquímicas, propiedades tecnofuncionales y actividad antioxidante y seleccionar las de mayor potencial tecnológico.

(iii) Evaluar el potencial funcional de las harinas de caqui seleccionadas, para el desarrollo de alimentos, a través de estudios de digestión y fermentación in *vitro.*

2.2.2. Aplicación de las harinas de caqui a matrices alimentarias de origen animal

(i) Desarrollar alimentos de origen animal (paté de hígado de cerdo) adicionados con diferentes concentraciones de las harinas de caqui seleccionadas de ambos cultivares, "Rojo Brillante" y "Triumph".

(ii) Caracterizar química, fisicoquímica, y sensorialmente los alimentos desarrollados.

(iii) Evaluar la influencia de las harinas de caqui en la calidad del alimento desarrollado.

2.2.3. Aplicación de las harinas de caqui a matrices alimentarias de origen vegetal

(i) Desarrollar alimentos de origen vegetal (espaguetis de trigo duro) adicionados con diferentes concentraciones de las harinas de caqui seleccionadas de ambos cultivares, "Rojo Brillante" y "Triumph".

(ii) Caracterizar química, fisicoquímica, y sensorialmente los alimentos desarrollados.

(iii) Evaluar la influencia de las harinas de caqui en la calidad del alimento desarrollado.

2.2.4. Evaluación funcional de la interacción de los compuestos bioactivos de las harinas de caqui con las diferentes matrices alimentarias durante la digestión gastrointestinal "*in vitro*"

(i) Evaluar el efecto de la matriz alimentaria (cárnica y amilácea) sobre la estabilidad de los compuestos (poli)fenólicos durante su digestión *in vitro*.

(ii) Evaluar la actividad antioxidante de los compuestos (poli)fenólicos presentes en la pasta durante su digestión *in vitro*.

(iii) Evaluar la digestibilidad del almidón en la pasta y la estabilidad de la grasa en el paté durante su digestión *in vitro*.

(iv) Evaluar la oxidación lipídica en los productos cárnicos durante su digestión *in vitro*.

Capítulo 3.- MATERIALES Y MÉTODO

En el siguiente apartado se expone un resumen de los procesos, ensayos, materiales y métodos llevados a cabo para la realización de la presente Tesis, los cuales serán agrupados por secciones. La metodología completa y con una descripción más detallada aparece en las publicaciones correspondientes.

3.1.- OBTENCIÓN DE LAS HARINAS DE CAQUI A PARTIR DE LOS COPRODUCTOS DE SU INDUSTRIALIZACIÓN

Con el fin de poder estudiar por separado los coproductos de los dos cultivares de caqui "Rojo Brillante" y "Triumph", se procedió a la obtención de zumo de caqui en la planta piloto de la Universidad Miguel Hernández, situada en la Escuela Politécnica Superior de Orihuela (EPSO), siguiendo el proceso industrial. La extracción del zumo y posterior acondicionamiento de los coproductos se efectuó con caquis de los cultivares "Rojo Brillante" y "Triumph" comprados en supermercados locales (Orihuela, Alicante, España) durante su temporada comercial (septiembre-enero) cuando estos presentaban un color naranja-rojizo (Figura 2). La manipulación de la fruta se realizó bajo correctas prácticas de manipulación de alimentos, y con una desinfección previa de la planta piloto, así como de los utensilios y aparatos a usar con hipoclorito de sodio (5%) y alcohol (70%). Los caquis se separaron por cultivar y por lotes de 5 kg cada uno. Primeramente, los caquis fueron lavados y acondicionados (eliminación del pedúnculo). Posteriormente fueron cortados y licuados, separando el zumo de la pulpa y la piel. Tanto el zumo como los coproductos se pesaron para determinar el rendimiento del proceso de elaboración. Una vez procesado todo el lote, se procedió a distribuir la pulpa y la piel del caqui en bandejas de aluminio. El grosor de la capa de pulpa y piel de caqui fue inferior a 1 cm, para favorecer la transmisión de calor y la evaporación del agua del producto de forma homogénea. Las bandejas con el producto se colocaron en un horno mixto (MYCHEF, Distform S.L, Lleida, España) a 45 ºC y 0% de humedad relativa. La muestra se deshidrató durante 36 horas. Una vez deshidratada, la torta de pulpa y piel fue troceada y molida con ayuda de una Termomix® (TM6, Vorwerk, Wuppertal, Alemania) (primera molienda) y un molinillo de café (Taurus SP-7407, Electrodomésticos Taurus S.L, Lleida, España) (molienda fina). Con la ayuda de tres tamices con diferente luz de malla (701, 417 y 210 µm) se procedió al tamizado y separación de la harina de caqui por tamaño de partícula. Las diferentes harinas producidas fueron empaquetadas en bolsas de vacío debidamente etiquetadas y en cantidades variables (100, 250 y 500 g). Éstas fueron termoselladas y almacenadas en refrigeración (6-8 ºC) (Figura 7). Las muestras se envasaron a vacío parcial, porque un vacío total ocasionaba la aglomeración de la harina y su endurecimiento, ocasionando dificultades en su manipulación y disminuyendo su calidad.

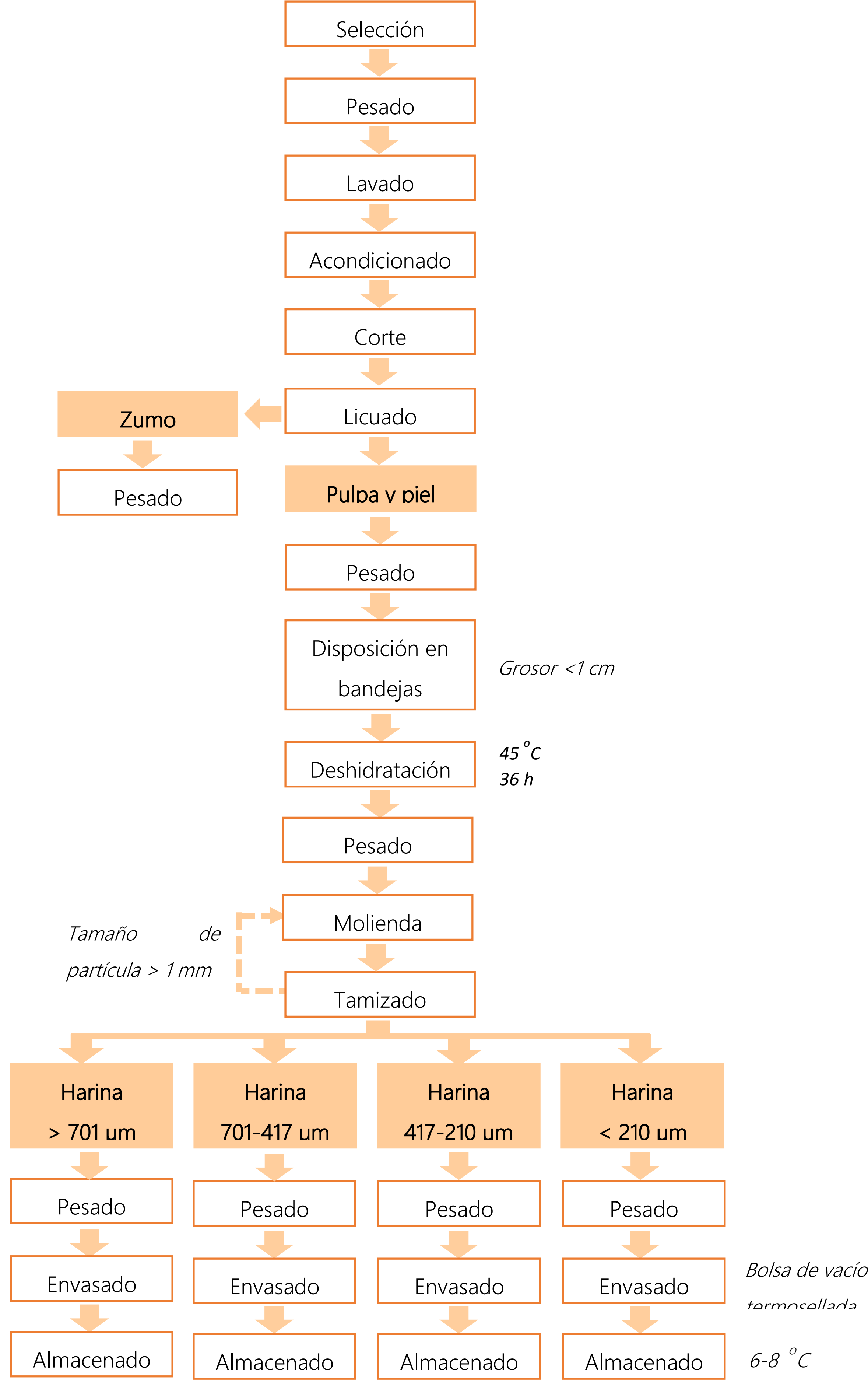


Figura 7.- Diagrama de flujo de la obtención de harina de caqui.

3.2.- CARACTERIZACIÓN DE LAS HARINAS DE CAQUI

Del apartado anterior se deduce que se obtuvieron harinas de dos cultivares de caquis ("Rojo Brillante" y "Triumph"), y de cada uno, cuatro tamaños de partícula, es decir que se procedió a la caracterización de 8 muestras diferentes de harinas de caqui.

3.2.1.- Composición química de las harinas de caqui

3.2.1.1.- Humedad

La humedad se determinó siguiendo la correspondiente norma AOAC (2007), donde se depositaba una cantidad conocida de la muestra y se deshidrataba durante 24 h a 105 ºC. Se expresaron los resultados como g agua/100 g muestra.

3.2.1.2.- Grasa total

La grasa total de las muestras se determinó en un extractor Soxhlet automático (SOXTHERM® SOX 6-place C., Gerhardt GmbH & Co. KG, Königswinter, Alemania) usando como agente extractante éter de petróleo siguiendo las directrices de su correspondiente norma AOAC (2007). Los resultados se expresaron como g grasa/100 g muestra.

3.2.1.3.- Proteína cruda

El método Kjeldahl se utilizó para la determinación de la proteína. Aproximadamente, un gramo de la muestra se digirió (Büchi Digestion Unit Modelo 426, BUCHI Ibérica, Barcelona, España) con ayuda de 15 mL de H_2SO_4 (98%) y dos pastillas catalizadoras, a 400 ºC durante 1 hora. Después la muestra digerida se destiló y neutralizó con ayuda de un analizador automático (Analizador Kjeltec TM 8400, Foss Iberia, Barcelona, España). Los resultados se expresaron como % de proteína, usando el factor de conversión de nitrógeno a proteína cruda de 6,25.

3.2.1.4.- Cenizas totales

Las cenizas totales se determinaron siguiendo el correspondiente método de la AOAC (2007), en el cual se produce una incineración de las muestras a 550 ºC durante 8 horas en una Mufla (MOD. 12 PR/300 SERIE 8B, Forns Hobersal, Barcelona, España). Los resultados se expresaron en g/100 g de producto.

3.2.1.5.- Fibra dietética total, fibra dietética insoluble y fibra dietética soluble

El contenido de fibra dietética total (FDT) y la fibra dietética insoluble (FDI) de las muestras se determinó por el método enzimático-gravimétrico propuesto por la AOAC (2007). Los resultados se expresaron en g/100 g de producto. El contenido de la fibra dietética soluble

(FDS) se obtuvo por diferencia, siguiendo la ecuación 1. Todos los valores se expresaron en g fibra/100 g de producto.

$$FDS = FDT - FDI \quad (Ecuación\ 1)$$

3.2.1.6.- Contenido de azúcares y ácidos orgánicos

Para la extracción de la muestra se siguió el siguiente proceso: 1,000 ± 0,005 g de muestra se mezcló con 40 mL de agua ultrapura en tubos de centrífuga de polietileno con tapón (80 mL). La solución se homogenizó durante 1 minuto a 12.000 rpm con ayuda de un Ultra-Turrax (Dispersador T 25 Basic ULTRA-TURRAX®, IKA®, Deutschland, Alemania). Posteriormente la solución homogenizada se centrifugó a 5.000 *g*, a 4 ºC durante 10 minutos (SIGMA 3-16PK, Sartorius, Goettingen, Alemania). El sobrenadante fue cuidadosamente recogido y filtrado con filtros de nailon con un tamaño de poro de 0,45 μm (Millipore Corporation, Bedford, USA). Las muestras se analizaron por cromatografía líquida de alta eficiencia (HPLC) en un equipo HP-1100 Hewlett-Packard (Hewlett-Packar, Woldbronn, Alemania) acoplado a un detector de diodos UV-visible G1315A (ajustado a 210 nm) y un detector de índice de refracción G-1362. Se usó una columna de intercambio iónico (Supelcogel C-610H, 300 × 7.8 mm; Supelco, Bellefonte, USA) acoplada a una precolumna (Supelguard-H, 50 × 4.6 mm, Supelco, Bellefonte, USA). Se trabajó con flujo isocrático (0,5 mL/min) a 30 ºC de la fase móvil compuesta por agua acidificada con ácido fosfórico al 98% (0,1 %). Se inyectaron 10 μL de muestra y el tiempo del método fue de 30 minutos. Los compuestos fueron identificados por coincidencia del tiempo de retención con los patrones de azúcares (glucosa, fructosa y sacarosa) y de ácidos orgánicos (L -ascórbico, cítrico, fumárico, málico, oxálico, succínico y tartárico) que fueron previamente analizados en las mismas condiciones que las muestras. La cuantificación se llevó a cabo usando las ecuaciones de la recta de los diferentes patrones estudiados. Los resultados para los azúcares se expresaron como g azúcar/100 g muestra, mientras que para los ácidos orgánicos se expresaron como mg ácido orgánico/100 g muestra.

3.2.1.7.- Contenido total de carotenoides

La extracción de las muestras se llevó a cabo siguiendo el protocolo descrito por Pfeifhofer (1989) con algunas modificaciones. La extracción se llevó a cabo en oscuridad, para ello el material fue cubierto con papel de aluminio. Se pesó con exactitud 1,000 ± 0,005 g en tubos de centrífuga de polietileno con rosca y seguidamente se añadieron 15 mL de acetona fría con 0,01 % del antioxidante Butilhidroxitolueno (BHT). Esta mezcla se homogeneizó a 8000 rpm durante 1 minuto con baño de hielo recubriendo el tubo de centrífuga para evitar aumentar la temperatura de la muestra. Después se centrifugó (Centrífuga SIGMA 3-16PK, Sartorius, Goettingen, Alemania) la muestra durante 10 minutos a 5.000 *g* y 4 ºC. El sobrenadante se recogió cuidadosamente y se

depositó en un balón de fondo plano de 100 mL. Al pellet se le volvió a adicionar otros 15 mL de acetona fría con 0,01 % de BHT y se repitieron los mismos pasos de homogenización y centrifugación descritos previamente. Los sobrenadantes fueron evaporados, con ayuda de un rotavapor (BÜCHI Rotavapor R-200, BUCHI Ibérica, Barcelona, España) hasta alcanzar la sequedad. Los sólidos resultantes fueron resuspendidos con 5 mL de acetona grado HPLC fría. Las muestras fueron filtradas con filtros de nailon con un tamaño de poro de 0,45 µm (Millipore Corporation, Bedford, USA). La lectura de las muestras se realizó en un espectrofotómetro HP 8451 (Hewlett Packard, Cambridge, Reino Unido) a 440 nm de absorbancia. El contenido de carotenos se cuantificó usando la ecuación de la recta realizada con 5 concentraciones diferentes de β-caroteno puro (Sigma-Aldrich; C4582). El contenido total de carotenoides se expresó como mg de β-caroteno equivalentes/g de muestra.

3.2.1.8.- Extracción de compuestos bioactivos (extractos metanólicos)

Para la extracción de los compuestos bioactivos se pesaron 3,000 $\pm$ 0,005 g de muestra en tubos de centrífuga de polietileno con rosca. A continuación, se añadieron a la muestra 10 mL de metanol grado HPLC (80%) y la mezcla fue homogenizada durante 1 minuto a 12.000 rpm (Dispersador T 25 Basic ULTRA-TURRAX®, IKA®, Deutschland, Alemania). Posteriormente se centrifugó la muestra 10 minutos a 5.000 g y 4 ^{o}C. El sobrenadando se depositó en un matraz de fondo plano de 100 mL y al pellet se le adicionaron 10 mL de acetona grado HPLC (70 %). La muestra volvió a ser homogenizada y centrifugada en las mismas condiciones previamente descritas. Los sobrenadantes se mezclaron y se llevó a desecación la muestra en un rotavapor (BÜCHI Rotavapor R-200, BUCHI Ibérica, Barcelona, España). Los sólidos fueron resuspendidos en 5 mL de metanol grado HPLC, filtrados a través de un filtro de nailon con un tamaño de poro de 0,45 µm (Millipore Corporation, Bedford, USA) y almacenados a -20 ^{o}C.

3.2.1.9.- Contenido de fenoles totales

El contenido de fenoles totales en los extractos metanólicos se evaluó usando el reactivo de Folin–Ciocalteu's y siguiendo el proceso descrito por Singleton y Rossi (1965). La cuantificación se realizó espectrofotométricamente, (espectrofotómetro HP 8451; Hewlett Packard, Cambridge, Reino Unido), realizando las lecturas a 760 nm y usando como patón el ácido gálico (Sigma-Aldrich, 8.42649). Los resultados se expresaron como mg ácido gálico equivalente/g muestra.

3.2.1.10.- Contenido de flavonoides totales

Los extractos metanólicos se usaron para cuantificar el contenido de flavonoides totales de las muestras, el cual se realizó siguiendo las directrices propuestas por Blasa y col. (2006). La lectura de las muestras se realizó con un espectrofotómetro HP 8451 (Hewlett Packard,

Cambridge, Reino Unido) a 510 nm. Como patrón se usó la rutina (Sigma-Aldrich, R5143). Los resultados se presentaron como mg rutina equivalente/g muestra.

3.2.1.11.- Perfil de compuestos fenólicos

Para este análisis se realizó también una extracción de los compuestos fenólicos siguiendo el mismo proceso descrito en el Apartado 3.2.1.8, pero con las siguientes modificaciones: se pesó 1,000 ± 0,005 g de muestras; el extracto una vez resuspendido se pasó a través de un cartucho C18 (Thermo Scientific™ Cartuchos HyperSep™ C18, Fisher Scientific, Madrid) previamente acondicionado con 5 mL de metanol, 5 mL de agua ultrapura y 5 mL de HCl (0,01 N). El cartucho se lavó con 5 mL de agua ultrapura y la muestra fue eluida con 5 mL de metanol grado HPLC acidificado. Las muestras fueron depositadas en un vial de HPLC y almacenadas a -20ºC. Las muestras se analizaron con ayuda de un equipo Hewlett-Packard HPLC series 1200 al que se le acopló una columna C18 (Mediterranean Sea18, 25× 0,4 cm, tamaño de partícula 5 µm; Teknokroma, Barcelona, España). Las condiciones del método fueron las mismas que describen Genskowsky y col. (2016). Para la fase móvil A se usó agua ultrapura acidificada con ácido fórmico grado analítico (98 %) (Agua:Fórmico; 99:1) y para la fase móvil B se usó acetonitrilo grado HPLC. El gradiente de elución fue de 1 mL/min y se inyectaron 20 µL de muestra. Se trabajó a temperatura ambiente con tres longitudes de onda: 280, 320 y 360 nm. Previamente se inyectaron 19 patrones (ácido gálico, ácido protocateico, catequina, ácido 4-hidroxibenzoico, epicatequina, ácido clorogénico, ácido vanílico, ácido cafeico, ácido siríngico, vanillina, ácido *p*-cumárico, ácido ferúlico, ácido sinápico, quercetina, rutina, kaempferol, miricitina, luteína y luteína-o-glicosilada) siguiendo las mismas condiciones. Los compuestos fueron identificados comparando el tiempo de retención y el espectro de absorbancia con los patrones. La cuantificación se realizó por regresión lineal. Los resultados se expresaron como µg compuestos fenólicos/g muestra.

3.2.1.12.- Perfil de compuestos fenólicos libres y unidos

La extracción de los compuestos fenólicos libres de la harina de caqui se realizó sobre 2,000 ± 0,005 g siguiendo el protocolo descrito por Pellegrini y col. (2017), con algunas modificaciones; se aumentó el tiempo de sonicación (30 minutos) y el proceso se realizó a 4-8 ºC; las muestras se resuspendieron en agua ultrapura (15 mL) y se pasaron por un cartucho C18 (Thermo Scientific™ Cartuchos HyperSep™ C18, Fisher Scientific, Madrid) como se describe en el Apartado 3.2.1.11, no obstante, la muestra fue eluida con 1,5 mL de metanol acidificado (1 %). El pellet que quedó tras la extracción de los compuestos libres se usó para la extracción de los compuestos fenólicos unidos. La cual consiste en una hidrolisis alcalina seguida de una hidrolisis ácida, para finalizar con una extracción líquido-líquido usando acetato de etilo como agente extractante. El proceso se llevó a cabo siguiendo el protocolo descrito por Krygier y col. (1982)

con las recomendaciones de Mpofu y col. (2006). El extracto se llevó a desecación, se resuspendió en 2 mL de metanol puro y se filtró (filtros de nailon de 0,45 µm; Millipore Corporation, Bedford, USA). La detección, identificación y cuantificación de ambas fracciones de compuesto, libres y unidas se determinó siguiendo las indicaciones del Apartado 3.2.1.11. Con la inclusión de los siguientes patrones: catequina-3-galato, (-) - epicatequina-3-galato, (-) - epigalocatequina-3-galato, (-) - galocatequina, (-) - galocatequina-3-galato, pelargonidina-3-glucósido, cianidina-3-glucósido, peonidina-3-glucósido, delfinidina-3-glucósido, petunidina-3-glucósido, malvidina-3-glucósido, malvidina, malvidina-3,5-diglucósido, naringenina, hesperidina, neorecitrina y neohesperidina; y dos longitudes de onda, 325 y 520 nm.

3.2.2.- Propiedades fisicoquímicas de las harinas de caqui

3.2.2.1.- Determinación de la actividad de agua

La actividad de agua de las muestras se llevó a con el medidor de actividad de agua Sprint TH-500 Novasina Thermoconstanter (Novasina, Neuheimstrasse, Suiza) a 25 °C.

3.2.2.2.- Análisis del pH

Una solución de muestra y agua destilada (1:10) se preparó para medir el pH tras 10 minutos de reposo. Se usó el pH-metro modelo pH/Ion, Eutech (Instruments Pte Ltd., Singapur) para realizar las lecturas.

3.2.2.3.- Evaluación instrumental del color

Para la determinación de las propiedades de color de las muestras se seleccionó el espacio de color CIELAB y se utilizó un espectrofotocolorímetro CM-2600d (Minolta CameraCo, Osaka, Japón). Para las lecturas se seleccionó el iluminante D_{65}, observador 10º, en modo SCI, con apertura de 11 mm para el iluminador y 8 mm para la medida. Se determinaron las coordenadas de color luminosidad (L*), rojo/verde (a*) y amarillo/azul (b*) y a partir de ellas se calcularon los atributos de color, tono (h*) y croma (C*), siguiendo las ecuaciones 2 y 3. Además, también se evaluó el espectro de reflexión, tomando los valores de refracción en el espectro visible (360-740 nm).

$$h^* = Arctang\frac{b^*}{a^*} \quad (Ecuación\ 2)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \ (Ecuación\ 3)$$

3.2.3.- Propiedades tecnofuncionales de las harinas de caqui

3.2.3.1.- Capacidad de retención de agua (CRA) y capacidad de retención de aceite (CRO)

Ambos análisis se llevaron a cabo siguiendo las recomendaciones de Robertson y col. (2000). Para cada ensayo se usó 0,500 ± 0,005 g de muestra y 10 mL de agua o aceite de girasol. Los resultados se expresaron como g de agua o aceite retenidos/g muestra.

3.2.3.2.- Capacidad de hinchamiento (CH)

La capacidad de hinchamiento de las muestras se expresó como mL de agua/g de muestra y se determinó siguiendo la metodología descrita por Gómez-Ordoñez y col. (2010)

3.2.3.3.- Capacidad emulsificante y estabilidad de la emulsión (CE y EE)

La capacidad de las muestras para formar emulsiones, así como la estabilidad de la emulsión formada se estudiaron siguiendo el protocolo propuesto por Chau y col. (1997). Los resultados se expresaron en %.

3.2.4.- Propiedades fisiofuncionales de las harinas de caqui

3.2.4.1.- Capacidad de retención de bilis (CRB)

La capacidad de las harinas de caqui para retener bilis se estudió siguiendo la metodología descrita por Eastwood y col. (1973) con algunas modificaciones. En resumen, 0,160 ± 0,005 g de muestra se pesaron en tubos de centrífuga de 16 mL de volumen. A continuación, se añadieron 5 mL de bilis porcina fresca. La mezcla se homogenizó en un vortex durante 2 minutos y se dejó reposar 18 horas a temperatura ambiente. Transcurrido este tiempo, la muestra se centrifugó 20 minutos a 1.500 *g*. Se eliminó cuidadosamente el sobrenadante y se tomó el peso del pellet. Los resultados se muestran como g de bilis retenido/g muestra.

3.2.4.2.-Capacidad de retener grasa/aceite (FOB)

La capacidad de la muestra de retener aceite dentro de su matriz y bajo condiciones ácidas (simulando condiciones de digestión gástrica) se evaluó siguiendo el protocolo de López-Marcos y col. (2015). El resultado se expresó como g de aceite retenido/g de muestra.

3.2.5.- Análisis microbiológico de las harinas de caqui

Se realizó un muestreo microbiológico (en las muestras de harina de caqui con menor tamaño de partícula), siguiendo los métodos microbiológicos descritos por la Asociación Americana de Salud Pública (APHA, 2015). Se rastrearon los siguientes grupos de bacterias: bacterias mesófilas aeróbicas, enterobacteriáceas, coliformes totales, hongos, levaduras y

Clostridium sulfito reductores. Los resultados se expresan como Log_{10} de Unidades Formadoras de Colonias (UFC)/g muestra.

3.2.6.- Actividad antioxidante de las harinas de caqui

Para la evaluación de la actividad antioxidante de las harinas se aplicaron cuatro métodos diferentes. Todos los análisis se basaron en medidas espectrofotométricas, para lo cual se usó un espectrofotómetro HP 8451 (Hewlett Packard, Cambridge, Reino Unido).

3.2.6.1.- Método del radical 2,2'-Azino-bis (3-etilBenzoTiazolin)-6-Sulfonato de amonio (ABTS)

El protocolo propuesto por Leite y col. (2011) se utilizó para evaluar la capacidad de las muestras para capturar el radical catiónico $ABTS^{\bullet+}$. La absorbancia, tanto de las muestras, como del antioxidante de referencia (Trolox) a concentraciones descendentes, se evaluó a 734 nm. Los resultados se expresaron en mg Trolox equivalente/g muestra.

3.2.6.2.- Método del radical 2,2-Difenil-1-Picrilhidrazilo (DPPH)

La capacidad de las muestras para donar hidrógenos o atrapar el radical DPPH se evaluó siguiendo la metodología descrita por Brand-Williams y col. (1995). Las lecturas de absorbancia se realizaron a 517 nm. El antioxidante Trolox se tomó como referencia. Los resultados se expresaron en mg Trolox equivalente/g muestra.

3.2.6.3.- Método de la capacidad de quelación de iones ferrosos (FIC)

Se siguió la metodología de Carter (1971) para determinar la actividad quelante de las muestras. Las lecturas se tomaron a una longitud de onda de 562 nm. La actividad antioxidante se cuantificó por regresión lineal, con la ecuación de la recta elaborada con el ácido etilendiaminotetraacético (EDTA), un conocido quelante. Se expresaron los resultados como µg EDTA equivalente/g muestra.

3.2.6.4.- Método del poder antioxidante por reducción del ion férrico (FRAP)

Este ensayo, basado en la transferencia de electrones, mide la capacidad de las moléculas para reducir el complejo férrico. Se determinó dicha actividad siguiendo el método de Oyaizu (1986). Los cambios de color de las muestras se midieron a una longitud de onda de 700 nm. Se realizó una curva de calibrado en las mismas condiciones que las muestras con el antioxidante Trolox. Se expresaron los resultados como mg Trolox equivalente/g muestra.

3.3.- FUNCIONALIDAD DE LAS HARINAS DE CAQUI

Tras analizar los resultados de composición química, actividad antioxidante, propiedades tecnofuncionales y fisiofuncionales de los cuatro tamaños de partícula de ambas harinas de caqui (Rojo Brillante y Triumph), se seleccionaron las harinas de ambos cultivares con un tamaño de partícula inferior a 210 μm como las de mayor potencial para su aplicación como ingredientes funcionales. Es por ello, que la funcionalidad de las harinas se estudió únicamente en estas dos harinas.

3.3.1.- Digestión *in vitro* de las harinas de caqui

Las harinas de caqui Triumph y Rojo Brillante se sometieron a un proceso de digestión gastrointestinal *in vitro* siguiendo el protocolo descrito por Gullon y col. (2015a). Se simularon las 3 fases del proceso digestivo: oral, gástrica, e intestinal. Después, para simular el proceso de absorción de nutrientes se utilizó una membrana de diálisis. Al final de cada proceso, las muestras fueron congeladas y liofilizadas hasta su análisis.

3.3.1.1.- Estabilidad de los compuestos fenólicos tras la digestión *in vitro*.

Se determinó la estabilidad de los compuestos fenólicos presente en ambas harinas de caqui (Rojo Brillante y Triumph), después de cada una de las etapas de la digestión (oral, gástrica e intestinal). De la etapa intestinal se estudiaron dos fases: IN, correspondiente a la sección que quedaba dentro de la membrana de diálisis y OUT, que corresponde a la sección que difundía fuera de la membrana de diálisis (Figura 8).

La extracción de los compuestos fenólicos de las muestras sin digerir y digeridas se realizó siguiendo el protocolo descrito por Pellegrini y col. (2017). Se usaron como agentes extractantes metanol (80%) y acetona (70%) y los ultrasonidos (15 minutos) como fuerza para liberar los compuestos fenólicos de la matriz de la harina.

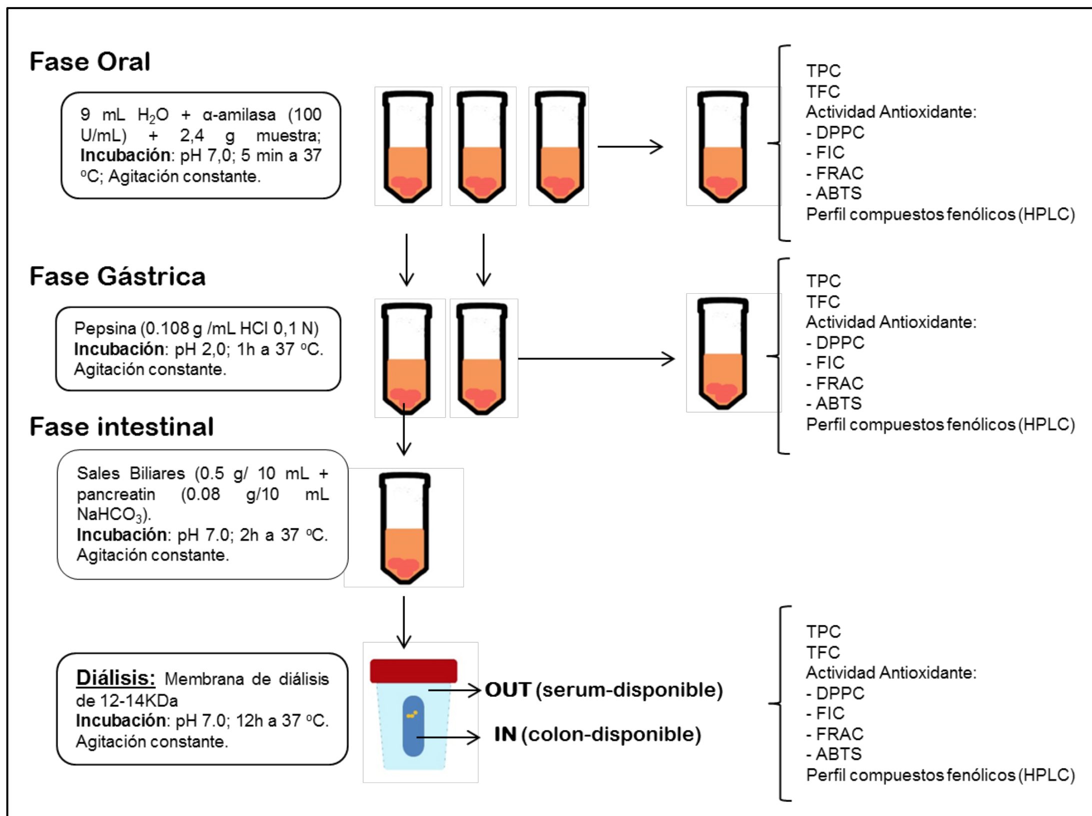


Figura 8.- Representación esquemática del proceso de digestión *in vitro* y las determinaciones llevadas a cabo.

3.3.1.1.1.- Contenido de fenoles y flavonoides totales

El contenido de fenoles y flavonoides totales en las muestras de harina digeridas y sin digerir se determinó siguiendo los procedimientos descritos en los Apartados 3.2.1.9. y 3.2.1.10., respectivamente. Los resultados también se expresaron como mg ácido gálico equivalentes/g muestra en el caso de los fenoles totales y como mg rutina equivalentes/g muestra para los flavonoides totales.

3.3.1.1.2.- Actividad antioxidante

Para determinar la actividad antioxidante de las muestras de harina digeridas y sin digerir se aplicaron los mismo 4 métodos (ABTS, DPPH, FIC y FRAP) descritos en los apartados 3.2.6.1., 3.2.6.2., 3.2.6.3. y 3.2.6.4. Los resultados para la actividad antioxidante según los métodos ABTS, DPPH y FRAP se expresaron en mg Trolox equivalente/g muestra. Para el ensayo FIC los resultados se expresaron como mg EDTA equivalente/g muestra.

3.3.1.1.3.-Perfil de compuestos fenólicos

La detección, identificación y cuantificación de los compuestos fenólicos de las muestras de harina no digeridas y digeridas se realizó de la misma forma que se describe en el Apartado

3.2.1.11. Los espectros de absorción ultravioleta de compuestos incluidos en las publicaciones de Medina-Medrano y col. (2015) y Martínez-Las Heras y col. (2016) se usaron para realizar una identificación tentativa de aquellos compuestos de los que se carecía de patrón de referencia.

3.3.1.1.4.- Índice de recuperación e índice de bioaccesibilidad de los compuestos fenólicos

El índice de recuperación de los compuestos fenólicos se define como la cantidad de compuestos recuperados tras la digestión, en cualquiera de sus fases, y se determina por comparación con el contenido de compuestos fenólicos presentes en las muestras antes de la digestión (ecuación 4). Mientras que, el índice de bioaccesibilidad está definido como la cantidad de compuesto que son capaces de atravesar la membrana de diálisis (ecuación 5). Ambos índices se calcularon tanto para el contenido de fenoles como de flavonoides totales de cada una de las fases digestivas estudiadas, siguiendo las directrices de Ortega y col. (2011).

$$\textbf{Índice de recuperación}\ (\%) = \frac{TPC\ o\ TFC_{fd}}{TPC\ o\ TFC_{h}} \times 100\ (\textit{Ecuación 4})$$

Donde,

TPC o TFC_{fd} = Contenido de fenoles o flavonoides totales en la fracción digerida (oral, gástrica o intestinal) (mg)

TPC o TFC_{fd} = Contenido de fenoles o flavonoides totales en la harina de caqui sin digerir (mg)

$$\textbf{Índice de bioaccesibilidad}\ (\%) = \frac{TPC\ o\ TFC_{OUT}}{TPC\ o\ TFC_{OUT+IN}} \times 100\ (\textit{Ecuación 5})$$

Donde,

TPC o TFC_{out} = Contenido de fenoles o flavonoides totales en la fracción de diálisis OUT (mg)

TPC o TFC_{OUT+IN} = Contenido de fenoles o flavonoides totales en la fracción intestinal dializada (IN + OUT) (mg)

3.4.- INCORPORACIÓN DE LAS HARINAS DE CAQUI A DIFERENTES MATRICES ALIMENTARIAS

Con el propósito de estudiar el potencial de las harinas de caqui Rojo Brillante y Triumph de tamaño de partícula < 210 µm, como ingredientes intermedios, se incorporaron en alimentos con diferente matriz alimentaria: una matriz cárnica (paté de hígado de cerdo) y otra vegetal (espaguetis de trigo duro). Las concentraciones a las que se incorporaron ambas harinas (3 y 6%) se seleccionaron en base a pruebas previas tentativas y a la experiencia del grupo de investigación en la elaboración de alimentos con otros coproductos agroindustriales.

3.4.1.- Elaboración de paté de cerdo con adicción de harinas de caqui

Se realizaron cinco formulaciones de paté de hígado de cerdo siguiendo la formulación que se especifica en la Tabla 3:

- Paté control (PC): ausencia de harina de caqui
- Paté de hígado de cerdo con un 3 % de harina de caqui Rojo Brillante (PR-3).
- Paté de hígado de cerdo con un 3% de harina de caqui Triumph (PT-3).
- Paté de hígado de cerdo con un 6 % de harina de caqui Rojo Brillante (PR-6).
- Paté de hígado de cerdo con un 3% de harina de caqui Triumph (PT-6).

Todas las formulaciones de paté de hígado de cerdo estudiadas en la presente Tesis se elaboraron en la planta piloto de la Escuela Politécnica Superior de Orihuela (Universidad Miguel Hernández). Se trabajó de acuerdo con las normas higiénicas para la manipulación de alimentos.

Se realizaron 5 lotes diferentes, correspondientes a cada una de las formulaciones estudiadas, de 1 kg cada uno (Figura 9). Antes de proceder con el picado y mezclado de los ingredientes se retiró la piel de la panceta y la papada, el hígado se puso a remojo durante 10 minutos en agua fría y la papada se coció durante 15 minutos a 100 ºC. A continuación, se pesaron los ingredientes y se trocearon las piezas cárnicas. Se utilizó una cutter (Cutter de mesa R 4 V V., Robot Coupe, Francia) para picar y mezclar los ingredientes. Primero se picó la panceta, la papada y la sal, hasta que el tamaño de las partículas fue $<$ 2 mm aprox., a continuación, se añadió el hígado, luego los aditivos, y por último las especias y la harina. Cada vez que se añadía un ingrediente se homogeneizaba durante 1 minuto con el resto de los ingredientes. La emulsión se formó con la agregación y mezclado de agua caliente (80 ºC). La pasta fina se trasvasó a la embutidora manual (Embutidora manual de acero inóxidable, Palumbo, Pavi, Italia.) y se embutió en tripa artificial Fibran-Pack (Fibran, Girona, España) de 5 cm diámetro. Se realizaron unidades de 10-15 cm de longitud que fueron cerradas con clips (Polyclip system/Niedecker, Hattersheim am Main, Alemania). La cocción del producto se realizó a 95 ºC durante aproximadamente 30-40 minutos en un baño con agua y sonda de temperatura (MA/125, M. Serra, Folgueroles, Barcelona). La cocción se dio por finalizada cuando el interior del producto alcanzó los 72 ºC. Posteriormente la temperatura del paté se bajó con ayuda de una abatidor (air-o-chill, Electrolux, Alcobendas, Madrid, España). Las muestras se almacenaron en refrigeración.

Tabla 3.- Formulación de los cinco lotes de paté de hígado de cerdo elaborados.

Ingredientes	PC	PR-3	PT-3	PR-6	PT-6
Papada (%)	65	65	65	65	65
Hígado (%)	25	25	25	25	25
Panceta (%)	10	10	10	10	10
Agua (%)*	15	15	15	15	15
Sal (%)*	2	2	2	2	2
Polifosfato (ppm)*	300	300	300	300	300
Nitrito (ppm)*	125	125	125	125	125
Ascorbato (ppm)*	500	500	500	500	500
Caseinato (%)*	1	1	1	1	1
Pimienta blanca (%)*	0,05	0,05	0,05	0,05	0,05
Nuez moscada (%)*	0,03	0,03	0,03	0,03	0,03
Tomillo (%)*	0,03	0,03	0,03	0,03	0,03
Harina Rojo Brillante < 210 μm (%)*	-	3	-	6	-
Harina Triumph <210 μm (%)*	-	-	3	-	6

*Los porcentajes de ingredientes no cárnicos se expresan en relación con el 100% de carne.

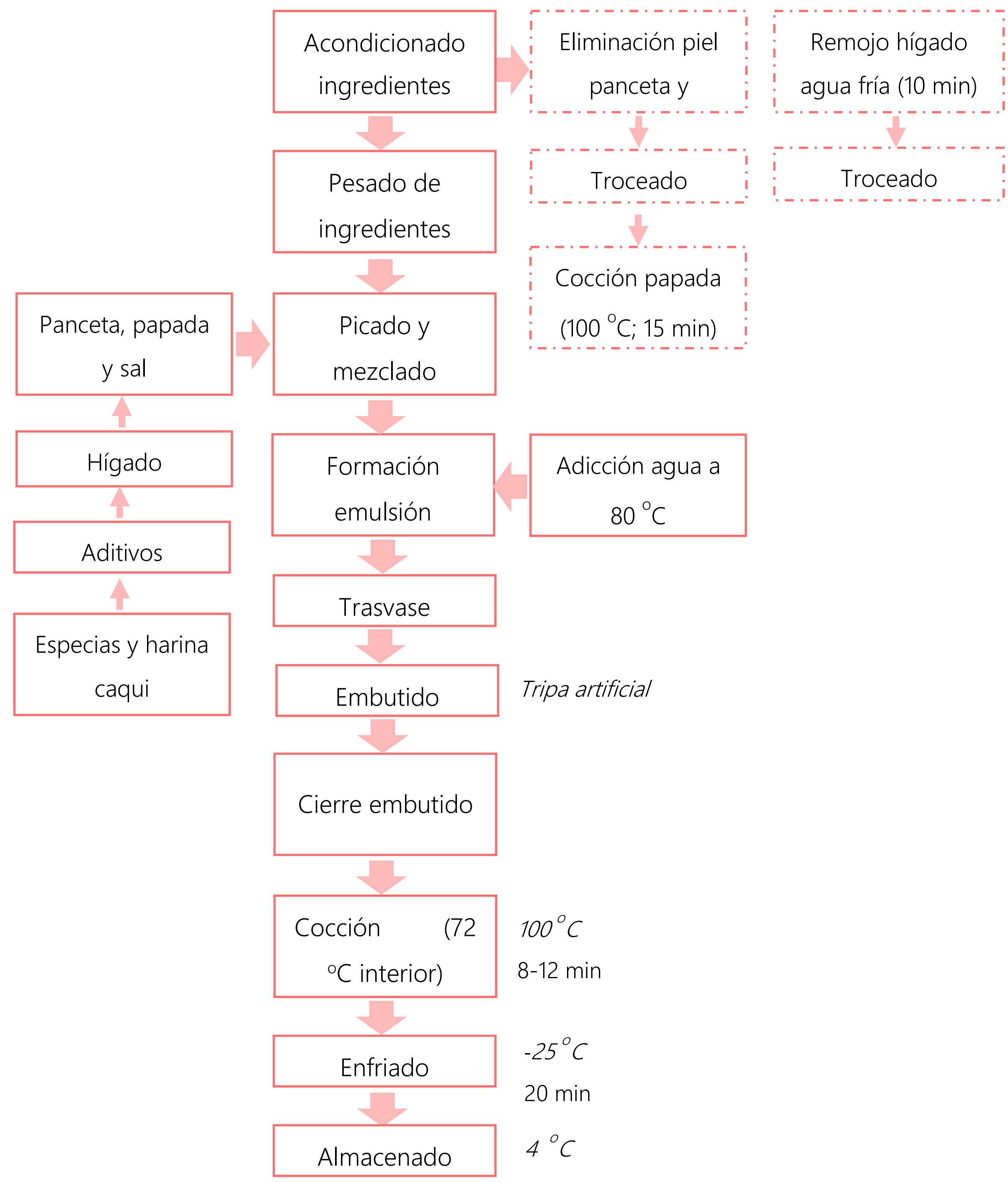


Figura 9.- Diagrama de flujo de la elaboración del paté de hígado de cerdo.

3.4.2.- Caracterización del paté de cerdo con harina de caqui

3.4.2.1.- Composición química del paté

3.4.2.1.1.- Composición proximal

La determinación de humedad, grasa total, proteína bruta y cenizas se realizó siguiendo los correspondientes métodos AOAC (2007). Todos los resultados se expresaron como g/100 g paté.

3.4.2.1.2.- Perfil de ácidos grasos

La extracción de grasa se realizó siguiendo el método Folch (Folch y col., 1957). Para la metilación de los ácidos grasos se usaron aproximadamente 100 µg de la grasa extraída. En resumen, se añadió 1 mL de metóxido de sodio (0,5 N) y 100 µL de diclorometano a la muestra. Tras agitar vigorosamente con la ayuda de un vortex la muestra se incubó durante 10 minutos a 90 ºC. Transcurrido el tiempo de incubación la muestra se enfrió con ayuda de hielo, y se añadió 1 mL de fluoruro de boro (14% en metanol), se agitó vigorosamente y se incubó en las mismas condiciones descritas anteriormente. Tras enfriar las muestras se añadió 1 mL de agua destilada y 0,6 mL de hexano. Se agitó la muestra durante 1 minuto en el vortex, y se dejó reposar hasta que las fases se separaron. Con ayuda de una pipeta Pasteur de vidrio se recuperó la fase superior (hexano + grasa) y se depositó en un vial opaco de HPLC. Las muestras se inyectaron en un cromatógrafo de gases serie HP - 6890 (Woldbronn, Alemania) equipado con un detector de ionización de llama (FID) y una columna capilar Suprawax 280 (30 m × 0,25 µm de espesor de película × 0,25 mm d.i.; Tecknokroma Barcelona, España) siguiendo las condiciones descritas por Botella-Martínez y col. (2021).

3.4.2.1.3.- Concentración de nitrito residual

El contenido de nitrito residual en la muestra se cuantificó siguiendo el estándar ISO/DIS 2918.26 (1975). Tras una extracción previa con agua y la precipitación de las proteínas con los reactivos Carrez I y Carrez II, el extracto se mezcló con el agente colorante y se dejó reposar en la oscuridad. La absorbancia se midió a 520 nm en un espectrofotómetro HP (HP 8451; Hewlett Packard, Cambridge, UK). Los resultados se expresaron en ppm de $NaNO_2$.

3.4.2.1.4.- Perfil de compuestos fenólicos libres y unidos a la matriz alimenticia

La extracción, detección e identificación de los compuestos fenólicos libres y unidos se realizó como se describe en el Apartado 3.2.1.12.

3.4.2.2.- Propiedades fisicoquímicas del paté

3.4.2.2.1.- Actividad de agua, pH y color

La actividad de agua, el pH y el color de las cinco muestras de paté de hígado de cerdo se realizaron siguiendo los procedimientos descritos en los Apartados 3.2.2.1., 3.2.2.2. y 3.2.2.3., respectivamente.

3.4.2.2.2.- Textura

La textura del paté se estudió mediante dos ensayos diferentes: un Análisis de perfil de textura (TPA), el cual simula el proceso de masticación, y un Ensayo de untabilidad. Para ambos estudios se utilizó el Texturómetro TA-XT2i (Stable Micro Systems, Surrey, Gran Bretaña).

3.4.2.2.2.1.- TPA

El análisis consistió en dos ciclos de compresión (75 %) a una velocidad de 5 mm/s, con la sonda cilíndrica (P100) de 100 mm de diámetro sobre las muestras de paté cortadas en cilindros de 1 cm de altura. Se obtuvieron los siguientes parámetros: dureza (g), elasticidad (mm), cohesividad (mm) y gomosidad (g) siguiendo las directrices de Bourne (1978).

3.4.2.2.2.2.- Ensayo de untabilidad

Para realizar este ensayo las muestras de paté se colocaron en los correspondientes soportes de la sonda de cono de 90º. Se comprimió el paté a una velocidad de 3 mm/s usando una célula de carga de 30 kg, la velocidad de retorno fue de 10 mm/s y la distancia de la sonda a la muestra de 23 mm. Los siguientes accesorios fueron utilizados para realizar el ensayo: Plataforma de esparcimiento TTC (HDP/SR*) y plataforma de trabajo pesado (HDP/90). Los resultados se expresaron como fuerza de corte (g*s).

3.4.2.3.- Oxidación lipídica

La oxidación lipídica del paté se estudió con el análisis de las sustancias reactivas al ácido 2-Tiobarbitúrico (TBARS) siguiendo el protocolo propuesto por Rosmini y col. (1996). La extracción del malondialdehido (MDA) en las muestras de paté se realizó con ácido tricloroacético. Los resultados se expresaron en mg MDA/kg paté.

3.4.2.4.- Estabilidad de la emulsión

La estabilidad de la emulsión de las muestras de paté se determinó midiendo los fluidos (agua y/o aceite) liberados de la matriz del paté tras ser sometida a estrés térmico y mecánico, siguiendo las directrices de Hughes y col. (1997). Se estudió la estabilidad de la emulsión de las muestras a dos temperaturas diferentes, 4 ºC y 30 ºC y los resultados se expresaron como el total de fluidos liberados (%).

3.4.2.5.- Análisis sensorial

Los atributos organolépticos de las cinco formulaciones de paté elaboradas fueron analizados por un panel de catadores formado por trabajadores y estudiantes de la UMH, seleccionados en base a su frecuencia de consumo de paté y su experiencia en el análisis sensorial. En total 30 panelistas (12 hombres y 18 mujeres) con un rango de edad de 20 a 55 años realizaron un análisis descriptivo cuantitativo (IFT, 1981) sobre las muestras de paté, presentadas en piezas rectangulares de aproximadamente 1,5 x 2 cm. Se dispuso de biscotes de pan tostados y agua para que los panelistas pudieran enjuagarse la boca entre muestra y muestra. Los atributos evaluados en el análisis fueron los siguientes: intensidad de color (1: extremadamente claro; 7: extremadamente oscuro), brillo (1: extremadamente mate; 7: extremadamente brillante), rancidez (1:imperceptible; 7: muy perceptible), dureza (1: extremadamente blando; 7: extremadamente duro), jugosidad (1: extremadamente seco; 7: extremadamente jugoso), detección de partículas (1: imperceptible; 7: extremadamente perceptible), cohesividad (1: extremadamente desintegrado; 7: extremadamente cohesivo), salado (1: imperceptible 7: extremadamente salado) y dulce (1: imperceptible; 7: extremadamente dulce). El ensayo se realizó en la sala de catas de la EPSO (Universidad Miguel Hernández).

3.4.3.- Elaboración de espaguetis de trigo duro con harinas de caqui

Se elaboraron cinco formulaciones de espaguetis de sémola de trigo duro (cv. "Marco Aurelio") en la planta piloto del Centro de Investigación en Ingeniería y Procesamiento Agroalimentario (Roma, Italia):

- Espaguetis control: formulación tradicional, sémola de trigo duro y agua (SC).
- Espaguetis con adición de 3 % de harina de caqui Rojo Brillante (SR-3).
- Espaguetis con adición de 3 % de harina de caqui de Triumph (ST-3).
- Espaguetis con adición de 6 % de harina de caqui Rojo Brillante (SR-6).
- Espaguetis con adición de 6 % de harina de caqui Triumph (ST-6).

Para la formulación de los espaguetis (Tabla 4) se tuvo en cuenta la capacidad de retención de agua de la sémola de trigo duro y de las harinas de caqui Rojo Brillante y Triumph, con el propósito de ajustar el agua añadida a la masa y alcanzar en la misma una cantidad de agua de 32 g/100 g. Se trataba de evitar así la aparición de posibles defectos en los espaguetis por falta de hidratación en la masa. Las cinco formulaciones estudiadas se elaboraron (Figura 10) por separado, en lotes de 2 kg usando una extrusora Namad (Roma Italia), compuesta por dos recipientes y un extrusor de tornillo sin fin único, con una longitud de cañón de 350 mm y un diámetro de 40 mm, provisto de un sistema de refrigeración por agua, al que se le acopló una placa de cobre redonda de 100 mm de diámetro provista de 18 insertos de bronce de 12 mm con 7

troqueles de 1,2 mm. Los ingredientes secos (sémola y harina de caqui) una vez pesados se mezclaron durante 15 minutos en el primer recipiente de la extrusora. Transcurrido el tiempo se añadió el agua (18 ºC) y los ingredientes se mezclaron durante 12 minutos en el primer recipiente y 10 minutos en el segundo recipiente. La masa formada se extruyó a una velocidad de 15 kg/h (la velocidad del tornillo sin fin se ajustó a 42 rpm). La velocidad de salida de la pasta fue de 1 m/min. Las hebras de pasta húmedas fueron cortadas (30 cm de longitud) y secadas a 50 ºC durante 24 horas en un secador experimental asistido por ventilación (AFREM, Lyon, Francia). Los espaguetis secos se empaquetaron en bolsas de plástico selladas y se dejaron estabilizar durante 10 días. Con el fin de evitar la absorción de agua fueron almacenados a temperatura ambiente en condiciones de baja humedad y oscuridad.

Tabla 4.- Ingredientes de las cinco formulaciones de espaguetis de sémola de trigo duro estudiadas en la presente Tesis.

	CS	SR-3	ST-3	SR-6	ST-6
Sémola de trigo duro, 280 μm (kg)	2,00	2,00	2,00	2,00	2,00
Agua (kg)	0,55	0,60	0,70	0,75	0,85
Harina Rojo Brillante, < 210 μm (kg)		0,06		0,12	
Harina Triumph, < 210 μm (kg)			0,06		0,12

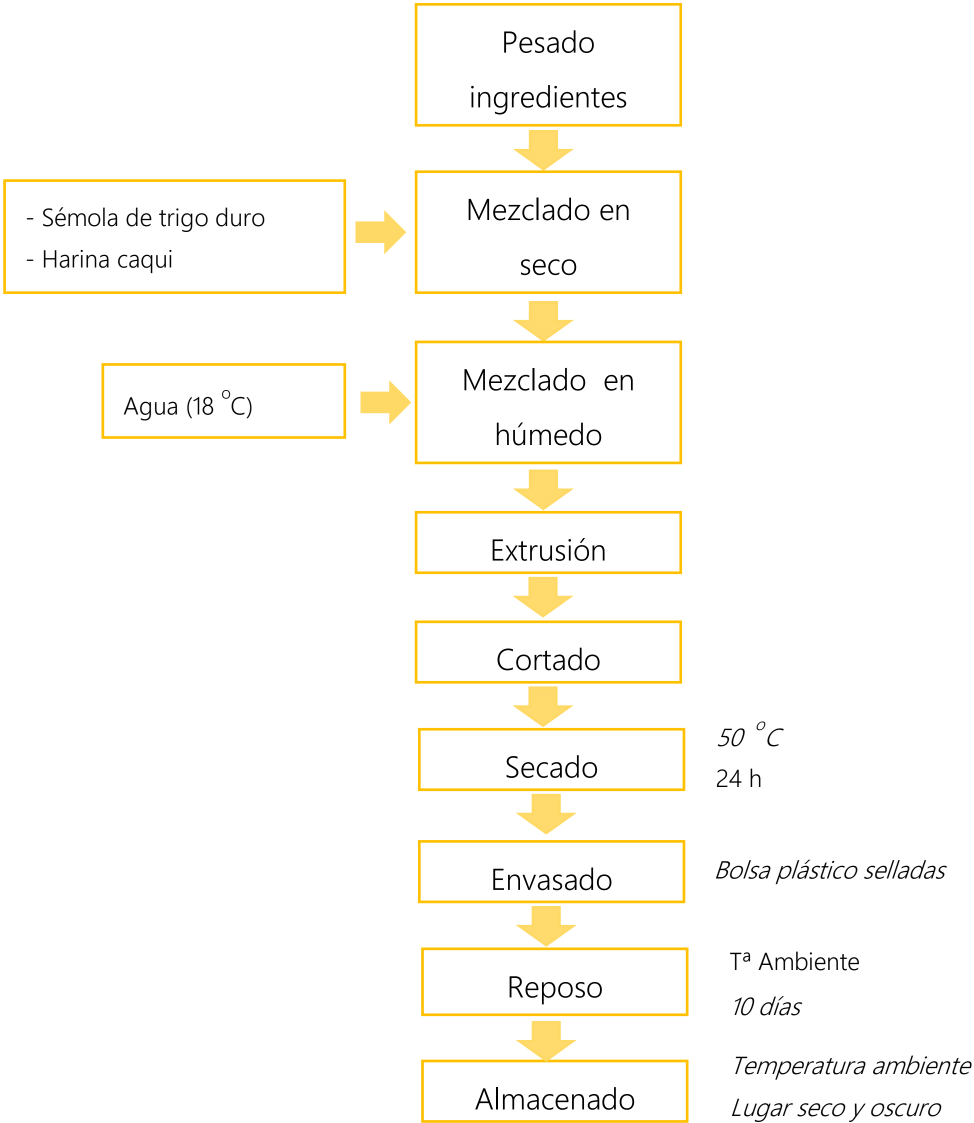


Figura 10.- Diagrama de flujo de la elaboración de espaguetis enriquecidos con harina de caqui.

3.4.4.- Caracterización de los espaguetis con harina de caqui

La caracterización de los espaguetis se realizó tanto en el alimento crudo como cocinado. En los espaguetis crudos se determinó su composición química, color, diámetro, fracturabilidad y análisis microbiológico. En los espaguetis cocinados se evaluó color, diámetro, propiedades de cocción, pegajosidad y firmeza. El contenido de compuestos fenólicos libres y unidos, así como su actividad antioxidante se determinó en los espaguetis crudos y cocinados.

3.4.4.1.- Composición química de los espaguetis

3.4.4.1.1.- Composición proximal

El contenido de humedad, proteína bruta, cenizas y fibra dietética total de los espaguetis crudos se determinó como se describe en los Apartados 3.2.1.1., 3.2.1.3. y 3.2.1.4. y 3.2.1.5., respectivamente. Todas las determinaciones se expresaron como g/100 g muestra.

3.4.4.1.2.- Almidón total

El contenido de almidón total de los espaguetis crudos se determinó siguiendo el método AOAC 996.11 (AOAC, 2007) con ayuda del K-TSTA Megazyme assay (Megazyme Ltd, Irlanda) para la determinación de almidón total. Los resultados se expresaron como g almidón/100 g muestra

3.4.4.1.3.- Azúcares totales

La determinación de los azúcares en las muestras de espagueti crudas se realizó siguiendo las indicaciones de Cavazza y col. (2013). Para la detección e identificación de los azúcares se usó la Cromatografía de intercambio aniónico de alto rendimiento con detección electroquímica pulsada (HPAEC-PED) usando el cromatógrafo iónico Dionex ICS 3000 (Thermo Scientific™ Dionex™, Madrid, España) acoplado a una precolumna Dionex Carbopac PA20 (3 × 30 mm) y columna Dionex Carbopac PA20 (3 × 150 mm). Se usaron los patrones de glucosa, fructosa, sacarosa y maltosa para realizar la identificación y cuantificación. Los resultados se expresaron como mg azúcar/100 g espaguetis.

3.4.4.1.4.- Pigmentos amarillos totales

El contenido de pigmentos amarillos totales (PAT) en los espaguetis crudos se realizó siguiendo el método aprobado internacional 14-50.01 (AACC, 1999). Los resultados se expresaron como mg α-caroteno equivalentes/100 g espaguetis.

3.4.4.1.5.- Perfil de compuestos fenólicos libres y unidos

El perfil de compuestos fenólicos libres y unidos se determinó tanto en los espaguetis crudos como en los espaguetis cocinados en su tiempo óptimo de cocción. Para la extracción de los compuestos fenólicos libres se usaron 2,000 ± 0,005 g de muestra (en las muestras cocinadas

se pesó la pasta en crudo). La extracción se realizó con 10 mL de metanol grado HPLC acidificado al 1% con HCl (37%; pureza). La solución se dejó en sonicación durante 2 horas (Ultrasons 330137, J.P SELECTA, Barcelona, España). Posteriormente el extracto se centrifugó (7100 *g* 10 min a 4 ºC) y se llevó a desecación por rotavapor (BÜCHI Rotavapor R-200, BUCHI Ibérica, Barcelona, España). El sólido se resuspendió, en 5 mL de metanol acuoso (50%), se filtró y almacenó a -20 ºC. El pellet se usó para la extracción de los compuestos fenólicos unidos, siguiendo la metodología descrita en el Apartado 3.2.1.12. con la diferencia que la muestra se resuspendió en metanol acuoso (50 %).

La detección de los compuestos fenólicos se realizó por HPLC usando el equipo Hewlett-Packard HPLC serie 1200 acoplado a una columna C18 Mediterranean Sea 18 (25 × 0.4 cm, 5 µm tamaño de partícula, Teknokroma, Barcelona, España). Para permitir la elución de la muestra inyectada (20 µL) se usó agua ultrapura acidificada con fórmico (1 %) (Fase móvil A) y metanol grado HPLC (fase móvil B). El gradiente que se usó durante los 45 minutos que duró el método fue el siguiente: 0 min- 5 % MeOH, a los 20 min- 25 % MeOH, a los 40 min-50 % MeOH; y a los 45 min-5 % MeOH. Se usaron cuatro longitudes de onda (270, 280, 325 y 360 nm) para la detección de los compuestos. La identificación y cuantificación se realizó comparando el tiempo de retención y el espectro de absorción UV con los patrones previamente inyectados bajo las mismas condiciones que las muestras (se realizó una recta de calibrado). Los patrones que se usaron para el estudio, así como las longitudes donde se observó su máxima absorción (270, 280 y 325 nm) fueron: ácido gálico, ácido protocateico, catequina, ácido 4-hidroxibenzoico, epicatequina, ácido clorogénico, ácido vanílico, ácido cafeico, ácido siríngico, vanillina, ácido *p*-cumárico, ácido ferúlico, ácido sinápico y L-triptófano (aminoácido aromático). Los resultados fueron expresados en µg compuestos fenólico/g muestra seca.

3.4.4.2.- Propiedades fisicoquímicas de los espaguetis

3.4.4.2.1.- Determinación del color

El estudio instrumental del color de los espaguetis (crudos y cocinados) se realizó bajo las mismas condiciones expuestas en el Apartado 3.2.2.3., pero con la salvedad que se usó el colorímetro Minolta CR300 (Tokio, Japón) y únicamente se estudiaron las coordenadas de color L*, a* y b*. Además, se evaluaron las diferencias de color (ΔE) de las muestras (m) con respecto a la muestra control (mc) siguiendo la ecuación 6.

$$\Delta E = \sqrt{(L_m^* - L_{mc}^*)^2 + (a_m^* - a_{mc}^*)^2 + (b_m^* - b_{mc}^*)^2} \quad (\textit{Ecuación 6})$$

3.4.4.2.2.- Diámetro

El diámetro de los espaguetis crudos y cocinados se determinó siguiendo la metodología propuesta por Cocci y col. (2008). Para ello se usó un dinamómetro eléctrico INSTRON U.T.M modelo 5542 (INSTRON, Pianezza, Italia).

3.4.4.2.3.- Fracturabilidad

Con la ayuda del Texturómetro TA.XT2i (Stable Micro Systems, Surrey, Gran Bretaña) plus equipado con una célula de 30 kg, se determinó la fracturabilidad de los espaguetis crudos. Para ello, cuatro hebras de espaguetis fueron comprimidas con una cuchilla a una velocidad de 1 mm/s y 3 mm de distancia entra la cuchilla y los espaguetis. Los resultados fueron expresados como la fuerza máxima requerida (N) para romper cuatros hebras de espagueti.

3.4.4.2.4.- Propiedades de cocción

La cocción de los espaguetis se realizó en una proporción 1:10 en vasos de precipitado sobre placas calefactoras. Concretamente para evaluar las propiedades de cocción se usaron 10 g de espaguetis crudos y 100 g de agua del grifo. Los espaguetis se cocinaban en agua hirviendo, 30 segundos antes de finalizar el tiempo óptimo de cocción establecido para cada formulación estudiada, los espaguetis se escurrían y se dejaban enfriar durante 10 minutos.

3.4.4.2.4.1.- Tiempo óptimo de cocción

El tiempo óptimo de cocción de cada uno de los espaguetis estudiados se determinó siguiendo las recomendaciones de Menger (1979) y Cocci y col. (2008). Los resultados se expresaron en minutos.

3.4.4.2.4.2.- Agua absorbida

La cantidad de agua absorbida por los espaguetis cocinados en su tiempo óptimo de cocción se estudió por diferencia de peso entre el espagueti crudo y el espagueti cocinado. Se plasmaron los resultados como porcentaje de agua absorbida.

3.4.4.2.4.3.- Pérdida de sólidos

Tras cocer los espaguetis en su tiempo óptimo de cocción, se estudió la cantidad de sólidos que habían sido lixiviados hacia el agua de cocción, siguiendo la metodología propuesta por Delcour y col. (2012). Los resultados se expresaron en g sólidos/100 g espaguetis.

3.4.4.2.4.4.- Material orgánico total (TOM)

El material orgánico total liberado por los espaguetis tras ser cocinado en su tiempo óptimo de cocción se determinó como indica D’Egidio y col. (1982). Se muestran los resultados como g materia orgánica/100 g espaguetis.

3.4.4.2.4.1.- Humedad después de cocinar

La humedad de los espaguetis cocinados se determinó por deshidratación en estufa de las muestras (aprox. 5,000 g) a 105 ºC hasta peso constante. Los resultados se expresaron como g agua/100 g espagueti.

3.4.4.2.5.- Textura

Dos análisis de textura se realizaron a los espaguetis cocinados en su tiempo óptimo de cocción: uno para la evaluación de la firmeza, la cual se determinó con el dinamómetro eléctrico INSTRON U.T.M modelo 5542 (INSTRON, Pianezza, Italia) equipado con una célula de 100 N; y otro para la evaluación de la pegajosidad, usando el Texturómetro TA.XT2i (Stable Micro Systems, Surrey, Gran Bretaña) equipado con una célula de 30 kg.

3.4.4.2.5.1.- Firmeza

Ocho hebras de espaguetis dispuestas una al lado de la otra, sin dejar huecos, se cortaron a una velocidad de 50 mm/s con el accesorio de cuchilla plana, el cual se encontraba a 10 mm por encima de los espaguetis testados. Los resultados se expresaron como la fuerza de corte por hebra de espagueti (N).

3.4.4.2.5.2.- Pegajosidad

Cuatro hebras de espaguetis dispuestas una al lado de la otra, sin dejar huecos fueron sometidas durante 2 segundos a una presión de 10 N con una sonda cilíndrica de 50 mm de diámetro (P/50). Los resultados se expresaron como el pico máximo de fuerza de retracción necesaria (N) para separar los espaguetis de la sonda cilíndrica (P/50).

3.4.4.3.- Análisis microbiológico

El muestreo y análisis microbiológico de las muestras crudas de espaguetis con mayor concentración de harina de caqui (SR-6 y ST-6) se realizó como se describe en el Apartado 3.2.5.

3.4.4.4.- Análisis sensorial: Prueba triangular

Se realizó una prueba triangular siguiendo la norma ISO 4120 (2004) donde se confrontó la muestra control cocinada en su tiempo óptimo de cocción (CS) con las cuatro formulaciones de espaguetis estudiadas (SR-3, ST-3, SR-6 y ST-6). Para ello se contó con 8 panelistas entrenados. Los ensayos se realizaron bajo luz roja en la sala de catas de la Universidad de Teramo (Teramo, Italia).

3.5.- EVALUACIÓN DE LA FUNCIONALIDAD DE LOS PATÉS DE HÍGADO DE CERDO Y LOS ESPAGUETIS DE SÉMOLA DE TRIGO DURO CON HARINAS DE CAQUI

Tras analizar los resultados, sobre la composición química, calidad y ensayos sensoriales de ambos alimentos, se decidió evaluar la funcionalidad de todas las muestras de espaguetis estudiadas (SC, SR-3, ST-3, SR-6 y ST-6) y de las muestras de paté, incluyendo únicamente las enriquecidas con harina de caqui Rojo Brillante (PC, PR-3 y PR-6). Se excluyeron los patés enriquecidos con harina de Triumph debido a la alta oxidación lipídica y desestabilización de la emulsión mostrada con respecto a los patés enriquecidos con harina de Rojo Brillante.

3.5.1.- Digestión gastrointestinal *in vitro*

La digestión gastrointestinal *in vitro* sobre las muestras de paté de hígado de cerdo y los de espaguetis de sémola de trigo duro se realizó siguiendo el protocolo harmonizado de la red INFOGEST (Minekus y col., 2014). En el caso concreto de las muestras de paté se simularon dos condiciones intestinales diferentes:

- **Fase Intestinal C1**: Se utilizó la pancreatina de Sigma-Aldrich. La actividad final de la lipasa en la fase intestinal fue de 8 U/mL.
- **Fase intestinal C2**: Se utilizó la pancreatina Kreon (25,000U) previamente usada por Calvo-Lerma y col. (2019). La actividad final de la lipasa en la fase intestinal fue de 2,000 U/mL.

Las digestiones se llevaron a cabo sobre 5,000 $\pm$ 0,005 g de muestra. En el caso de los espaguetis, éstos fueron cocinados hasta su tiempo óptimo de cocción, dejados enfriar durante 10 minutos mientras se cortaban con un cuchillo en trozos de 2 a 5 mm. Mientras que el paté se dejó atemperar unos minutos mientras se desmenuzaba con un tenedor. Posteriormente a estos tratamientos las muestras se pesaron en tubos Falcon de 50 mL de capacidad debidamente rotulados y pesados. Las muestras se sometieron a las tres fases digestivas, oral, gástrica e intestinal. La desactivación de las enzimas se realizó por shock térmico (cinco minutos en agua hirviendo), o congelación y posterior liofilización.

3.5.1.1.- Estabilidad de los compuestos fenólicos tras la digestión *in vitro*.

3.5.1.1.1.- Perfil de compuestos fenólicos libres y unidos

La estabilidad de los compuestos fenólicos libres y unidos presentes en las muestras de paté y espaguetis estudiados se evaluó en todas las fases simuladas de la digestión *in vitro*. Una vez finalizada cada fase de la digestión (oral, gástrica e intestinal) las muestras se centrifugaron a 7.200 *g*, durante 10 minutos y 4 ºC, con el fin de separar la parte líquida de la sólida. Las fracciones sólidas de ambos alimentos estudiados (paté y espaguetis) se liofilizaron y sus compuestos fenólicos se extrajeron siguiendo el protocolo para la extracción de compuestos fenólicos unidos

descritos en el Apartado 3.2.1.12. Mientras que, las fracciones líquidas de las muestras de espaguetis y paté se trataron de formas diferentes. En el caso de las muestras de paté la fracción líquida fue pasada en su totalidad por un cartucho C18 previamente activado como se describe en el Apartado 3.2.1.11. Para las muestras de espaguetis las fracciones líquidas, previamente liofilizadas fueron resuspendidas en 2, 5 y 10 mL de metanol acuoso (50%) (oral, gástrica e intestinal, respectivamente) tras estar 8 horas en contacto con la solución metanólica; después se centrifugaron (4.000 g durante 5 minutos a temperatura ambiente) y filtraron (filtro de nailon de 0,45 µm, Millipore Corporation, Bedford, USA).

La detección, identificación y cuantificación de los compuestos fenólicos, de ambas fracciones, se realizó como se indica en el Apartado 3.2.1.12. en el caso de las muestras de paté; y como se describe en el Apartado 3.4.4.1.5., para las muestras de espaguetis.

3.5.1.1.2.- Índice de bioaccesibilidad e índice de fracción colónica disponible en los espaguetis.

El contenido total de los compuestos fenólicos cuantificados cromatográficamente como se describe en el Apartado 3.5.1.1.1., de las diferentes fases digestivas, así como de las muestras sin digerir, se usaron para determinar el índice de bioaccesibilidad e índice de fracción colónica disponible, siguiendo las ecuaciones 5 y 7, respectivamente. Para calcular el índice de bioaccesibilidad se usa FL_i en lugar de TPC o TFC_{OUT}; y TP_C, en lugar TPC o TFC_{OUT+IN}.

$$\%\ \text{Índice de fracción colónica disponible} = \frac{FS_i}{BP_c} \times 100\ (\textit{Ecuación 7})$$

Donde,

FL_i: Suma de los compuestos (poli)fenólicos en la fracción soluble después de la fase intestinal (µg /g)
TP_C: Contenido compuestos fenólicos muestra cocida (µg/g)
FS_i: Suma de los compuestos (poli)fenólicos en la fracción sólida después de la fase intestinal (µg /g)
BP_C: Contenido total de los compuestos (poli)fenólicos unidos en la muestra cocida (µg/g)

3.5.1.1.3.- Actividad antioxidante de los espaguetis crudos, cocinados y tras ser sometidos a la digestión gastrointestinal in vitro

A los extractos de ambas fracciones de compuestos fenólicos, libres y unidos, estudiadas sobre todas las muestras de espaguetis (cruda, cocinada, y tras la digestión oral, gástrica e intestinal) se les determinó la actividad antioxidante usando dos ensayos diferentes, ABTS y FRAP. Los ensayos se realizaron siguiendo las directrices descritas en los Apartados 3.2.6.1. y 3.2.6.4., respectivamente. La capacidad de las muestras para atrapar al radical catiónico ABTS•+, se expresó como µg Trolox equivalente/g espaguetis en base seca; mientras que su habilidad para reducir el ion férrico se expresó como mg Trolox equivalente/g espaguetis en base seca.

3.5.1.2.- Cinética de digestión del almidón en espaguetis

La digestibilidad del almidón de los espaguetis durante la digestión *in vitro* se evaluó con el método establecido por Goñi y col. (1997) pero siguiendo el protocolo de Minekus y col. (2014) para simular la digestión *in vitro*, con la modificación del tiempo de digestión gástrica (1 h). Para evaluar la cinética de digestión del almidón de los espaguetis se tomaron alícuotas (100 µL) a 8 tiempos diferentes del proceso digestivo (2, 30, 60, 75, 90, 120, 150 y 180 min). Una vez desactivada la enzima, las alícuotas se diluyeron (1:25) y se sometieron a digestión con la amiloglucosida (3.300 U/L) (enzima que transforma la maltosa en glucosa). La determinación de glucosa en las muestras fue determinada espectrofotométricamente (espectrofotómetro HP 8451; Hewlett Packard, Cambridge, UK) a 510 nm tras incubar las muestras con el reactivo GOPOD. Los resultados se expresaron como porcentaje de almidón hidrolizado siguiendo la ecuación 8.

$$\%\, Almidón = A \times F \times V_D \times \frac{D}{W_d} \times 0{,}9 \quad \textit{(Ecuación 8)}$$

Donde,

A= Valor de absorbancia de la muestra

F= factor que convierte los valores de absorbancia a µg de D-glucosa (100 µg de D-glucosa dividido por el valor de absorbancia de 100 µg de D-glucosa tras reaccionar con el reactivo GOPOD)

VD= Volumen de la fase de digestión (mL)

D= Factor de dilución

Wd= peso de la muestra en base seca (mg)

0,90= Factor de conversión de glucose libre, como se determina a, glucosa anhidra, como ocurre en el almidón.

3.5.1.2.1.- Determinación del índice glicémico in vitro de los espaguetis.

El índice glicémico *in vitro* estimado (IG_e) se determinó, calculando el área debajo de la curva obtenida de la cinética de hidrólisis del almidón (ecuación 9 y 10), siguiendo las ecuaciones 11 y 12, las cuales han sido propuestas por Goñi y col. (1997). Se usó pan blanco como referencia y la concentración de almidón liberado a los 120 minutos como concentración final de la reacción.

$$C = C_\infty\,(1 - e^{-kt}) \textit{ (Ecuación 9)}$$

$$AUC = C_\infty(t_\infty - t_0) - (C_\infty/k)\,[1 - e^{[-k(t_\infty - t_0)]} \textit{ (Ecuación 10)}$$

$$HI = AUC_{Spaghetti}/AUC_{white\ bread} \times 100\ (\textit{Ecuación}\ 11)$$

$$IG_e = 39{,}71 + 0{,}549\ HI\ (\textit{Ecuación}\ 12)$$

Donde,

C= almidón hidrolizado (%)

$C\infty$= almidón hidrolizado en la concentración final (%)
k= constante cinética de reacción
$t\infty$ = tiempo final de la reacción (120 min)
t_0 = tiempo inicial reacción (0 min)

3.5.1.3.- Determinación de la oxidación lipídica durante la digestión *in vitro* en el paté

La determinación de la oxidación lipídica después de la fase gástrica y las dos intestinales (C1 y C2) se llevó a cabo siguiendo el protocolo descrito por Sobral y col. (2020). Los resultados se expresaron como µmol MDA/kg paté. El incremento de la oxidación lipídica se realizó comparando el valor de TBARS de las muestras crudas con el de las muestras digeridas siguiendo la ecuación 13.

$$Incremento\ oxidación\ lipídica\ (\%) = \frac{TBARS_d - TBARS_{sd}}{TBARS_{sd}} \times 100 \quad (\textit{Ecuación 13})$$

Donde,
$TBARS_d$= valor de TBARS en las muestras digeridas expresado en µmol MDA/kg paté
$TBARS_{sd}$= valor de TBARS en las muestras sin digerir expresado en µmol MDA/kg paté

3.5.1.4.- Determinación de la estabilidad de los ácidos grasos en el paté.

La estabilidad de los ácidos grasos se estudió tras ambas fases intestinales simuladas (fase intestinal C1 y C2). Para la extracción de la grasa se usó todo el contenido (40 mL) obtenido tras la fase intestinal (C1 y C2). La metilación, detección e identificación de los ácidos grasos se realizó como se describe en el Apartado 3.4.2.1.2. La variación de los ácidos grasos después de la digestión gastrointestinal *in vitro* se calculó mediante la ecuación 14.

$$Variación\ ácidos\ grasos\ (\%) = \frac{AG_d - AG_{sd}}{AG_{sd}} \times 100 \quad (\textit{Ecuación 14})$$

Donde,
AG_d: contenido de ácido graso en la muestra digerida (fase intestinal) expresado en mg/100 g grasa.
AG_{sd}: contenido de ácido graso en la muestra sin digerir expresado en mg/100 g grasa.

3.5.1.5.- Observación micelas de grasa con microscopio óptico

La observación de las micelas de grasa de las muestras de paté después de la digestión gastrointestinal *in vitro* se realizó siguiendo el protocolo descrito por Calvo-Lerma y col. (2019). Con la ayuda de un microscópico óptico (Olympus CX40RF200, OLYMPUS OPTICAL CO, LTD, Japón). Las imágenes se tomaron con una cámara acoplada al microscópico (COLOR VIDEO CAMERA, Hyper HAD, SONY CORPORTATION, Japón). Las imágenes se muestran con una resolución de 40X.

3.6.- ESTADÍSTICA

Todos los análisis, extracciones y ensayos se realizaron por triplicado. Los resultados están expresados como el valor medio y la desviación estándar de las tres repeticiones.

Los análisis estadísticos empleados para cada uno de los estudios estadísticos que se realizó durante todo el desarrollo de la Tesis fueron: ANOVA simple, ANOVA de dos factores, la prueba de Tukey y correlación de Person, (paquete estadístico STAT Graphic). La media y desviación estándar se realizaron con el programa de office Excel. La elección de cada uno de los ensayos está específicamente descrita en cada uno de los artículos que forman el cuerpo de esta Tesis.

Capítulo 4.- RESULTADOS Y DISCUSIÓN

En el presente apartado se muestra un resumen de los resultados más destacados obtenidos durante la realización de la Tesis. El conjunto completo de resultados, así como su discusión extensa se muestran en los correspondientes artículos recogidos en el Capítulo 7.

4.1 OBTENCIÓN DE COPRODUCTOS DERIVADOS DEL CAQUI

Los coproductos con los que se ha trabajado en la presente Tesis son la pulpa y la piel que derivan de la obtención de zumo de caqui. En la Tabla 5 se pueden observar los rendimientos del procesado tras el secado y la molienda de las dos cultivares de caqui estudiados. El cultivar "Triumph", presentó mayor rendimiento en el proceso de secado, lo que indica que se evaporó menos agua. Durante la molienda del coproducto desecado se observaron pérdidas en torno al 5-10 % en ambos cultivares de caqui estudiados.

Tras la molienda, se procedió a su envasado al vacío para tratar de conservar todas sus propiedades, sin embargo, el vacío no debe ser total, pues sino se crean agregaciones entre las partículas de harina.

Tabla 5.- Rendimiento obtención de harina de caqui a partir de coproductos del procesado del zumo de caqui (media ± sd).

Cultivar	ºBrix	Rendimiento secado (%)	Rendimiento molienda (%)
"Rojo Brillante"	15,48±1,01	21,11±1,57	90,39±5,26
"Triumph"	18,28±0,44	24,05±0,48	90,83±4,92

4.2.- EFECTO DEL TAMAÑO DE PARTÍCULA DE LAS HARINAS DE CAQUI SOBRE SU COMPOSICIÓN QUÍMICA, PROPIEDADES FISICOQUÍMICAS, TECNOFUNCIONALES, FISIOFUNCIONALES Y ACTIVIDAD ANTIOXIDANTE.

Los resultados de este trabajo han sido publicados en la revista Plant Foods for Human Nutrition (2017) 72:67–73 y en la revista Journal of the Science of Food and Agriculture (2018) 98:504–510.

Las harinas vegetales ricas en fibra, procedentes de cereales, legumbres, verduras, frutas, o sus coproductos, presentan diferentes propiedades tecnofuncionales, tales como, capacidad de retención de agua y aceite, actividad emulsificante, gelificante, antioxidante o espesante, de interés para la industria alimentaria en la formulación de alimentos (López-Vargas y col., 2013; López-Marcos y col., 2015; Fernández-López y col., 2018; Longato y col., 2019; Fernández-López y col., 2020b; Lorente-Mento y col., 2020; Nakov y col., 2020). Además, la fibra, definida como la mezcla de polímeros de carbohidratos que no pueden ser digeridos por las enzimas digestivas

humanas, ejerce efectos fisiológicos positivos en el organismo, como reducir las concentraciones plasmáticas de colesterol sanguíneo, modular los niveles de glucosa plasmática, regular el peso corporal, retardar el vaciado gástrico, estimular la fermentación colónica, aumentar el tránsito intestinal y la masa fecal, entre otras (Burton-Freeman 2000; Cummings, 2001; Anderson y col., 2009; Viuda-Martos y col., 2010b; Kaczmarczyk y col., 2012). Estas propiedades están determinadas, principalmente, por la naturaleza y composición química de la fibra presente en los productos vegetales. La fibra se puede clasificar en función del número de polimerización, la naturaleza de sus unidades monoméricas o su solubilidad en agua (Ellutech y col., 2011). El tamaño de partícula de las fibras también afecta a sus propiedades tecnológicas y fisiológicas. En este ámbito, no existe una tendencia regular que relacione tamaño de partícula con propiedades tecnológicas; el comportamiento es intrínseco al producto vegetal analizado (Ahmed y col., 2016; Ren y col., 2021). Por ejemplo, la reducción del tamaño de partícula de las harinas vegetales puede, tanto disminuir su capacidad de retención de agua, debido a los daños ocasionados por la molienda en las regiones con capacidad de unir agua, como incrementarla, a consecuencia del aumento del tamaño de superficie (Ellutech y col., 2011). Es por ello, que, si se quiere conocer el efecto del tamaño de partícula en las propiedades fisicoquímicas, tecnofuncionales o composición química de las harinas vegetales, se debe llevar a cabo un estudio experimental donde se analicen los cambios que ocasiona la molienda en las mimas.

4.2.1.- Composición química

Los efectos de la molienda sobre la composición química de las harinas de caqui Rojo Brillante y Triumph fueron dependientes del tipo de cultivar del que derivan las harinas. En todas las fracciones de ambas harinas de caqui estudiadas, sus componentes mayoritarios fueron los hidratos de carbono simples (fructosa y glucosa) y la fibra dietética, siendo la fracción insoluble mayoritaria, frente a la soluble (Tabla 6).

El tamaño de partícula no afectó al contenido de fibra dietética insoluble, pero sí lo hizo sobre la cantidad de fibra dietética soluble, y por tanto sobre el contenido de fibra dietética total ($p < 0{,}05$). Sin embargo, las mayores variaciones entre el contenido de FDT fueron debidas al cultivar. En todos los tamaños de partícula la harina de Triumph presentó mayor contenido de FDT y FDI que las fracciones de las harinas de Rojo Brillante ($p < 0{,}05$). El contenido de fructosa y glucosa también fue mayor en las harinas de Triumph ($p < 0{,}05$) y no se vio afectado por el tamaño de partícula, mientras que en las harinas Rojo Brillante la reducción del tamaño de partícula provocó una disminución de su contenido ($p < 0{,}05$).

Tabla 6.- Composición química (g/100g) de las harinas de caqui con distinto tamaño de partícula.

Tamaño de partícula (µm)		>701	701-417	417-210	<210
Harinas Rojo Brillante	Humedad	14,16±0,38[a]	13,46±0,66[c]	13,71±0,12[c]	15,53±0,35[a]
	Proteína	3,14±0,06[a]	3,24±0,02[a]	2,92±0,01[b]	3,07±0,07[a]
	Grasa	0,56±0,02[a]	0,34±0,00[c]	0,45±0,00[b]	0,49±0,03[b]
	Cenizas	2,07±0,51[a]	1,98±0,11[a]	1,82±0,06[b]	1,88±0,07[ab]
	FDT	33,44±1,05[d]	36.73±0,84[c]	37,07±0,51[c]	32,05±0,62[d]
	FDI	26,85±0,52[b]	26,76±0,35[b]	25,30±0,61[b]	22,20±0,29[b]
	FDS	6,59±0,29[c]	10,00±0,50[b]	11,77±0,49[a]	9,85±0,41[b]
	Fructosa	24,40±0,10[a]	24,45±0,32[a]	17,86±0,00[d]	19,57±0,80[c]
	Glucosa	24,06±0,15[a]	23,93±0,45[a]	16,39±0,00[d]	18,45±0,88[c]
Harinas Triumph	Humedad	13,06±0,52[c]	11,15±0,25[d]	12,39±0,46[d]	14,02±0,22[b]
	Proteína	2,78±0,00[c]	2,89±0,00[b]	2,86±0,04[b]	2,23±0,03[d]
	Grasa	0,37±0,04[c]	0,46±0,03[b]	0,52±0,01[a]	0,34±0,01[c]
	Cenizas	2,04±0,03[a]	1,86±0,07[b]	1,79±0,07[b]	1,86±0,01[b]
	FDT	42,68±1,48[a]	43,26±0,59[a]	43,57±0,48[a]	40,30±0,48[b]
	FDI	32,69±0,99[a]	32,08±0,71[a]	33,93±0,44[a]	34,22±0,49[a]
	FDS	9,99±0,52[b]	11,18±0,30[a]	6,64±0,33[c]	6,08±0,22[c]
	Fructosa	22,45±0,36[b]	22,09±0,44[b]	21,94±0,16[b]	22,57±0,12[b]
	Glucosa	20,66±0,38[b]	20,36±0,40[b]	20,16±0,16[b]	20,25±0,13[b]

FDT: Fibra dietética total; FDI: Fibra dietética insoluble; FDS: Fibra dietética soluble.
Los valores en la misma fila y para la misma determinación, seguidos de la misma letra (a-h) no son significativamente diferentes ($p > 0,05$) según la prueba de rango múltiple de Tukey.

Con respecto a los fitoquímicos presentes en las harinas de caqui, destaca su contenido en ácido gálico (Tabla 7), el cual ronda los 3,0-4,0 mg/g en las harinas de Rojo Brillante y 1,5-3,0 mg/g en las harinas de Triumph. En ambos cultivares de caqui estudiados, las fracciones de harinas con menor tamaño de partícula (<210 µm) presentaron los valores más altos para dicho compuesto fenólico ($p < 0,05$). Esta misma tendencia fue observada para el contenido de carotenos totales: a menor tamaño de partícula, mayor contenido ($p < 0,05$). No ocurre lo mismo con los ácidos orgánicos presentes en las harinas, ya que, salvo excepciones, su contenido disminuye con la reducción del tamaño de partícula.

Tabla 7.- Ácidos orgánicos, compuestos (poli)fenólicos y contenido total de fenoles, flavonoides y carotenos presentes en las diferentes harinas de caqui estudiadas con distinto tamaño de partícula.

	Harinas Rojo Brillante				Harinas Triumph			
Tamaño partícula (µm)	**>701**	**701-417**	**417-210**	**<210**	**>701**	**701-417**	**417-210**	**<210**
Ácidos orgánicos (mg/g)								
Ácido málico	17,65±0,41^{a}	16,01±1,32^{a}	13,18±0,41^{b}	14,15±0,56^{b}	17,04±0,18^{a}	16,08±1,53^{a}	16,96±0,17^{a}	17,39±0,67^{a}
Ácido succínico	6,11±0,18^{a}	5,74±0,01^{b}	4,61±0,14^{d}	4,81±0,15^{d}	4,89±0,13^{d}	4,78±0,05^{d}	5,26±0,23^{c}	5,48±0,14^{bc}
Ácido fumárico	2,03±0,02^{d}	1,92±0,07^{de}	0,96±0,03^{e}	1,59±0,06^{de}	8,28±0,27^{b}	7,22±0,10^{c}	8,23±0,15^{b}	10,13±0,09^{a}
Ácido cítrico	2,36±0,08^{a}	2,28±0,05^{ab}	1,79±0,06^{c}	1,89±0,10^{bc}	2,21±0,00^{a}	2,20±0,06^{a}	2,24±0,13^{a}	2,23±0,11^{a}
Ácido tartárico	0,36±0,02^{bc}	0,37±0,03^{b}	0,35±0,00^{bc}	0,24±0,01^{d}	0,32±0,03^{c}	0,34±0,01^{bc}	0,38±0,00^{b}	0,54±0,03^{a}
Ácido oxálico	0,04±0,00^{cd}	0,03±0,00^{d}	0,10±0,00^{b}	0,18±0,01^{a}	0,05±0,00^{c}	0,04±0,00^{cd}	0,04±0,00^{c}	0,04±0,00^{c}
(Poli)fenoles (µg/g)								
Ácido gálico	2.959±229^{b}	3.234±99^{b}	3.582±84^{b}	4.236±114^{a}	1.554±118^{c}	1.718±40^{c}	1.823±86^{c}	2.956±258^{b}
Ácido elágico	2,88±0.25^{ab}	2,41±0,11^{bc}	2,38±0,21^{b}	2,98±0,27^{ab}	3,21±0,49^{a}	3,27±0,24^{ac}	2,46±0,15^{b}	3,38±0,25^{a}
Quercetina	1,21±0,08^{b}	1,32±0,05^{b}	1,51±0,00^{b}	1,52±0,00^{b}	3,55±0,28^{a}	3,75±0,22^{a}	3,38±0,27^{a}	3,61±0,08^{a}
Kaempferol	0,54±0,04^{b}	0,55±0,02^{b}	0,66±0,00^{b}	0,71±0,01^{b}	1,59±0,13^{a}	1,68±0,08^{a}	1,65±0,00^{a}	1,60±0,05^{a}
Epicatequina	4,08±0,27^{b}	ND	ND	6,81±0,69^{a}	3,23±0,00^{b}	2,83±0,00^{b}	3,18±0,03^{b}	4,35±0,00^{b}
Catequina	2,73±0.02^{a}	1,89±0,01^{b}	1,21±0,01^{e}	1,50±0,03^{d}	0,45±0,00^{f}	0,34±0,00^{g}	ND	1,62±0,05^{c}
CFT (mg ácido gálico eq./g)	1,39±0,15^{b}	1,20±0,02^{bc}	1,06±0,02^{c}	1,75±0,13^{a}	1,00±0,09^{c}	1,00±0,05^{c}	1,00±0,04^{c}	1,06±0,06^{c}
CFLT (mg rutina eq./g)	1,61±0,12^{a}	1,85±0,07^{a}	1,28±0,10^{b}	1,87±0,07^{a}	1,21±0,08^{b}	1,06±0,03^{b}	1,04±0,06^{b}	1,19±0,10^{b}
CCT (mg β-caroteno eq./g)	1,60±0,02^{c}	1,76±0,03^{b}	1,59±0,04^{c}	1,85±0,02^{ab}	1,56±0,01^{c}	1,55±0,00^{c}	1,10±0,07^{d}	1,92±0,00^{a}

CFT: contenido de fenoles totales; CFLT: Contenido de flavonoides totales; CCT: contenido de carotenos totales. ND: no detectado Los valores en la misma fila seguidos de la misma letra (a-h) no son significativamente diferentes ($p > 0,05$) según la prueba de rango múltiple de Tukey.

4.2.2.- Propiedades fisicoquímicas y tecnofuncionales

El color de las harinas de caqui se vio afectado por la molienda (Figura 11). Se observó que a menor tamaño de partícula mayor luminosidad y tono (h*) y menor valor de rojez (a*), en ambos tipos de harina estudiadas ($p < 0,05$). El incremento de la luminosidad en las harinas con menor tamaño de partícula está asociado a una mayor área superficial, lo que implica mayor reflexión de la luz (Ahmed y col., 2015); mientras que la reducción en la rojez podría deberse a la pérdida de pigmentos (Ahmed y col., 2016).

Entre las propiedades tecnofuncionales de las harinas de caqui destacan sus propiedades de hidratación (capacidad de retención de agua y capacidad de hinchamiento) (Tabla 8), las cuales, en la mayoría de los casos presentan valores mayores que los mostrados por otros coproductos de frutas (Tabla 9). Ambas propiedades se vieron afectadas por el tamaño de partícula y por el cultivar. La CRA de las harinas de Triumph presentaron mayores valores que las harinas de Rojo Brillante ($p < 0,05$). El efecto de la molienda sobre las propiedades de hidratación de ambas harinas estudiadas (Rojo Brillante y Triumph) fue diferente. Los valores más altos en la CRA para la harina Rojo Brillante se mostró en los tamaños de partícula más grandes, mientras que en la harina de Triumph se observó lo contrario. Además, en ninguna de las dos harinas se observó una relación lineal con la reducción del tamaño de partícula. La mayor CRA mostrada por la harina de Triumph, puede deberse a su mayor contenido de fibra insoluble, pues diversos autores apuntan a que, a mayor contenido de fibra insoluble, mayor habilidad para retener agua (Bchir y col., 2014). Por otro lado, como previamente se había comentado, la molienda causa modificaciones en el área superficial y puede modificar la porosidad y densidad de la fibra, lo que está asociado a variaciones en las propiedades de hidratación (Cadden 1987; Lario y col., 2004a).

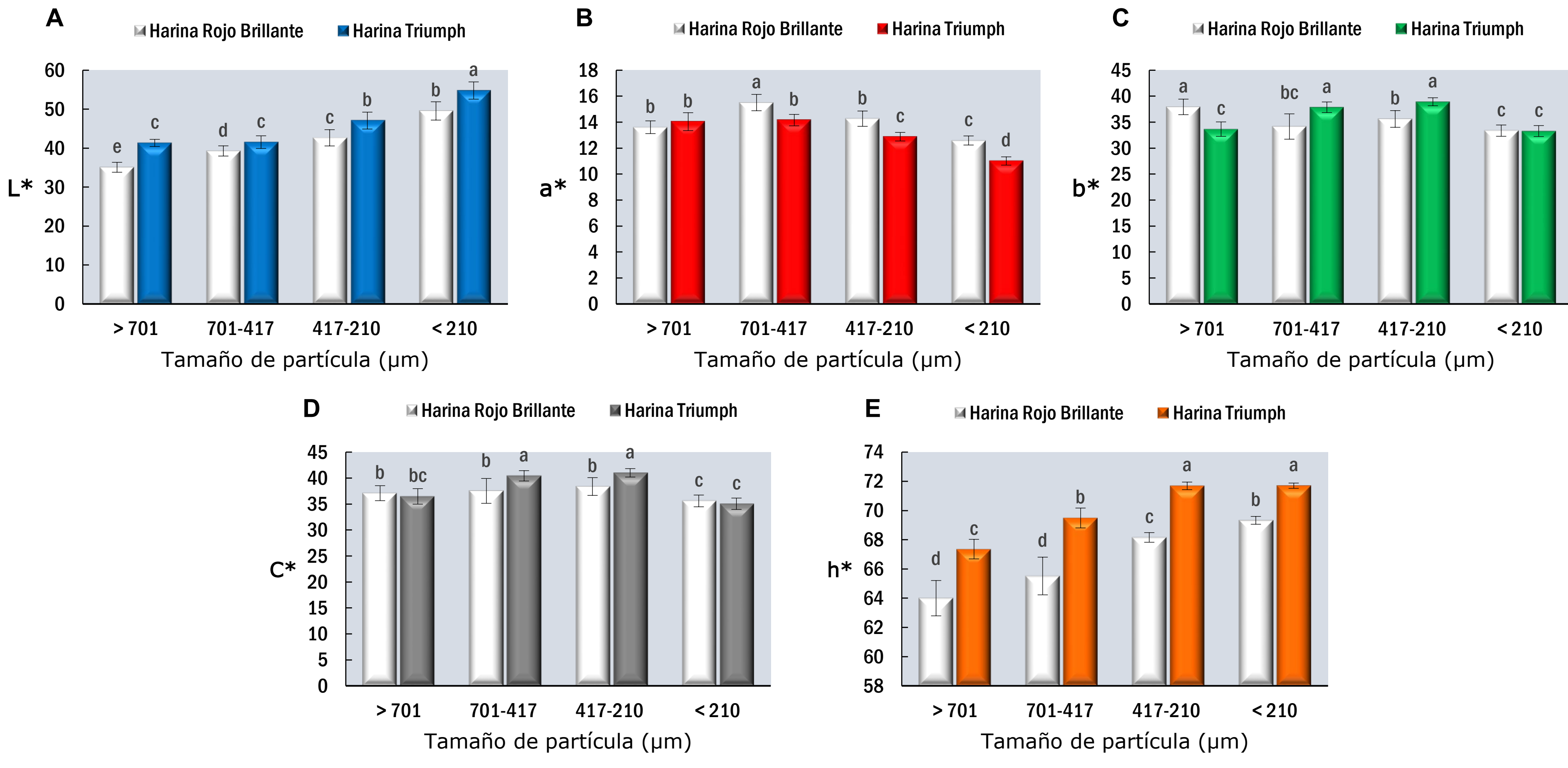


Figura 11.- Coordenadas de color y magnitudes de color de las harinas de caqui estudiadas. A) Luminosidad (L*); B) coordenada rojo-verde (a*), C) coordenada amarillo-azul (b*), D) Croma (C*) y E) Tono (h*).

Diferentes letras (a-h) indican diferencias estadísticamente significativas ($p < 0{,}05$) según la prueba de rango múltiple de Tukey.

Tabla 8.- pH, actividad de agua (a_W) y propiedades tecnofuncionales de las harinas de caqui estudiadas con diferentes tamaños de partícula.

	Tamaño de partícula (µm)	> 701	701-417	417-210	< 210
Harinas Rojo Brillante	pH	4,87±0,01^{c}	4,90±0,02^{c}	4,90±0,02^{c}	4,89±0,05^{c}
	a_w	0,426±0,00^{ab}	0,381±0,00^{d}	0,351±0,00^{e}	0,384±0,00^{d}
	CRA (g/g)	8,04±0,09^{d}	5,87±0,21^{f}	6,64±0,41^{e}	7,37±0,73^{de}
	CRO (g/g)	1,72±0,07^{c}	1,67±0,03^{c}	1,88±0,12^{b}	2,15±0,08^{a}
	CH (mL/g)	11,52±1,12^{a}	11,51±0,86^{a}	11,41±0,42^{a}	10,27±0,36^{b}
	AE (mL/mL)	69,33±0,47^{a}	74,00±4,97^{a}	69,00±1,41^{a}	69,00±0,82^{a}
	EE (%)	92,51±0,77^{bc}	91,84±2,76^{c}	94,12±0,07^{b}	93,93±1,29^{b}
Harinas Triumph	pH	5,28±0,06^{b}	5,27±0,04^{b}	5,26±0,05^{b}	5,42±0,00^{a}
	aw	0,427±0,00^{ab}	0,418±0,00^{c}	0,430±0,00^{a}	0,438±0,00^{a}
	CRA (g/g)	10,57±0,25^{b}	9,47±0,21^{c}	12,19±0,19^{a}	11,23±0,56^{ab}
	CRO (g/g)	1,92±0,03^{b}	2,07±0,09^{a}	2,18±0,07^{a}	2,26±0,06^{a}
	CH (mL/g)	7,95±0,16^{d}	9,90±0,59^{c}	11,39±0,40^{a}	11,58±0,33^{a}
	AE (mL/mL)	60,33±0,47^{b}	59,00±2,16^{b}	57,33±1,70^{b}	57,67±1,25^{b}
	EE (%)	99,44±0,79^{a}	71,78±1,35^{d}	51,85±2,70^{f}	64,08±3,76^{e}

CRA: Capacidad de retención de agua; CRO: Capacidad de retención de aceite; CH: Capacidad de hinchamiento; AE: Actividad emulsificante; EE: Estabilidad de la emulsión. Los valores en la misma fila y para la misma determinación, seguidos de la misma letra (a-h) no son significativamente diferentes ($p > 0.05$) según la prueba de rango múltiple de Tukey.

Tabla 9.- Propiedades de hidratación de diferentes materiales vegetales.

Coproductos ricos en fibra	CRA (g agua /g)	CH (mL/g)	CRO (g aceite/g)
Limón[1]	7,96±0,11	5,96±0,10	1,69±0,13
Uva[1]	6,38±0,40	6,50±0,09	2,30±0,09
Granada[1]	4,43±0,20	1,92±0,11	1,73±0,03
Albedo de limón[1]	5,56±0,56	5,70±0,36	2,23±0,77
Chufa[1]	3,76±0,43	1,93±0,42	3,60±0,22
Manzana[2]	1,62±0,10	6,59±0,05	0,95±0,05
Naranja[2]	1,65±0,20	6,11±0,05	1,81±0,05
Dátil[3]	5,70±0,20	3,90±0,10	2,30±0,10
Pera[3]	4,90±0,10	5,90±0,10	2,10±0,10

Valores expresados en peso seco.[1] López-Marcos y col. (2015); [2] Figuerola y col. (2005); [3] Bchir y col. (2014); CRA: Capacidad de retención de agua; CRO: Capacidad de retención de aceite; CH: Capacidad de hinchamiento

4.2.3.- Actividad antioxidante y propiedades fisiofuncionales

La actividad antioxidante de las harinas se determinó mediante cuatro ensayos diferentes (Tabla 10). El fundamento de los métodos FRAP, DPPH y ABTS, se basa en reacciones de transferencia de un electrón, y el del método FIC en la capacidad quelante de compuestos prooxidantes. Los valores más altos de la actividad antioxidante de las harinas de caqui fueron obtenidos mediante el método FRAP. Con estos resultados, pese a excepciones, podría indicarse que la molienda de las harinas de caqui no modificó su actividad antioxidante ($p > 0,05$). Por otro lado, las fracciones de Rojo Brillante estudiadas mostraron los mayores valores de actividad antioxidante para los cuatro métodos usados ($p < 0,05$).

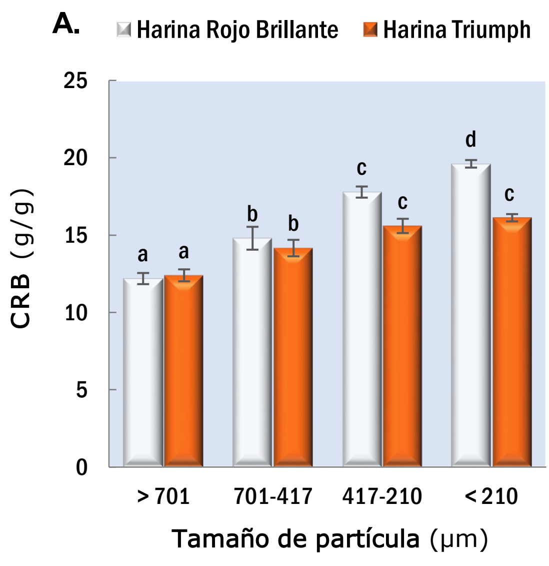
La capacidad antioxidante mostrada por las harinas de caqui podría ejercer tanto efectos tecnológicos, evitando las reacciones de oxidación durante el procesado y almacenado de los alimentos, como fisiológicos. Sin embargo, pese a lo extendido que están estos métodos en la comunidad científica y la relación de los efectos beneficiosos del consumo de compuestos polifenólicos con sus propiedades antioxidantes (Granato y col., 2020), cabe destacar, que paulatinamente su uso es menor, debido a las evidencias cada vez más sólidas, de que los antioxidantes dietéticos, como los compuestos (poli)fenólicos, no ejercen actividades de captación de electrones *in vivo* (Forman y col., 2014; Croft, 2016). Su actividad beneficiosa está más relacionada con la señalización celular, la modulación de la actividad de las enzimas redox o el incremento de la defensa endógena no enzimática del organismo. (Upadhyay y col., 2015; Croft, 2016; Kerimi y Williamson, 2016). Algunos científicos plantean la posibilidad de prohibir los métodos *in vitro* para evaluar la actividad antioxidante de los alimentos (Granato y col., 2018), debido a que estos métodos no tienen en cuenta ni las condiciones, ni las concentraciones fisiológicas del organismo; se basan en reacciones genéricas, donde otros compuestos, como ácidos orgánicos, aminoácidos aromáticos o azúcares reductores, pueden interaccionar y, por ende, se sobreestima la capacidad antioxidante de los compuestos (poli)fenólicos (Becker y col., 2004; Tresserra-Rimbau y col., 2018); y no contemplan las transformaciones que se producen en el hígado e intestino que conllevan la modificación de la estructura química de los compuestos (poli)fenólicos, y por lo tanto, sus propiedades antioxidantes (Hunyadi, 2019; Morand y col., 2020). Estas técnicas pueden verse como técnicas preliminares de screening, rápidas, sencillas y baratas, para comparar entre diferentes muestras, pero con los actuales avances científicos, no se deberían usar para proclamar los efectos beneficiosos de un compuesto o ingrediente.

Tabla 10.- Actividad antioxidante mostrada por las harinas de caqui con diferentes métodos antioxidantes.

	Tamaño de partícula (µm)	DPPH (µg Trolox eq./g)	FIC (µg EDTA eq./g)	FRAP (mg Trolox eq./g)	ABTS (mg Trolox eq./g)
Harinas Rojo Brillante	> 701	0,79±0,02[ab]	0,08±0,00[a]	2,68±0,19[b]	1,92±0,00[a]
	701-417	0,87±0,01[a]	0,08±0,00[a]	3,37±0,21[a]	1,87±0,28[a]
	417-210	0,83±0,02[a]	0,08±0,00[a]	3,76±0,29[a]	1,72±0,05[b]
	< 210	0,84±0,01[a]	0,08±0,00[a]	3,96±0,24[a]	2,12±0,16[a]
Harinas Triumph	> 701	0,73±0,02[bc]	0,08±0,00[a]	2,23±0,14[b]	1,31±0,10[c]
	701-417	0,73±0,04[bc]	0,08±0,00[a]	2,18±0,14[b]	1,16±0,02[c]
	417-210	0,70±0,03[c]	0,08±0,00[a]	2,34±0,09[b]	1,16±0,12[c]
	< 210	0,72±0,01[bc]	0,08±0,00[a]	2,74±0,17[b]	1,20±0,02[c]

eq.: equivalentes. Los valores en la misma columna seguidos de la misma letra (a-h) no son significativamente diferentes ($p > 0,05$) según la prueba de rango múltiple de Tukey.

La molienda de las harinas de caqui mejoró su capacidad de retención de bilis (Figura 12). La relación observada fue lineal e inversa: a mayor capacidad de retención de bilis menor tamaño de partícula ($p < 0,05$). Esta relación fue más acusada en las harinas Rojo Brillante, que además presentaron mayores valores que las harinas de Triumph ($p < 0,05$). La mejora de la capacidad de retención de bilis por parte de ingredientes vegetales ha sido mostrada por otros autores (Huang y col., 2018). Cabe destacar que los resultados obtenidos son superiores a los observados en otros coproductos de frutas (López-Marcos y col., 2015). Esta propiedad fisiofuncional está relacionada con mejorar el perfil lipídico en sangre y la homeostasis de la glucosa (Houten y col., 2006). Esto es debido a que la excreción por las heces de ácidos biliares (principales componentes de la bilis) activa la síntesis de ácidos biliares a partir de colesterol (su precursor), principalmente en el hígado (Houten y col., 2006). La activación de esta ruta metabólica se asocia con la mejora del metabolismo lipídico, glucídico y proteínico (Chiang, 2009). Pese a que estos resultados son *in vitro*, y no se han reproducido las condiciones fisiológicas, Matsumoto y col. (2010) han relacionado la capacidad de prevenir y mejorar las enfermedades cardiovasculares derivadas del consumo de caqui en ratas, con su capacidad para retener bilis.



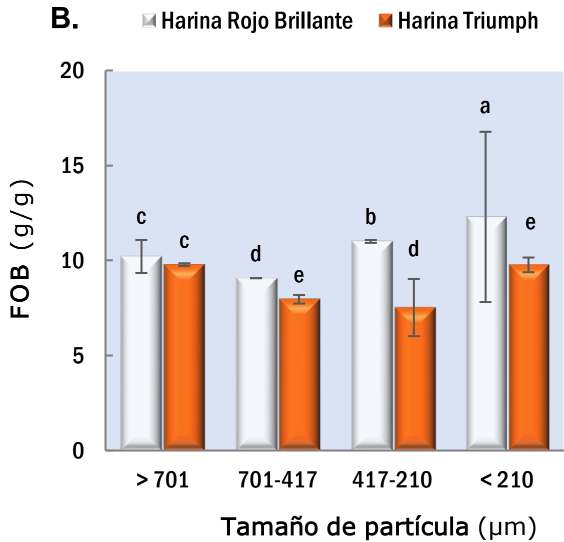


Figura 12.- Propiedades fisiofuncionales de las harinas de caqui estudiadas A. capacidad de retención de bilis (CRB); B. Capacidad de retención de grasa y aceite (FOB).

Diferentes letras (a-h) indican diferencias estadísticamente significativas ($p > 0{,}05$) según la prueba de rango múltiple de Tukey.

Teniendo en cuenta los resultados mostrados en el presente apartado, se seleccionaron las harinas de caqui de ambos cultivares con menor tamaño de partícula (< 210 µm), para ser agregadas a diferentes matrices alimentarias. Previamente se realizó un análisis microbiológico para comprobar su estabilidad microbiana, y se sometieron a la digestión *in vitro* para estudiar la estabilidad de sus compuestos (poli)fenólicos, como se detallará en el siguiente apartado.

4.3.-ESTUDIO DE LA FUNCIONALIDAD DE LAS HARINAS DE CAQUI

Los resultados de este trabajo han sido publicados en la revista Food Chemistry 256 (2018) 252-258.

La biodisponibilidad se define como "la cantidad de un nutriente o compuesto dietético disponible para algún proceso fisiológico". La disponibilidad está determinada por la bioaccesibilidad, "cantidad de un compuesto que está disponible para ser absorbido", y la bioactividad, "transformaciones biomoleculares necesarias a las que es sometido un nutriente para alcanzar tejidos diana de forma activa o para volverse bioactivo" (Fernández-García y col., 2009; Lorenzo y col., 2019). En consecuencia, para estimar o predecir la actividad biológica de un compuesto (poli)fenólico presente en un alimento o ingrediente, determinar exclusivamente su cantidad en el alimento no es suficiente. Por tanto, estudiar su bioaccesibilidad y su biodisponibilidad es un paso más para comprender la estabilidad de dichos compuestos a los

jugos digestivos y el efecto de la matriz alimentaria en la protección y liberación de los (poli)fenoles.

Para las harinas de caqui seleccionadas (harinas Rojo Brillante y Triumph con un tamaño de partícula < 210 μm), se analizaron tanto la bioaccesibilidad como el índice de recuperación de los compuestos fenólicos y los flavonoides, así como las modificaciones de su actividad antioxidante tras ser sometidas a procesos de digestión *in vitro*. Como previamente se ha descrito en la introducción, estos ensayos simulan las condiciones físicas y químicas que ocurren durante la digestión humana.

Los resultados obtenidos mostraron diferencias significativas ($p < 0,05$) en la bioaccesibilidad e índice de recuperación de los compuestos fenólicos y los flavonoides presentes en las dos harinas de caqui estudiadas (Figura 13 y 14).

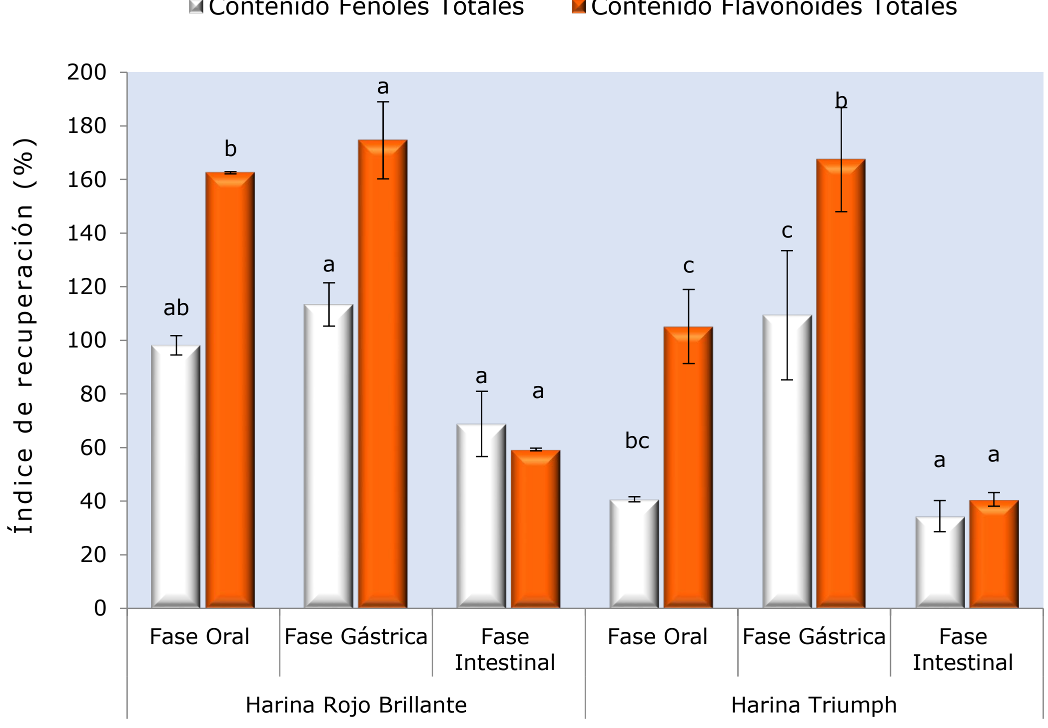


Figura 13.- Índice de recuperación del contenido de fenoles y flavonoides totales presentes en las harinas de caqui (Rojo Brillante y Triumph) en las tres fases digestivas simuladas.

Diferentes letras (a-h) entre cada determinación indican diferencias estadísticamente significativas ($p > 0.05$) según la prueba de rango múltiple de Tukey.

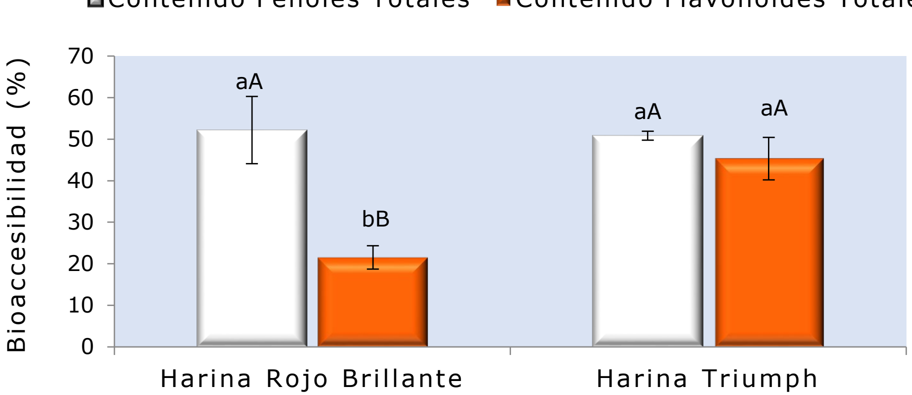


Figura 14.- Bioaccesibilidad del contenido de fenoles y flavonoides totales de las harinas de caqui. Diferentes letras minúsculas (a-b) indican diferencias estadísticamente significativas entre los tipos de harina, mientras que diferentes letras mayúsculas (A-B) expresan diferencias significativas entre ambos compuestos estudiados ($p > 0,05$) según la prueba de rango múltiple de Tukey.

Estos resultados indicarían que diferencias en la composición química, especialmente en el contenido y tipo de fibra presente en las harinas, determina la liberación de los compuestos (poli)fenólicos de la matriz alimentaria, y su posterior estabilidad tras los procesos digestivos. En este sentido, Viuda-Martos y col. (2018) mostró que la estabilidad de los compuestos (poli)fenólicos presentes en extractos de maqui, integrados en diferentes tipos de fibra, tras la simulación de la fase intestinal, variaba en función del tipo de fibra usado, siendo dicha estabilidad mayor cuando se aplicaron gomas, que cuando se aplicaron otras fibras, como inulina, pectina o celulosa. Otros autores han manifestado también la influencia de la matriz del alimento, así como el procesado, en la bioaccesibilidad de los compuestos (poli)fenólicos (Stanisavljević y col., 2015; Pešić y col., 2019; Tomás-Barberán y Espín, 2019). Por ejemplo, Pineda-Vadillo y col. (2017) mostraron que la bioaccesibilidad de las antocianinas varía en función de la matriz a la que sean añadidas (matrices lácteas y de huevo) y concluyeron que la matriz alimentaria protege la degradación de las antocianinas en el intestino en comparación con una solución de agua y antocianinas. Por otro lado, McClements y Xiao (2017) sugieren que la bioaccesibilidad de los compuestos bioactivos hidrófobos puede mejorarse incrementando su solubilidad mediante la inclusión de ácidos grasos en la matriz del alimento.

En relación con el perfil de compuestos (poli)fenólicos, este fue sutilmente diferente ente ambas harinas de caqui estudiadas (Tabla 11). La harina de Triumph mostró mayor cantidad y variedad de flavonoides que la harina de Rojo Brillante, mientras que la harina de Rojo Brillante presentó mayor contenido y variedad de ácidos fenólicos.

Además, aunque exista una tendencia de aumento del contenido de compuestos fenólicos en la fase gástrica ($p < 0,05$), y una disminución significativa en la fase intestinal ($p < 0,05$), de forma individual, los compuestos fenólicos se comportaron de manera diferente. Tras la fase intestinal, el ácido gálico, compuesto mayoritario en ambas harinas, no fue detectado, mientras que el ácido 4-hidroxibenzoico vio incrementada su concentración. Igualmente, los flavonoides glicosilados fueron más estables a los procesos digestivos que sus agliconas. Estos resultados permiten dilucidar que la liberación de los compuestos fenólicos de la matriz del caqui, así como su estabilidad, es intrínseca a cada compuesto. La baja biodisponibilidad de los compuestos (poli)fenólicos ha sido previamente observada por otros autores, tanto en ensayos *in vitro* (Lucas-González y col., 2016; Pellegrini y col., 2017; Viuda-Martos y col., 2018; Podio y col., 2019; Chait y col., 2020) como en ensayos *in vivo* (Murota y col., 2010; Neacsu y col., 2017; Tena y col., 2020). Pese a su baja disponibilidad, existe evidencia en humanos de que el consumo de poli(fenoles) naturalmente presentes en los alimentos, promueve la salud (Jacques y col., 2013; Zamora-Ros y col. 2013; Zhang y col., 2018). Sin embargo, también hay estudios que muestran que altas dosis de (poli)fenoles, suministradas principalmente como suplementos dietéticos, pueden tener efectos negativos sobre la salud (Granato y col., 2020). En este sentido, la Autoridad Europea de Seguridad Alimentaria (EFSA), solo reconoce efectos beneficiosos derivados del consumo de los flavanoles del cacao y los (poli)fenoles del aceite de oliva (Reglamento 432/2012). Esta controversia en el estado de arte de los (poli)fenoles y la promoción de la salud, podría deberse a varios factores concomitantes; por un lado, las biotransformaciones de los (poli)fenoles, tanto por las enzimas endógenas como por la microbiota intestinal, generan metabolitos desconocidos que pudieran ser responsables de la acción beneficiosa para el organismo. Recientes investigaciones en este campo apuntan a que los metabolitos generados por la microbiota intestinal se absorben mejor que los (poli)fenoles precursores y persisten en sangre más tiempo (García-Villalba y col., 2020; Oracz y col., 2020). Si tenemos en cuenta que los metabolitos producidos son generados por el organismo y los microorganismos, se entiende por qué algunos individuos no responden de igual manera al consumo de los mismos alimentos ricos en compuestos (poli)fenólicos, debido a la interindividualidad (Morand y col., 2020; Oracz y col., 2020).

Tabla 11.- Perfil de compuestos (poli)fenólicos (µg/g) de las muestras de las harinas de caqui Rojo Brillante (R) y Triumph (T), sin digerir y digeridas (oral, gástrica e intestinal)

		Sin digerir	Fase oral	Fase gástrica	FI (IN)	FI (OUT)	Total
Ácido gálico (mg/g)	R	3,40 ±0,99^{c}	5,70 ±0,35^{b}	6,93 ±0,70^{a}	ND.	ND.	-
	T	2,19 ±0,29^{b}	3,97 ±0,11^{c}	4,40 ±0,42^{c}	ND.	ND.	-
Ácido 4-hidroxibenzoico	R	193,35 ±18,01 ^{c}	222,37 ±13,15^{c}	304,25 ±12,53^{b}	265,62 ±22,19^{x}	243,96 ±14,87^{x}	509,58 ±37,06^{a}
	T	106,22 ±8,69^{d}	88,67 ±14,73^{d}	189,99 ±6,63^{c}	110,75 ±6,36^{y}	232,24 ±38,67^{x}	342,99 ±32,30^{b}
Ácido elágico	R	7,63 ±0,12^{b}	6,25 ±1,05^{b}	16,00 ±2,12^{a}	4,26 ±0,99^{x}	1,78 ±0,10^{y}	6,04 ±0.90^{b}
	T	7,62 ±1,99^{b}	3,75 ±0,55^{c}	4,07 ±0,26^{c}	1,39 ±0,23^{x}	1,28 ±0,19^{x}	2,76 ±0.40^{c}
Ácido cumárico-*o*-hexósido	R	23,91 ±4,64^{a}	20,54 ±1,09^{a}	20,27 ±3,34^{a}	ND.	ND.	-
	T	ND.	ND.	ND.	ND.	ND.	-
Epicatequina	R	36,50 ±3,68^{a}	21,80 ±1,96^{b}	19,00 ±1,71^{b}	ND.	ND.	-
	T	33,41 ±3,35^{a}	18,49 ±3,67^{b}	22,66 ±2,76^{b}	ND.	ND.	-
Quercetina-*o*-hexóxido	R	ND.	ND.	ND.	ND.	ND.	-
	T	108,70 ±15,75^{a}	45,48 ±2,40^{c}	67,23 ±1,28^{b}	11,42 ±0,30^{x}	13,62 ±1,99^{x}	25,04 ±2,28^{c}
Quercetina-*o*-pentósido I	R	54,09 ±0,80^{c}	44,46 ±1,24^{d}	36,43 ±1,77^{f}	14,60 ±3,85^{x}	16,15 ±3,11^{x}	30,76 ±0,74^{g}
	T	111,27 ±16,24^{a}	44,68 ±4,31^{d}	71,10 ±1,72^{b}	14,31 ±0,35^{y}	18,90 ±2,85^{x}	33,22 ±2,50^{g}
Kaempferol-3-*o*-glucósido	R	4,78 ±0,51^{a}	4,85 ±0,09^{a}	4,29 ±0,60^{a}	ND.	ND.	-
	T	ND.	ND.	ND.	ND.	ND.	-
Quercetina-*o*-pentósido II	R	ND.	ND.	ND.	ND.	ND.	-
	T	23,50 ±3,10^{a}	16,38 ±0,74^{b}	16,77 ±0,57^{b}	ND.	ND.	-
Kaempferol-*o*-hexósido I	R	ND.	ND.	ND.	ND.	ND.	-
	T	18,92 ±3,10^{a}	7,94 ±0,51^{bc}	12,03 ±0,03^{b}	3,48 ±0,77^{x}	3,09 ±0,20^{x}	6,56 ±0,58^{c}
Kaempferol-*o*-ramnósido	R	12,17 ±0,75^{a}	5,39 ±0,40^{c}	4,43 ±0,53^{c}	3,25 ±0,21^{x}	1,49 ±0,22^{y}	4,82 ±0,44^{c}
	T	14,48 ±2,35^{a}	7,73 ±1,37^{b}	9,34 ±0,26^{b}	2,57 ±0,01^{x}	2,10 ±0,08^{y}	4,67 ±0,07^{c}
Quercetina	R	1,56 ±0,06^{c}	1,36 ±0,00^{c}	1,57 ±0,46^{c}	ND.	ND.	-
	T	16,81 ±3,36^{a}	8,89 ±0,00^{b}	11,57 ±0,09^{b}	ND.	ND.	-
Kaempferol	R	ND.	ND.	ND.	ND.	ND.	-
	T	2,05 ±0,45^{a}	1,70 ±0,02^{a}	2,08 ±0,07^{a}	ND.	ND.	-

R: Rojo Brillante; T: Triumph. Para el mismo (poli)fenol, los valores con la misma letra (a-g) indica que no hay diferencias significativas entre la fase y el tipo de harina ($p > 0,05$), según la prueba de rango múltiple de Tukey. Para el mismo (poli)fenol y diferente fracción intestinal (IN y OUT) los valores con la misma letra (x-y) no son significativamente diferentes ($p > 0,05$), según la prueba de rango múltiple de Tukey. ND.: No detectado.

Además, aunque exista una tendencia de aumento del contenido de compuestos fenólicos en la fase gástrica ($p < 0,05$), y una disminución significativa en la fase intestinal ($p < 0,05$), de forma individual, los compuestos fenólicos se comportaron de manera diferente. Tras la fase intestinal, el ácido gálico, compuesto mayoritario en ambas harinas, no fue detectado, mientras que el ácido 4-hidroxibenzoico vio incrementada su concentración. Igualmente, los flavonoides glicosilados fueron más estables a los procesos digestivos que sus agliconas. Estos resultados permiten dilucidar que la liberación de los compuestos fenólicos de la matriz del caqui, así como su estabilidad, es intrínseca a cada compuesto. La baja biodisponibilidad de los compuestos (poli)fenólicos ha sido previamente observada por otros autores, tanto en ensayos *in vitro* (Lucas-González y col., 2016; Pellegrini y col., 2017; Viuda-Martos y col., 2018; Podio y col., 2019; Chait y col., 2020) como en ensayos *in vivo* (Murota y col., 2010; Neacsu y col., 2017; Tena y col., 2020). Pese a su baja disponibilidad, existe evidencia en humanos de que el consumo de poli(fenoles) naturalmente presentes en los alimentos, promueve la salud (Jacques y col., 2013; Zamora-Ros y col. 2013; Zhang y col., 2018). Sin embargo, también hay estudios que muestran que altas dosis de (poli)fenoles, suministradas principalmente como suplementos dietéticos, pueden tener efectos negativos sobre la salud (Granato y col., 2020). En este sentido, la Autoridad Europea de Seguridad Alimentaria (EFSA), solo reconoce efectos beneficiosos derivados del consumo de los flavanoles del cacao y los (poli)fenoles del aceite de oliva (Reglamento 432/2012). Esta controversia en el estado de arte de los (poli)fenoles y la promoción de la salud, podría deberse a varios factores concomitantes; por un lado, las biotransformaciones de los (poli)fenoles, tanto por las enzimas endógenas como por la microbiota intestinal, lo cual genera metabolitos desconocidos que pudieran ser responsables de la acción beneficiosa para el organismo. Recientes investigaciones en este campo apuntan a que los metabolitos generados por la microbiota intestinal se absorben mejor que los (poli)fenoles precursores y persisten en sangre más tiempo (García-Villalba y col., 2020; Oracz y col., 2020). Si tenemos en cuenta que los metabolitos producidos son generados por el organismo y los microorganismos, se entiende por qué algunos individuos no responden de igual manera al consumo de los mismos alimentos ricos en compuestos (poli)fenólicos, debido a la interindividualidad (Morand y col., 2020; Oracz y col., 2020).

En este contexto, García-Villalba y col. (2019) descubrió un nuevo metabolito derivado de los elagicotaninos y el ácido elágico en muestras de heces y orina en 9 pacientes de 47, poniendo de manifiesto que no todos los individuos del estudio presentaban el/los microrganismo/s responsables de la producción de dicho metabolito. Por último, las limitaciones metodológicas, relacionadas tanto con la ausencia de técnicas de detección para los nuevos metabolitos, como con la degradación de los compuestos cuando las muestras están congeladas (Manach y col., 2005) juegan un papel importante en las limitaciones del estudio del binomio (poli)fenoles -salud.

En relación con la actividad antioxidante mostrada por las harinas de caqui, esta varió en función de la fase digestiva simulada, el método de análisis empleado y el tipo de harina estudiada ($p < 0{,}05$) (Figura 15). De forma general, la actividad antioxidante se vio disminuida tras el proceso digestivo. No obstante, la actividad de ambas harinas aumentó tras la fase intestinal (método FIC) y tras la fase gástrica (método ABTS). Sin embargo, debido a lo anteriormente comentado (Apartado 4.2.3.) y a las posibles interacciones de otros nutrientes, como los aminoácidos aromáticos (ver Apartado 4.5.1.1), los resultados mostrados no tienen por qué tener una correlación directa con lo que ocurre *in vivo*.

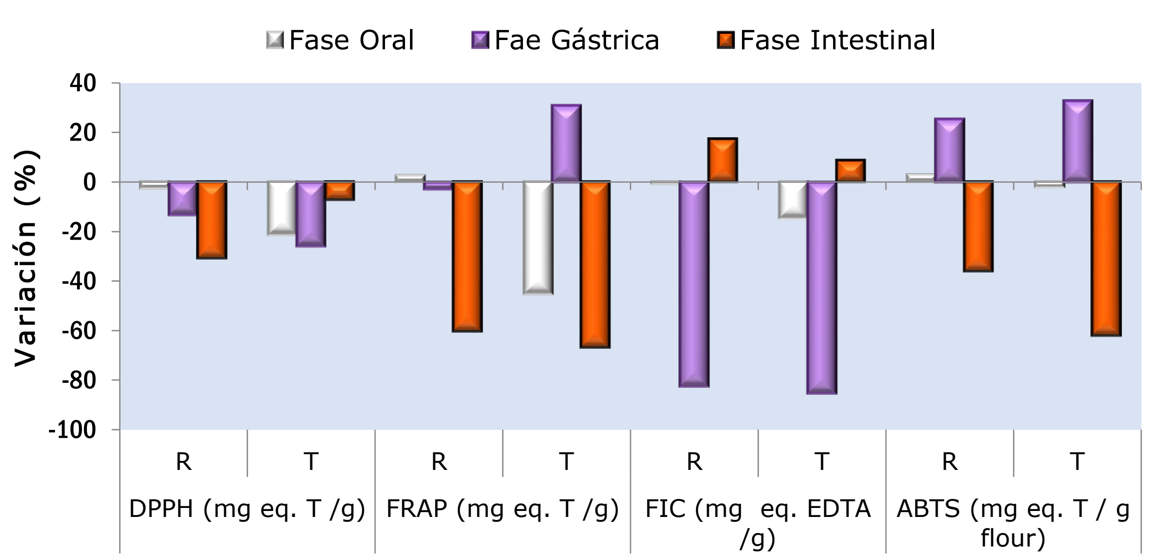


Figura 15.- Variación de la actividad antioxidante de las harinas de caqui durante el proceso de digestión *in vitro* (La determinación se ha realizado usando los valores medios).

R: Harina de caqui Rojo Brillante; T: Harina de caqui Triumph.

En las determinaciones del perfil de compuestos (poli)fenólicos de las harinas de caqui previamente mostradas (Tabla 7 y 11) no se tuvo en cuenta las diferentes fracciones de (poli)fenoles, libres, soluble-conjugados e insoluble-unidos que se pueden encontrar en los alimentos. Se entiende por (poli)fenoles libres aquellos que se encuentran libres dentro de la vacuola de la célula (Zhang y col., 2020b), mientras que los compuestos fenólicos conjugados son aquellos que están esterificados o eterificados a azúcares, ácidos orgánicos, aminas, lípidos u otros compuestos fenólicos (Chandrasekara, 2019). Por último, los compuestos fenólicos unidos son aquellos compuestos insolubles que se encuentran unidos covalentemente a proteínas estructurales y compuestos que conforman la pared celular de los productos vegetales como celulosa, hemicelulosa, lignina o pectina (Acosta-Estrada y col., 2014). Dado la actual relevancia en la promoción de la salud que presentan los compuestos (poli)fenólicos unidos (Zhang y col., 2020b), sobre todo derivado de su capacidad de modular la microbiota intestinal (Sharma y

Padwad, 2020), junto con la naturaleza de la harina de caqui, (gran parte de los compuestos solubles del caqui están presentes en el zumo), se realizó de nuevo la determinación del perfil de compuestos fenólicos de la harina de caqui, diferenciando entre la fracción libre y unida (Tabla 12). Esta caracterización únicamente se llevó acabo en la harina Rojo Brillante, debido a que, la inclusión de esta harina en los alimentos enriquecidos, espaguetis y paté, produjo menor desestabilización de la matriz, probablemente por su menor contenido de fibra insoluble, y es, por tanto, mejor candidata a ser usada como ingrediente intermedio, que la harina de Triumph.

En la Tabla 13 se presenta el contenido de los compuestos (poli)fenólicos libres y unidos presentes en la harina de Rojo Brillante con un tamaño de partícula <210 μm. El refinamiento de las técnicas de extracción junto con el fraccionamiento del extracto permitió detectar un total de 42 compuestos (Tabla 12), de los cuales se pudieron identificar y cuantificar 37 (Tabla 13). Los compuestos número 6, 12, 36, 39 y 41 presentaron espectros de absorción diferentes a los patrones disponibles (Apartado 3.2.1.12) y no pudieron ser identificados . En la Figura 16, se pueden observar los espectros de absorción de algunos de los (poli)fenoles que fueron tentativamente identificados o no se pudieron identificar, pues únicamente 12 (poli)fenoles fueron confirmados con el estándar (ácido gálico, catequina, ácido cafeico, epigalocatequina-3-galato, galocatequina-3-galato, ácido *p*-cumárico, epicatequina-3-galato, ácido elágico, ácido ferúlico, miricitina, quercetina y kaempferol).

Los flavonoides fueron la familia de compuestos más numerosa cualitativamente, en la harina de caqui, mientras que los ácidos fenólicos fueron más abundantes cuantitativamente. Estos resultados estuvieron en concordancia con otros autores, los que también mostraron que el ácido gálico es el componente mayoritario en el caqui tanto del cultivar “Rojo Brillante” como de otros cultivares (Baltacioğlu y col., 2013; Ancillotti y col., 2019).

La fracción unida representó en torno al 95% del total de los (poli)fenoles detectados, resultado esperado, teniendo en cuenta que la harina de caqui deriva de un coproducto, donde la mayor parte de los (poli)fenoles libres estarán presentes en el zumo.

Estos resultados ponen de manifiesto la diversidad de compuestos (poli)fenólicos en la harina de caqui y su potencial para enriquecer matrices alimentarias con fitoquímicos unidos, especialmente ácido gálico.

Tabla 12.- Especificaciones de los compuestos polifenólicos detectados en la harina de caqui Rojo Brillante.

Nº	Tr (min)	ʎmax (nm)	Identificación tentativa	F	RC
1	4,2	234/268	Ácido gálico glicosilado[b]	U	Ácido gálico
2	6,9	234/270	Ácido gálico[a]	U	Ácido gálico
3	8,7	236/270	Gallocatequina glicosilada[b]	U	Galocatequina
4	9,5	236/300	Ácido cumárico glicosilado[b]	U	Ácido *p*-cumárico
5	10,2	236/288sh338	Flavanona glicosilada I[b]	U	Hesperidina
6	11,2	236/sh274/344/sh456	Desconocido	U	Sin cuantificar
7	12,3	236/280	Catequina glicosilada I[b]	U	Catequina
8	12,8	236/280	Catequina glicosilada II[b]	U	Catequina
9	13,1	236/280	Catequina glicosilada III[b]	U	Catequina
10	13,5	236/280	Catequina glicosilada VI[b]	U	Catequina
11	14,0	236/280/330	Vanillina glicosilada[b]	U	Vanillina
12	14,5	244sh286/334	Desconocido	U	Sin cuantificar
13	15,0	236/280	Catequina[a]	L	Catequina
14	15,2	236/284sh338	Flavanona glicosilada II[b]	U	Hesperidina
15	16,9	236/278	Epigalocatequina-3-galato glicosilada[b]	U	Epigalocatequina-3-galato
16	17,3	246 sh298 324	Ácido cafeico[a]	U	Ácido cafeico
17	17,8	236/278	Epigalocatequina-3-galato[a]	U	Epigalocatequina-3-galato
18	17,9	242/272/sh334/494	Antocianina[b]	U	Malvidina-3-O-glucosa
19	18,5	236/278	Galocatequina-3-galato glicosilada[b]	U	Galocatequina-3-galato
20	19,2	236/278	Galocatequina-3-galato[a]	U	Galocatequina-3-galato
21	19,9	236/288sh334	Flavanona glicosilada III[b]	L	Hesperidina
22	20,7	252/358	Quercetina glicosilada I[b]	L	Rutin
23	21,0	236/288sh334	Flavanona glicosilada IV[b]	U	Hesperidina
24	21,2	264/360	Quercetina glicosilada II[b]	L	Rutina
25	22,0	240/sh300/310	Ácido *p*-Cumárico[a]	U	Ácido *p*-Cumárico
26	22,6	238/286sh334	Flavanona glicosilada V[b]	L	Hesperidina
27	22,6	236/278	Epicatequina-3-galato[a]	U	Epicatequina-3-galato
28	23,2	254/362	Ácido elágico[a]	L	Ácido elágico
29	23,5	258/358	Quercetina glicosilada III[b]	L/U	Rutina
30	23,7	242/sh296/324	Ácido ferúlico[a]	U	Ácido ferúlico
31	24,0	262/358	Quercetina glicosilada IV[b]	L	Rutina
32	25,1	266/348	Kaempferol glicosilado I[b]	L	Kaempferol
33	25,5	240/sh290/308/400	Ácido cumárico derivado[b]	U	Ácido cumárico
34	25,9	266/350	Kaempferol glicosilado II[b]	L/U	Kaempferol
35	26,4	268/350	Kaempferol glicosilado III[b]	L	Kaempferol
36	26,6	246/260/300	Desconocido[b]	U	Sin cuantificar
37	27,6	266/344	Kaempferol glicosilado IV[b]	L	Kaempferol
38	28,5	254/372	Mirecetina[a]	L	Mirecetina
39	28,8	238sh302/346	Desconocido	L/U	Sin cuantificar
40	33,2	256/370	Quercetina[a]	L/U	Quercetina
41	37,1	254sh264/312sh374	Desconocido	U	Sin cuantificar
42	37,8	258/372	Kaempferol[a]	L	Kaempferol

Tr: tiempo de retención; F: Fracción; U: unidos; L: libres; MI: Modo de identificación; RC: recta de calibrado usada para la cuantificación. [a]Compuesto confirmado mediante espectro de absorbancia y tiempo de retención con el patrón correspondiente. [b]compuestos que presenta un espectro similar o igual al compuestos o familia que se le ha asignado.

Tabla 13.- Compuestos (poli)fenólicos libres y unidos (µg/g peso seco) de la harina de caqui				
Familia	**Nº**	**Compuesto**	**Libre**	**Unido**
Ácidos fenólicos	16	Ácido cafeico		23,80±4,09
	28	Ácido elágico	7,30±1,88	
	30	Ácido ferúlico		21,08±3,65
	2	Ácido gálico		10.074,64±1049,51
	1	Ácido gálico glicosilado		6,24±0,56
	25	Ácido *p*-cumárico		39,40±6,06
	4	Ácido cumárico-o-hexósido		77.35±5.37
	33	Ácido cumárico derivado		15,71±3,93
	11	Vanillina glicosilada		80,93±25,09
Total			**7,30±1,88**	**10.339,15±1.098,26**
Flavanonas	5	Flavanona glicosilada I		221,08± 50,98
	14	Flavanona glicosilada II		222,41±15,49
	21	Flavanona glicosilada III	28,21±6,74	
	23	Flavanona glicosilada IV		1233,34±104,81
	26	Flavanona glicosilada V	23,65±3,38	
Total			**51,86±10,12**	**1.676,83±171,29**
Flavanoles	13	Catequina	37,10±5,42	
	7	Catequina glicosilada I		540,52±46,86
	8	Catequina glicosilada II		122,69±4,75
	9	Catequina glicosilada III		158,00±39,70
	10	Catequina glicosilada IV		133,59±32,04
	27	Epicatequina-3-galato		30,84±2,16
	17	Epigalocatequina-3-galato		38,81±6,37
	3	Galocatequina glicosilada		327,24±1,23
	20	Galocatequina-3-galato		34,17±8,85
	19	Galocatequina-3-galato glicosilada		50,00±9,49
	15	Epigalocatequina-3-galato glicosilada		173,24±43,80
Total			**37,10±5,42**	**1.628,47±185,46**
Flavonoles	42	Kaempferol	2,96±1,29	
	32	Kaempferol glicosilado I	4,03±0,34	
	34	Kaempferol glicosilado II	11,84±1,10	9,94±2,19
	35	Kaempferol glicosilado III	2,47±0,48	
	37	Kaempferol glicosilado IV	2,00±0,64	
	40	Quercetina	7,40±5,64	7,67±1,24
	22	Quercetina glicosilada I	6,07±0,66	
	24	Quercetina glicosilada II	6,30±0,47	
	29	Quercetina glicosilada III	29,76±4,82	32,73±1,02
	31	Quercetina glicosilada IV	10,74±0,11	
	38	Mirecetina	3,61±0,54	
Total			**87,18±13,09**	**50,34±4,46**
Antocianinas	18	Antocianina		76,67±6,59
Total Flavonoides			**176,14±28,63**	**3.432,31±367,79**
		Total	**183,44±30,51**	**1.3958,7±1.489,2**

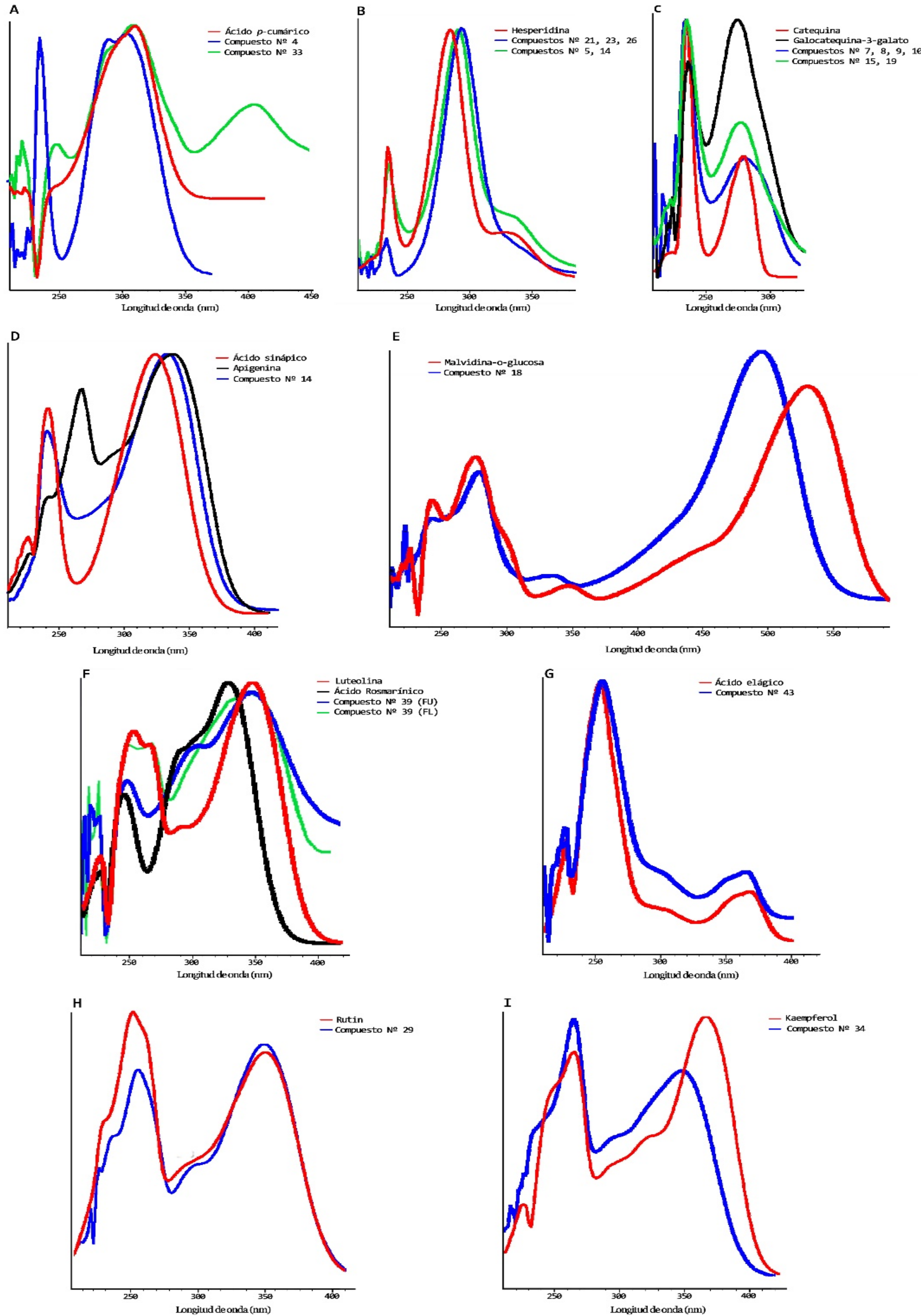


Figura 16.- Espectro de absorción de algunos de los compuestos (poli)fenólicos detectados en la harina de caqui.

4.4. INCORPORACIÓN DE LAS HARINAS DE CAQUI A DIFERENTES MATRICES ALIMENTARIAS

Los resultados del presente trabajo han sido publicados en la revista International Journal of Food Science and Technology (2019) 54:1232-1239 y la revista Journal of Food Measurement and Characterization (2020) 14:1634–1644.

Teniendo en cuenta la composición nutricional y las propiedades tecnofuncionales de las harinas de caqui estudiadas (Rojo Brillante y Triumph) con menor tamaño de partícula (<210 µm), se optó por introducir las harinas en dos matrices alimentarias diferentes, una cárnica, paté de hígado de cerdo y otra vegetal, espaguetis de sémola de trigo duro. En ambos casos se enriquecieron los alimentos con dos concentraciones diferentes, 3 y 6%.

4.4.1.- Elaboración de paté de hígado de cerdo con adición de harina de caqui

El paté de hígado de cerdo es un producto cárnico cocido tipo emulsión, cuyos ingredientes principales son el hígado, las partes grasas del cerdo (tocino, papada, panceta), el agua, la sal y los aditivos (Tabla 14). Se caracteriza por contener altas cantidades de hierro, ácido fólico y grasa (Brito y col., 2006). Esta composición química, junto a las operaciones unitarias (corte, picado, mezcla, cocción) a las que son sometidos sus ingredientes, hacen al paté de hígado de cerdo un alimento susceptible a la oxidación lipídica. Además, en su formulación se usan diferentes aditivos como el nitrito de sodio, el cual, rara vez no es incluido, debido a su papel antimicrobiano, antioxidante y fijador del color; o los antioxidantes, como el ascorbato de sodio, eritorbato de sodio o extractos ricos en tocoferol (Tabla 14).

La inclusión de harina de caqui en el paté de hígado de cerdo, varío significativamente su composición química ($p < 0,05$) (Tabla 15). Sin embargo, desde un punto de vista dietético, las diferencias mostradas no tienen un efecto nutricional, pues los valores más alejados al paté control distan unos 3 g por 100 g, con respecto a la grasa y la humedad, y en el caso de las proteínas apenas hay diferencias de 1,5g.

Tabla 14.- Comparación de diferentes patés de hígado de cerdo disponibles en el mercado (2020).

Fabricante/Marca	Precio (€/kg)	Ingredientes	G	G.S	Sal	H.C	P	H*
FRIGOLOURO, S.A./ Eroski	3,70	Tocino de cerdo, hígado de cerdo (23%), agua, harina de trigo, cebolla, proteína animal, azúcar, sal, aroma [colorante (E-160c) y antioxidante(E-306)] y conservador (E-250).	20,6	8,1	1,4	5,9	11,5	60,6
El Corte Inglés Hermosilla/ Aliada	6,21	Tocino, hígado de cerdo (31%),agua, carne de cerdo, harina de trigo, proteínas de la leche, sal, estabilizante (E-451i), aromas, azúcar, proteína animal y conservador (E-250)	33	13	1,5	1,6	11	52,9
Inds. Carnicas Tello S.A./Mina	6,95	Hígado de cerdo (24%), tocino, agua, harina de trigo, sal, especias, azúcar, antioxidante (E-331, E-316), aromas, estabilizador (E-415) y conservador (E-250).	11	3,6	1,5	5	8,5	74
FRIGOLOURO, S.A./ CARREFOUR	8,29	Hígado de cerdo (28%), tocino y carne de cerdo, agua, sal, azúcar, proteína animal, aromas [potenciador del sabor (E-621, E-627, E-631 y ácido (l-glutámico), corrector de acidez (E-330) y contiene hidrolizado de proteína de trigo], dextrosa de maíz, antioxidante (E-301), potenciador de sabor (E-621) y conservador (E-250)	27	10,5	1,5	1,3	12	58,2
Huerta dehesa "El Alcornocal"/ Iberitos.*	8,4	Tocino de cerdo 27%, hígado de cerdo ibérico 14%, hígado de cerdo 14%, papada de cerdo 14%, agua, leche, cebolla (cebolla, aceite de oliva, corrector de acidez (E-330), almidón, vino Pedro Ximénez 3%, sal, pasas 2% (pasas, aceite de girasol), proteína de soja, especias, aromas, estabilizantes (E-450, E-452), dextrosa, potenciador de sabor (E-621), antioxidante: (E301), conservador (E-250) colorante (E-120i)	29	7,08	2,44	5,48	10,5	52,6
n.d/ La piara Tapa Negra	12,04	Carne, hígado, tocino de cerdo, agua, harina de trigo, fibra vegetal, sal, estabilizantes, (E-451,E-407), potenciador del sabor (E-621), azúcar, especias, aromas y conservador (E-250)	24	8,1	1,7	1,4	11	61,9
Embutidos Munar S.L/ Munar Mallorca	13,68	Tocino, hígado (43%) y magro de cerdo; sal, especias, vino de Jerez y conservante (E-250)	44	17	2,6	1,24	11	41,1
n.d/ Domaines Kilger	21,2	80% Carne de cerdo, 20% Hígado de cerdo, Sal de cocina, especias, Cebolla, Manzana	n.d	n.d	n.d	n.d	n.d	n.d
Embutidos Munar S.L/ Munar Mallorca**	23,61	Tocino, hígado (39%) y magro de cerdo; sal, especias y conservante (E-250)	36	12	2,8	1,85	16	43,7

Valores expresados en g/100 g . G: grasa; GS: grasa saturada; H.C: hidratos de carbono; P: proteína; H: humedad.; n.d: no disponible.; E-451. tripolifósfato sódico; E-407 carragenanos; E-621 glutamato monosódico; E-250 nitrito sódico; E-627 guanilato sódico; E-631 inosinato sódico; (E-330 ácido cítrico); E-301 ascorbato sódico. E-450 difosfatos. E-452 polifosfatos. E-120i Rojo carmin E-331. Citratos sódicos. E-316 Eritorbato de sodio E-415 Goma Xantana. E160c- Extracto de pimentón, capsantina y capsorrubina E-360 Adipato de magnesio. E-306. Extracto rico en tocoferoles. *Valor estimado (100-suma de G, P, sal e H.C).

Tabla 15.- Composición química del paté control y los pates enriquecidos con harina de caqui.

	PC	PR-3	PT-3	PR-6	PT-6
Humedad	56,96±0,26[a]	53,97±0,26[b]	54,77±0,51[a]	56,15±2,09[a]	57,40±0,40[a]
Grasa	27,49±0,34[b]	30,69±0,60[a]	28,55±0,70[b]	27,19±0,79[b]	24,67±0,02[c]
Proteínas	13,45±0,36[a]	12,42±0,13[ab]	13,00±0,21[ab]	12,28±0,32[b]	12,46±0,14[ab]
Cenizas	1,55±0,03[c]	1,70±0,02[c]	2,17±0,02[b]	2,19±0,03[b]	2,45±0,08[a]
Nitrito residual*	28,96±0,30[a]	22,49±0,21[b]	14,20±0,19[c]	11,61±0,14[d]	10,58±0,14[e]

Valores expresados en g/100 g; *NaNO2 ppm; PC: paté control; PR-3: paté con 3% de harina Rojo Brillante; PT-3: paté con 3% de harina Triumph; PR-6: paté con 6% de harina Rojo Brillante; PT-6: paté con 6% de harina Triumph. Diferente letra (a-e) dentro de la misma fila indica diferencias estadísticamente significativas ($p < 0,05$) según la prueba de rangos múltiples de Tukey.

Estas mismas variaciones pueden ser debidas a la diferente composición de las piezas de carne (hígado, panceta, papada) usadas. El efecto más destacado se produce sobre el contenido de nitrito residual, el cual, se vio reducido por acción de las harinas de caqui incluidas en la matriz cárnica. La reducción fue concentración dependiente, y estuvo influenciada por el tipo de harina. La harina Triumph mostró mayor capacidad para disminuir el contenido total de nitrito residual que la harina Rojo Brillante ($p < 0,05$). La reducción del nitrito por parte de las harinas podría atribuirse a su contenido en (poli)fenoles, como han indicado otros autores al estudiar la inclusión de ingredientes vegetales en productos cárnicos (Viuda-Martos y col., 2009). Este efecto observado en los patés enriquecidos se puede considerar como un efecto positivo del enriquecimiento del paté con harina de caqui. El nitrito de sodio añadido al paté reacciona con la mioglobina formando nitrosomioglobina, la cual durante el tratamiento térmico se transforma en nitrosilhemocromo o nitrosoferrohemocromo, pigmento proteico responsable del color rosado característico del paté (Ventanas y col., 2004). El nitrito que no reacciona con la mioglobina queda libre para reaccionar con otros compuestos y formar compuestos N-nitrosos como las nitrosaminas (Viuda-Martos y col., 2009). La Agencia Internacional para la Investigaciones sobre el Cáncer (IARC) de la OMS cataloga a los nitritos o nitratos ingeridos a través de la dieta que puedan originar nitrosación endógena, como posibles sustancias cancerígena (Grupo 2A) para el ser humano (IARC, 2020). Estos compuestos junto con la aparición de otras sustancias cancerígenas en los productos cárnicos procesados, derivados de los procesos de ahumado o de la oxidación lipídica, pueden estar detrás de la relación del consumo de productos cárnicos con el desarrollo de cáncer colorrectal (Bouvard y col., 2015; IARC, 2018). Es por todo lo expuesto que el uso de ingredientes con capacidad para interaccionar con los nitritos, y reducir así su contenido residual en los productos cárnicos, es una estrategia para producir productos cárnicos seguros y saludables.

Todas las muestras de paté enriquecidas con harina de caqui mostraron valores menores de malondialdehído (MDA), producto final de la reacción de oxidación lipídica, en comparación con el control ($p < 0{,}05$) (Figura 17). Los patés enriquecidos con un 3% de ambas harinas de caqui, presentaron valores de MDA más bajos que las formulaciones con la máxima concentración de harina de caqui estudiada. Dadas las connotaciones negativas para el consumidor de los productos rancios (alta oxidación lipídica) la reducción de estos procesos oxidativos permite mantener durante más tiempo la calidad organoléptica del producto, así como su valor nutricional.

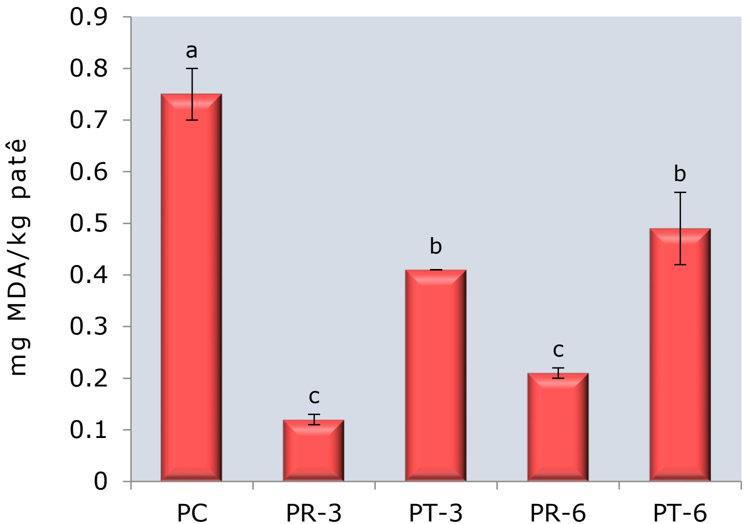


Figura 17.- Oxidación lipídica (TBARS) de las diferentes formulaciones de paté estudiadas.
Diferente letra (a-e) indica diferencias estadísticamente significativas ($p < 0{,}05$), según la prueba de rangos múltiples de Tukey.

No obstante, la harina de caqui también tuvo repercusiones negativas en la estabilidad del paté. Como puede observarse en la Figura 18, la adición de harina de caqui al paté produjo un aumento del porcentaje de exudado (agua y grasa) cuando el paté es sometido a estrés, tanto térmico como mecánico. La pérdida de estabilidad de la emulsión fue dependiente de la concentración y del tipo de harina de caqui ($p < 0{,}05$). La harina de Triumph mostró mayor capacidad para desestabilizar la emulsión cárnica, que la harina Rojo Brillante ($p < 0{,}05$). Estas diferencias pueden estar influenciadas por la composición química de ambas harinas, en especial por su contenido de fibra dietética insoluble.

El enriquecimiento del paté con las harinas de caqui tuvo otros efectos positivos tanto organolépticos como tecnológicos. El color es un atributo sensorial con especial repercusión en la aceptación o rechazo de los alimentos por parte de los consumidores. Los patés enriquecidos con harina de caqui vieron incrementada su rojez (a*) (Tabla 16), la cual era apreciable a simple vista. Este aumento fue concentración dependiente ($p < 0{,}05$). El aumento de color fue percibido por los panelistas encuestados como algo positivo, (Tabla 17 y Figura 19) pues los patés enriquecidos

obtuvieron mejor puntuación global que el paté control, siendo de entre las cinco formulaciones, el PR-3 el que obtuvo la mejor puntuación, y el único atributo donde se mostraron diferencias significativas fue el del color.

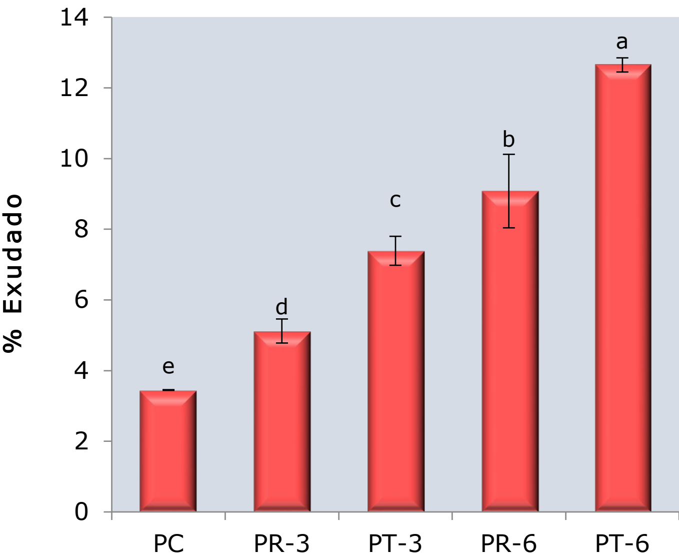


Figura 18.- Estabilidad de la emulsión de las formulaciones de paté estudiadas.

Diferente letra (a-e) indica diferencias estadísticamente significativas ($p < 0{,}05$), según la prueba de rangos múltiples de Tukey. PC: Paté control; PR-3: Paté con 3% de harina Rojo Brillante; PT-3: Paté con 3% de harina Triumph; PR-6: Paté con 6 % de harina Rojo Brillante; PT-6: Paté con 6% de harina Triumph.

Tabla 16.- Propiedades fisicoquímicas de los patés de hígado de cerdo estudiados

	CP	PR-3	PT-3	PR-6	PT-6
aw	0,933±0,004[a]	0,926±0,001[ab]	0,925±0,002[b]	0,929±0,001[a]	0,921±0,002[b]
pH	6,43±0,02[a]	6,37±0,02[ab]	6,38±0,04[ab]	6,24±0.01[c]	6,34±0,05[bc]
L*	64,15±0,42[a]	59,59±0,45[b]	54,87±0,99[d]	56,38±0,33[c]	54,46±0,21[d]
a*	4,25±0,31[c]	5,20±0,42[b]	6,88±0,72[a]	6,79±0,15[a]	7,41±0,53[a]
b*	14,05±0,32[b]	13,31±0,71[c]	15,43±0,42[a]	13,45±0,24[bc]	15,59±0,42[a]
Dureza (kg)	2,12±0,00[b]	2,96±0,56[a]	1,70±0,03[bc]	1,86±0,00[bc]	1,24±0,08[c]
Adhesividad (g/seg)	-64,21±20,80[b]	-111,12±30,94[ab]	-171,52±1,98[a]	-161,79±0,00[a]	-91,75±7,13[b]
Elasticidad (mm)	0,21±0,02[a]	0,26±0,01[a]	0,15±0,01[b]	0,14±0.01[b]	0,15±0,01[b]
Cohesividad	0,48±0,07[b]	0,76±0,05[a]	0,34±0,02[cd]	0,44±0,02[bc]	0,27±0,01[d]
Gomosidad (g)	934,67±47,41[a]	809,35±70,03[b]	604,14±17,60[c]	769,41±20,30[b]	337,67±31,45[d]
Untabilidad (kg*seg)	14,37±1,29[a]	16,50±1,46[a]	14,94±0,26[a]	13,84±1,09[a]	12,18±0,13[b]

PC: Paté control; PR-3: Paté con 3% de harina Rojo Brillante; PT-3: Paté con 3% de harina Triumph; PR-6: Paté con 6 % de harina Rojo Brillante; PT-6: Paté con 6% de harina Triumph. Diferente letra (a-e) dentro de la misma fila indica diferencias estadísticamente significativas ($p < 0,05$), según la prueba de rangos múltiples de Tukey.

Tabla 17.- Valores de los atributos “Aceptación general” y “color” de los diferentes patés y diferencias estadísticamente significativas con respecto al paté control.

	Aceptación general		Atributo de color
	Valor	*P* valor	*P* valor
PC	4,53[b]		
PR-3	7,16[a]	0,009	0,00012
PT-3	6,72[a]	0,044	0,00012
PR-6	7,06[a]	0,013	0,00012
PT-6	6,94[a]	0,021	0,00012

PC: Paté control; PR-3: Paté con 3% de harina Rojo Brillante; PT-3: Paté con 3% de harina Triumph; PR-6: Paté con 6 % de harina Rojo Brillante; PT-6: Paté con 6% de harina Triumph. Diferente letra (a-e) indican diferencias estadísticamente significativas ($p < 0,05$), según la prueba de rangos múltiples de Tukey.

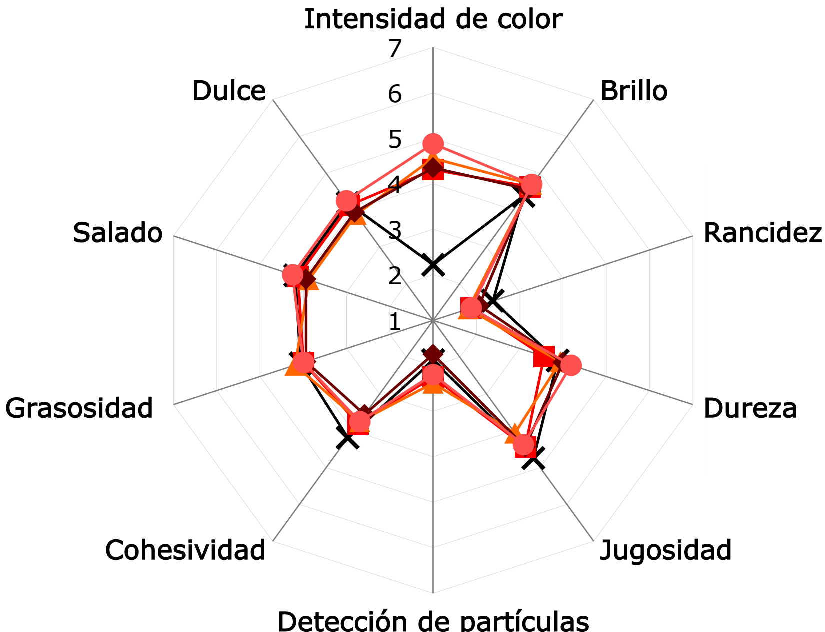


Figura 19.- Evaluación sensorial: resultados del análisis descriptivo cuantitativo llevado a cabo para las cinco formulaciones de paté de hígado de cerdo estudiadas.

CP: paté control; PR-3: paté con 3% de harina Rojo Brillante; PT-3: paté con 3% de harina Triumph; PR-6: paté con 6% de harina Rojo Brillante; PT-6: paté con 6% de harina Triumph;

4.4.2.- Elaboración de espaguetis de trigo duro con adición de harina de caqui

La pasta es ampliamente consumida en todas las partes del mundo por ser un alimento versátil, puede ir acompañado con casi cualquier ingrediente, ya sea carne, pescado, verduras o en combinación, y de fácil y rápida preparación. Se elabora mediante la formación de una masa compuesta por sémola de trigo duro y agua, que posteriormente es extruida, en la forma y tamaño deseados, y deshidratada. Durante el mezclado se configura la red gluten-almidón, en la que el almidón granulado queda embebido en la red que conforman las proteínas gluteína y gliadina (gluten) (Delcour y col., 2010). Esta red es la responsable de las propiedades texturales y nutritivas propias de la pasta (Bonomi y col., 2010). Durante los procesos de secado se producen daños en los gránulos de almidón (Bonomi y col., 2010), lo que puede ocasionar defectos en la pasta como aparición de puntos blancos o que las hebras se peguen. También se genera la

polimerización de las proteínas, lo cual tienen una gran importancia en la calidad de la pasta cocida, la cual depende en gran medida de cómo la red de proteínas resiste la hinchazón del almidón, el cual gelifica durante la cocción, y evita su posterior lixiviación al agua de cocción (Delcour y col., 2012). Por lo tanto, la calidad de la pasta está determinada, tanto por la composición química de la sémola, principalmente su contenido en proteína (Deng y col., 2017), como por los procesos de deshidratación (D'egidio y col., 1990). La inclusión de cualquier ingrediente diferente a la sémola de trigo puede ocasionar disrupciones en la red molecular del gluten o competencia por el agua, provocando un detrimento en la calidad de la pasta (Aravind y col., 2012). No obstante, actualmente existen en el mercado gran variedad de pasta a la que se le ha añadido, en pequeñas cantidades, otros ingredientes como el chocolate, tomate, espinacas, zanahorias, algas, calabaza o garbanzos (Tabla 18). Las formulaciones de espaguetis elaboradas para este estudio en el Centro Tecnológico de Cereales (Roma, Italia) cumplen las normas higiénico-sanitarias conforme a la legislación italiana (Ministero della Sanità, 1985) para pasta alimentaria, pues mostraron ausencia de bacterias mesófilas, hongos y *salmonella sp*.

La composición química de las cinco formulaciones de espaguetis elaboradas (Tabla 19) está en concordancia con el valor nutricional de la pasta que se puede encontrar en el mercado (Tabla 18). La incorporación de la harina de caqui provocó una ligera disminución del contenido de proteínas y un aumento de la fibra dietética, el azúcar y los pigmentos amarillos totales (PAT). El incremento fue dependiente de la concentración y tipo de harina ($p < 0,05$). La harina de Triumph aportó más fibra y PAT que la harina Rojo Brillante ($p < 0,05$). El incremento de fibra en las cuatro formulaciones de espaguetis enriquecidas permite etiquetarlas como fuente de fibra, bajo el amparo del Reglamento Europeo No 1924/2006 (Reglamento 1924/2006).

Tabla 18.- Comparación de diferentes pastas alimentarias de sémola de trigo duro adicionada con otros ingredientes de origen vegetal disponibles en el mercado (2020).

Fabricante/*Marca*	**Precio** (€/kg)	**Tipo**	**Ingredientes**	**TC** (min)	**G**	**G.S**	**H.C**	**A**	**P**	**F**
COMERCIAL GALLO S.A.U. *Pasta Fina*	3,38	Espaguetis	Sémola de trigo duro, espinacas deshidratadas (2,5%); sémola de trigo duro, tomate concentrado 28/30ºBrix (5%).	3	2,0	0,5	67,0	4,0	12	5,0
Biogran *El granero Intergral*	5,90	Espaguetis*	Sémola de trigo dura (85%) y harina de mijo (15%)	14	2,0	0,1	74,0	2,0	12	3,5
Biográ *pasta con verdura*	6,52	Espaguetis*	Sémola de Trigo Duro , Espinacas Deshidratadas (3%) , Tomate Deshidratado (2%) , Pimienta (1%)	15	1,5	0,06	75	2	12	4,1
Ceralis-Productos Alimentares *la Villa*	1,06	Fusilli	Sémola de trigo duro, espinacas en polvo (0,7%) y tomate en polvo (0,7%)	11	1,7	0,4	70,0	6,0	13	n.d
Comercial gallo S.A.U *Nature multivegetales*	4,12	Fusilli	Sémola de trigo duro (93,5%), concentrado de tomate 28/30 ºbrix (4%), remolacha (2,5%); Sémola de trigo duro (99%), espirulina (1%); sémola de trigo duro (98,5%), cúrcuma molida (1,5%).	10-12	2,0	0,6	70,0	4,0	12	6,2
COMERCIAL GALLO S.A.U. *Nature multivegetales*	4,12	Fusilli	Sémola de trigo duro (93%), zanahoria (4%), pimentón dulce (3%); sémola de trigo duro (97%), kale (3%); sémola de trigo duro (99%), espirulina (1%).	12-12	2,4	0,7	68,0	4,0	12	6,9
CASTAGNO BRUNO MOLINO E PASTIFICIO	n.d	Fusilli*	Semola de trigo duro, mezcla de algas en proporciones variables 4,5% (Nori (Porphyra Sp.) Kombu (Laminaria Japonica))	5-7	1,4	0,4	71,0	4,1	11,0	3,5
COMERCIAL GALLO S.A.U. *Nature multicereal*	3,12-4,12	Macarrones	Sémola de trigo duro (90%), harina de quínoa (10%); sémola de trigo duro (97%), harina de malta de centeno tostado (3%); sémola de trigo duro(98,5%), cúrcuma molida (1,5%).	6-8	0.9	0.5	72	1,2	12	4,4
CASTAGNO BRUNO MOLINO E PASTIFICIO	8,24	Marziani*	Sémola integral trigo duro, amaranto, garbanzos y perejil	5-7	3,6	0,8	64	3,5	13	7,3
Kürbishof Koller/ *Kurbisnudeln*	6,49	Noodels**	Sémola de trigo, pulpa de calabaza y cúrcuma	n.d	1,4	0,3	69	0,6	11	n.d
Pasta d´Alba/ *Cacao*	9,00	Tagliatelle*	Sémola de trigo duro, cacao en polvo (2%), agua.	4-6	1,5	0,3	75,0	4,9	11,0	3,5

Valores expresados en g/100g. *De cultivo ecológico; **Elaboración tradicional; n.d: no disponible. TC: Tiempo de cocción; G: grasa; GS: grasa saturada; H.C: hidratos de carbono; A: Azúcar; P: proteína; F: fibra. n.d: no disponible.

Fisicoquímica y tecnológicamente, puede decirse que los efectos más destacables de la inclusión de harina de caqui a los espaguetis de sémola de trigo duro fueron: su variación de color (Tabla 20), pues al igual que se observó en los patés de hígado de cerdo enriquecidos, se observó una reducción en la luminosidad (L*) y un aumento de la coordenada rojo-verde (a*), la cual fue concentración-dependiente; y la reducción de su tiempo óptimo de cocción (Tabla 21). Los SR-6 y ST-6 fueron los que requerían menor tiempo de cocción, concretamente tres minutos menos que la muestra control.

Tabla 19.- Composición química de las diferentes formulaciones de espaguetis de sémola de trigo duro elaboradas.

	SC	SR-3	ST-3	SR-6	ST-6
Humedad	9,99±0,08[a]	9,42±0,06[b]	9,48±0,06[b]	9,15±0,05[c]	8,96±0,07[d]
Almidón	62,98±2,91[a]	63,03±3,54[a]	62,84±1,62[a]	56,03±2,79[b]	56,72±1,37[b]
Proteína	11,20±0,23[a]	11,33±0,16[a]	10,51±0,00[b]	10,69±0,51[ab]	10,34±0,03[b]
FDT	2,96±0.12[e]	3,57±0,14[d]	3,94±0,01[c]	4,39±0,30[b]	4,87±0,26[a]
Cenizas	0,54±0,02[a]	0,58±0,02[a]	0,61±0,03[a]	0,61±0,05[a]	0,64±0,02[a]
Maltosa	6,24±0,22[a]	6,03±0,07[b]	5,77±0,11[c]	5,33±0,06[d]	5,33±0,03[d]
Fructosa	0,39±0,01[c]	1,72±0,03[b]	1,88±0,00[b]	2,37±0,03[a]	3,02±0,02[a]
Glucosa	0,39±0,01[c]	1,55±0,02[b]	1,64±0,00[b]	2,23±0,02[a]	2,92±0,04[a]
Sacarosa	0,06±0,00[a]	tr[b]	tr[b]	tr[b]	tr[b]
PAT (mg/100g)	1,37±0,10[e]	2,27±0,16[d]	2,97±0,04[c]	3,72±0,25[b]	4,49±0,31[a]

Valores expresados en (g/100g); tr: trazas; SC: Espaguetis control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph. FDT: Fibra dietética total; PAT: Pigmentos amarillos totales. Diferente letra (a-e) dentro de la misma fila indica diferencias estadísticamente significativas ($p < 0,05$), según la prueba de rangos múltiples de Tukey.

Tabla 20.- Diámetro, fracturabilidad y coordenadas de color de los espaguetis crudos; Coordenadas de color de los espaguetis cocinados en su tiempo óptimo de cocción; y Diferencias de color ($\Delta E*$) para los espaguetis crudos y cocidos, en comparación con los espaguetis control.

	Espaguetis Crudos						Espaguetis Cocidos			
	Diámetro (mm)	Fracturabilidad (N)	L*	a*	b*	ΔE*	L*	a*	b*	ΔE*
SC	1,70±0,00[a]	68,18±6,18[a]	63,54±1,74[a]	-7,85±0,35[c]	44,48±2,89[c]		66,25±0,84[a]	-9,03±0,38[d]	23,22±1,38[d]	
SR-3	1,65±0,04[ab]	66,97±5,68[a]	59,83±1,56[b]	-0,34±0,60[b]	47,23±1,37[b]	8,81	60,17±1,16[b]	-4,21±0,34[c]	26,55±1,22[c]	8,45
ST-3	1,61±0,01[b]	65,88±8,53[a]	58,73±2,39[b]	0,27±0,29[b]	49,75±1,96[a]	10,81	61,08±0,91[b]	-4,57±0,31[c]	27,70±0,98[bc]	8,17
SR-6	1,66±0,02[ab]	55,69±7,19[ab]	55,13±1,85[c]	4,62±0,37[a]	45,26±1,58[c]	15,09	57,64±1,16[c]	-1,72±0,55[a]	28,44±1,18[b]	12,45
ST-6	1,50±0,02[c]	48,09±8,31[b]	54,57±1,92[c]	4,04±0,43[a]	48,64±1,33[ab]	15,46	58,85±1,09[c]	-2,57±0,53[b]	31,80±1,48[a]	13,05

SC: Espaguetis control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph. Diferente letra (a-e) dentro de la misma columna indica diferencias estadísticamente significativas ($p < 0,05$), según la prueba de rangos múltiples de Tukey.

En base a los valores de calidad de la pasta cocida establecidos por D'Egidio y col. (1982) en función de su valor de la materia orgánica total (<1,4% alta calidad, 1,4-2,1% buena calidad >2,1 baja calidad) todas las formulaciones desarrolladas presentaron buena calidad de cocción (Tabla 21). Igualmente, la calidad de las formulaciones SR-3 y ST-3 no se vio comprometida, pues los resultados obtenidos, tanto para los parámetros determinados en crudo como en cocido, fueron similares a los observados para los espaguetis control. De igual modo, a excepción del color, las propiedades organolépticas de ambas formulaciones de espaguetis enriquecidos con un 3% de harina de caqui, no se vieron afectadas, pues tras la prueba triangular los panelistas no distinguieron entre la muestra control y los SR-3 o ST-3 ($p > 0{,}01$) (Tabla 22). Sin embargo, el incremento de la concentración, y el tipo de harina, especialmente la harina de Triumph, provocó disrupciones en la estructura molecular del espagueti, lo cual se hipotetiza al observar mayor fracturabilidad y menor diámetro en los SR-6 y ST-6 (Tabla 20). Esto pone de manifiesto que durante el proceso de secado se evaporaría una mayor cantidad de agua en estos espaguetis, lo que conlleva la disminución de su diámetro y la fragilidad de su estructura. Estos defectos, también pueden ser derivados de la competencia de agua por parte de la sémola de trigo y de la harina de caqui y el menor contenido de proteínas de estas formulaciones.

Igualmente, los panelistas detectaron diferencias entre las formulaciones ST6 y SR-6 y el control, derivadas de un sabor dulce (Tabla 22). Los ST-6 también mostraron los mayores valores de pegajosidad con respecto al resto de formulaciones estudiadas ($p < 0{,}05$), probablemente debido a que no se ha generado una red continua de gluten, lo que permite que los gránulos de almidón escapen y queden en la superficie.

Tabla 21.- Propiedades de cocción de los espaguetis elaborados con harina de caqui.

	SC	SR-3	ST-3	SR-6	ST-6
TCO (min)	8,50	6,00	7,50	5,50	5,50
Diámetro (mm)	2,15±0.15[a]	2.11±0.15[a]	2,15±0,09[a]	2,09±0,16[a]	2,10±0,13[a]
Firmeza (N)	3,51±0,18[a]	3,40±0,35[a]	3,45±0,30[a]	3,56±0,24[a]	3,41±0,25[a]
Pegajosidad (N)	1,95±0,08[b]	2,38±0,31[b]	2,13±0,05[b]	2,03±0,15[b]	2,65±0,13[a]
AA (%)	132,95±0,78[a]	115,46±3,03[b]	130,03±1,81[a]	103,46±3,28[c]	105,99±4,11[c]
PC (g/100g)	8,93±0,24[a]	8,15±0.05[a]	8,69±0,42[a]	8,06±0,44[a]	8,93±0,41[a]
MTO (g/100g)	2,05±0,20[b]	1,85±0,06[ab]	1,60±0,14[a]	1,99±0,02[b]	1,93±0,01[ab]
Humedad (g/100g)	62,57±0,37[a]	59,19±1,49[b]	61,98±0,92[ab]	58,36±1.51[b]	58,78±0,30[b]

TCO: tiempo óptimo de cocción; % AA: Absorción de agua; CL: pérdidas de cocción; MOT: materia orgánica total. SC: Espaguetis control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph.

Diferente letra (a-e) dentro de la misma fila indica diferencias estadísticamente significativas ($p < 0,05$), según la prueba de rangos múltiples de Tukey.

Tabla 22.- Resultados de la prueba triangular realizada sobre los espaguetis enriquecidos con harina de caqui en comparación con los espaguetis control (SC).

Test	Nº total de respuestas	Diferencia en el nº de respuestas correctas	Frecuencia en la que se detectó sabor dulce
SR-3 vs. SC	8	1	0/1
SR-6 vs. SC	8	6*	6/6*
ST-3 vs. SC	8	0	0/0
ST-6 vs. SC	8	6*	6/6*

SC: Espaguetis control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph. * Nivel de significancia $p < 0,01$.

4.5.- EVALUACIÓN DE LA FUNCIONALIDAD DE LOS PATÉS DE HÍGADO DE CERDO Y LOS ESPAGUETIS DE SÉMOLA DE TRIGO DURO ENRIQUECIDOS CON HARINA DE CAQUI

Los resultados del presente trabajo han sido publicados en la revista Food Chemistry (2021) 338:128142 y en la revista Nutrients 2021, 13:1332.

Como se puede extrapolar de los apartados anteriores, la incorporación de un ingrediente sin refinar, como es el caso de la harina de caqui, permite aportar nutrientes y fitoquímicos que no están presentes en el alimento tradicional enriquecido. Sin embargo, al igual que la incorporación de harina de caqui a las matrices alimentarias produce cambios en sus propiedades físico-químicas, estructurales y organolépticas, también puede conllevar modificaciones en la actividad biológica de sus componentes. De ahí la necesidad de evaluar la funcionalidad de los alimentos, y no basarse únicamente en la composición nutricional, pues los alimentos son más que la suma de sus nutrientes, y aspectos como la matriz alimentaria tienen mucho peso, tanto en cómo los alimentos son digeridos y absorbidos, como en la respuesta postprandial que estos ejercen en el organismo.

En consecuencia, tras someter las formulaciones de espaguetis de sémola de trigo duro y de paté de hígado de cerdo a los ensayos de digestión gastrointestinal *in vitro* se determinó la bioaccesibilidad de los compuestos (poli)fenólicos en ambos alimentos, la digestibilidad *in vitro* del almidón en los espaguetis y la oxidación lipídica y estabilidad de los ácidos grasos en los patés. En el caso de los patés solo se sometieron a la digestión *in vitro* los enriquecidos con 3 y 6% de harina Rojo Brillante, puesto que debido a la gran cantidad de digestiones que se debían realizar se escogieron sólo dos formulaciones para investigar la funcionalidad de los patés. El criterio de elección fue la estabilidad de la emulsión, puesto que, en estos ensayos, además de realizar tres digestiones independientes para evaluar cada parámetro (estabilidad de (poli)fenoles, estabilidad de ácidos grasos y oxidación lipídica) se simularon dos condiciones intestinales diferentes, una con baja actividad de la lipasa (8 UL/mL; pancreatina Sigma) (siguiendo el estándar del método descrito en (Minekus y col., 2014) y otra con actividad normal de la lipasa (2,000 UL/mL; pancreatina Kreon) para digestiones con alimentos ricos en grasa. Con este experimento se perseguía estudiar si la bioaccesibilidad de los compuestos fenólicos, oxidación lipídica y estabilidad de los ácidos grasos, se veía afectada por el incremento de la lipólisis. La razón de usar la pancreatina Kreon en lugar de las enzimas individuales, como recomienda Minekus y col., (2014) es por cuestiones económicas, pues la colipasa, necesaria para el correcto funcionamiento de la lipasa, tiene un precio elevado, que no permite el estudio de diversas formulaciones. Otros autores como Calvo-Lerma y col. (2019) ya han usado previamente esta enzima para simular digestiones *in vitro*.

4.5.1.- Evaluación de los espaguetis de sémola de trigo duro enriquecidos con harina de caqui

4.5.1.1.- Biodisponibilidad y bioaccesibilidad de los compuestos fenólicos

Los cereales se caracterizan por presentar mayor contenido de compuestos (poli)fenólicos unidos que libre (Zhang y col., 2020b). Este hecho se observó en las cinco formulaciones de espaguetis de sémola de trigo duro estudiadas (Figura 20). En los espaguetis enriquecidos se detectaron 12 compuesto unidos (Tabla 23) de los cuales, dos de ellos fueron aportados por la harina de caqui, el ácido gálico y el ácido *p*-cumárico. El aporte de ambos ácidos fenólicos fue concentración dependiente ($p < 0{,}05$). En el caso de los SC el ácido ferúlico fue el (poli)fenol mayoritario, resultados que están en concordancia con otros autores (Biney y col., 2014; Ciccoritti y col., 2017), mientras que en los cuatro espaguetis enriquecidos fue el ácido gálico ($p < 0{,}05$).

Respecto a los compuestos fenólicos libres, se detectaron 6 compuestos (Tabla 24), todos ellos, salvo el ácido gálico, provenientes de la sémola de trigo duro. Otro hallazgo importante fue la detección del aminoácido aromático L-Triptófano en la fase libre. Este compuesto es el responsable del incremento de la actividad antioxidante (Tabla 25) en la fase libre, pues se observa, en ambos métodos antioxidantes estudiados, una correlación superior al 90% con un grado de significancia de $p < 0{,}001$. Esto refuerza la discusión del Apartado 4.2.3. en cuanto a las interferencias entre los métodos antioxidantes y otros compuestos como los aminoácidos aromáticos. Otros autores como Podio y col. (2019) y Pešić y col. (2019) han observado inferencias entre los aminoácidos y los métodos antioxidantes.

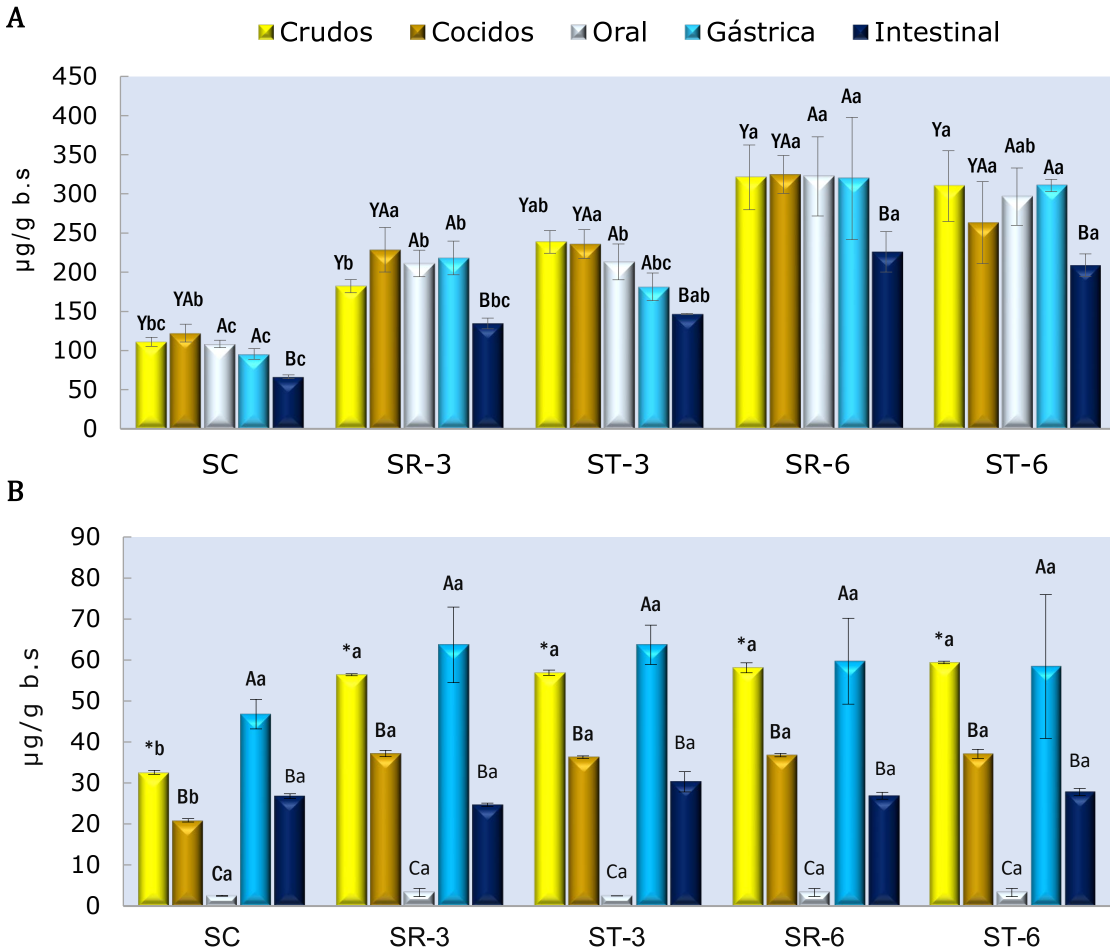


Figura 20.- Contenido total de los compuestos (poli)fenólicos (A) unidos y (B) libres de las muestras de espaguetis crudas, cocidas y digeridas (fase oral, gástrica e intestinal) estudiadas.

SC: Espagueti control; SR-3: espagueti con 3% de harina Rojo Brillante. ST-3: espagueti con 3% de harina de Triumph. SR-6: espagueti con 6% de harina Rojo Brillante. ST-6: espagueti con 6% de harina de Triumph. Diferentes letras indican diferencias estadísticamente significativas, según la prueba de rangos múltiples de Tukey.

Tabla 23.- Perfil de compuestos (poli)fenólicos unidos de los espaguetis crudos, cocidos y digeridos.

		Crudos	Cocidos	Oral	Gástrica	Intestinal
Ácido Gálico	SC	n.d^{d}	n.d^{c}	n.d^{c}	n.d^{c}	n.d^{d}
	SR-3	64,1±8,6^{Yc}	95,9±21,8^{YAb}	106,7±10,3^{Ab}	113,8±27,4^{Ab}	40,0±0,0^{Bc}
	ST-3	105,0±9,2^{Ybc}	111,0±17,7^{YAb}	110,7±14,2^{Ab}	83,2±4,6^{Ab}	53,2±4,2^{Bbc}
	SR-6	193,6±37,5^{Ya}	195,8±19,5^{YAa}	217,7±45,9^{Aa}	222,2±87,7^{Aa}	127,1±29,4^{Ba}
	ST-6	186,2±40,1^{Yab}	156,8±39,7^{YAab}	196,1±28,4^{Aab}	208,9±7,3^{Aa}	120,5±10,2^{Bab}
Ácido cumárico-*o*-hexósido	SC	n.d^{c}	n.d^{b}	n.d^{c}	n.d^{c}	n.d^{c}
	SR-3	2,4±0,2^{Yb}	2,4±0,2^{YAa}	1,1±0,0^{Ca}	1,4±0,2^{Ba}	1,1±0,0^{Bb}
	ST-3	3,3±0,1^{Yab}	2,3±0,1^{YAa}	1,2±0,1^{Ca}	1,4±0,2^{Ba}	1,4±0,0^{Bab}
	SR-6	3,2±0,1^{Yab}	3,2±0,4^{YAa}	1,4±0,2^{Ca}	1,8±0,5^{Ba}	1,9±0,3^{Ba}
	ST-6	3,4±0,5^{Ya}	3,0±0,6^{YAa}	1,2±0,2^{Ca}	2,0±0,3^{Ba}	1,9±0,2^{Ba}
Ácido protecateico	SC	6,8±1,4^{Ya}	5,7±0,6^{ZAa}	2,4±0,5^{Ca}	2,9±0,1^{Ba}	2,3±0,3^{Ca}
	SR-3	6,0±0,5^{Ya}	6,1±0,8^{ZAa}	2,4±0,0^{Ca}	3,2±0,2^{Ba}	1,9±0,0^{Ca}
	ST-3	6,1±0,1^{Ya}	5,5±0,3^{ZAa}	2,7±0,2^{Ca}	3,2±0,4^{Ba}	2,3±0,4^{Ca}
	SR-6	7,0±0,6^{Ya}	6,4±1,0^{ZAa}	2,6±0,3^{Ca}	3,4±1,0^{Ba}	2,0±0,0^{Ca}
	ST-6	6,9±0,8^{Ya}	5,1±0,2^{ZAa}	2,9±0,2^{Ca}	3,3±0,3^{Ba}	1,9±0,0^{Ca}
Ácido Vanílico	SC	3,5±0,0^{Ya}	2,8±0,0^{ZAb}	1,3±0,0^{Ba}	1,3±0,1^{Bb}	1,2±0,0^{Ba}
	SR-3	3,1±0,2^{Ya}	3,0±0,1^{YAab}	1,1±0,0^{Ca}	1,5±0,1^{Bab}	1,5±0,3^{Ba}
	ST-3	3,4±0,1^{Ya}	3,0±0,0^{YAab}	1,3±0,1^{Ca}	1,5±0,1^{Bab}	1,3±0,1^{Ca}
	SR-6	3,4±0,3^{Ya}	3,5±0,2^{YAa}	1,2±0,0^{Ca}	1,4±0,0^{Bab}	1,2±0,1^{Ca}
	ST-6	3,2±0,1^{Ya}	3,0±0,1^{YAab}	1,2±0,1^{Ca}	1,7±0,1^{Ba}	1,3±0,1^{Ca}
Ácido 4-Hidroxibenzoico	SC	6,0±0,5^{Ya}	5,6±0,1^{ZAa}	2,5±0,0^{Ba}	2,4±0,0^{Bb}	2,5±0,3^{Bb}
	SR-3	6,2±0,5^{Ya}	5,6±0,2^{ZAa}	2,3±0,0^{Ba}	2,4±0,1^{Bb}	2,4±0,3^{Bb}
	ST-3	6,2±0,8^{Ya}	5,6±0,1^{ZAa}	2,4±0,0^{Ba}	2,4±0,0^{Bb}	2,5±0,2^{Bb}
	SR-6	6,8±0,4^{Ya}	5,8±0,2^{ZAa}	2,4±0,1^{Ba}	2,4±0,1^{Bb}	3,1±0,0^{Ba}
	ST-6	6,6±0,8^{Ya}	5,5±0,2^{ZAa}	2,4±0,0^{Ba}	2,9±0,3^{Ba}	2,8±0,0^{Bab}
Catequina	SC	6,1±0,6^{Ya}	7,0±1,5^{YAa}	6,1±0,5^{Ba}	4,8±0,1^{Bb}	2,1±0,4^{Ca}
	SR-3	7,1±0,2^{Ya}	7,7±1,1^{YAa}	5,1±0,7^{Ba}	9,9±2,2^{Ba}	2,6±0,6^{Ca}
	ST-3	8,8±0,0^{Ya}	7,8±0,3^{YAa}	5,0±1,3^{Ba}	5,2±2,3^{Bab}	2,1±0,0^{Ca}
	SR-6	8,4±0,3^{Ya}	7,8±0,9^{YAa}	4,4±1,1^{Ba}	4,3±0,2^{Bab}	2,9±1,6^{Ca}
	ST-6	6,5±0,0^{Ya}	6,4±0,4^{Ya}	4,1±0,8^{Ba}	3,9±0,5^{Bb}	3,3±0,4^{Ca}
Vanillina	SC	2,7±0,4^{Ya}	2,9±0,0^{YAa}	2,0±0,1^{Ba}	1,9±0,1^{Bb}	1,5±0,1^{Ba}
	SR-3	2,9±0,5^{Ya}	3,6±0,2^{YAa}	1,7±0,1^{Ba}	2,1±0,1^{Bb}	2,1±0,5^{Ba}
	ST-3	3,0±0,5^{Ya}	3,8±0,5^{YAa}	2,1±0,1^{Ba}	2,2±0,0^{Bb}	2,0±0,1^{Ba}
	SR-6	3,9±0,7^{Ya}	3,8±0,7^{YAa}	2,1±0,0^{BCa}	2,5±0,2^{Bb}	1,7±0,2^{Ca}
	ST-6	3,7±0,9^{Ya}	4,3±0,5^{YAa}	1,7±0,0^{BCa}	3,8±0,5^{Aa}	1,5±0,0^{Ca}
Ácido *p*-Cumárico	SC	2,8±0,6^{Ya}	2,7±0,3^{YAa}	1,6±0,1^{Ba}	1,5±0,1^{Ba}	1,2±0,0^{Bb}
	SR-3	3,1±0,6^{Ya}	3,2±0,3^{YAa}	1,8±0,3^{Ba}	1,7±0,0^{Ba}	1,7±0,1^{Bab}
	ST-3	3,4±0,6^{Ya}	2,8±0,1^{YAa}	1,7±0,2^{Ba}	1,7±0,2^{Ba}	1,9±0,0^{Ba}
	SR-6	3,4±0,4^{Ya}	2,9±0,1^{YAa}	1,8±0,1^{BCa}	1,6±0,3^{Ca}	2,2±0,5^{Ba}
	ST-6	3,5±0,8^{Ya}	2,8±0,3^{YAa}	1,8±0,1^{Ba}	1,8±0,0^{Ba}	1,9±0,3^{Ba}
Ácido Ferúlico	SC	57,5±3,9^{Ya}	70,2±9,0^{abxB}	78,7±4,5^{aA}	68,5±5,3^{aAB}	45,8±5,8^{bB}
	SR-3	61,3±1,8^{ax}	73,9±5,8^{axB}	75,9±4,2^{aA}	69,7±0,1^{aAB}	69,2±4,8^{aB}
	ST-3	72,8±5,6^{ay}	68,2±5,3^{abxB}	72,2±6,8^{aA}	67,8±16,7^{aAB}	68,3±5,4^{aB}
	SR-6	65,3±4,6^{ax}	68,4±3,2^{abxB}	74,9±1,6^{aA}	65,6±18,2^{aAB}	72,8±3,8^{aB}
	ST-6	62,3±9,0^{ax}	51,5±10,7^{bxB}	71,9±6,4^{aA}	68,2±2,3^{aAB}	62,9±8,9^{abB}
Ácido Sinápico	SC	9,3±0,2^{Ya}	9,1±0,8^{YAa}	6,4±0,2^{Ba}	4,5±0,7^{Ca}	3,4±0,2^{Da}
	SR-3	9,7±0,1^{Ya}	10,3±0,1^{YAa}	6,5±0,2^{Ba}	4,5±0,1^{Ca}	3,3±0,3^{Da}
	ST-3	109±0,4^{Ya}	9,1±0,8^{YAa}	6,2±0,1^{Ba}	5,1±0,3^{Ba}	3,4±0,1^{Ca}
	SR-6	10,6±0,1^{Ya}	10,5±0,9^{YAa}	6,5±0,0^{Ba}	5,1±1,0^{Ba}	3,3±0,1^{Ca}
	ST-6	10,5±1,4^{Ya}	9,0±0,6^{YAa}	6,5±0,4^{Ba}	5,3±0,1^{Ba}	3,5±0,0^{Ca}
Ácido Ferúlico derivado I	SC	8,0±0,0^{Ya}	8,0±0,1^{YAa}	3,4±0,2^{Ba}	3,5±0,1^{Ba}	3,3±0,1^{Ba}
	SR-3	7,9±0,1^{Ya}	8,3±0,1^{YAa}	3,1±0,2^{Ba}	3,4±0,2^{Ba}	3,3±0,1^{Ba}
	ST-3	8,0±0,1^{Ya}	8,2±0,1^{YAa}	3,4±0,2^{Ba}	3,4±0,2^{Ba}	3,5±0,0^{Ba}
	SR-6	8,2±0,2^{Ya}	8,4±0,3^{YAa}	3,1±0,2^{Ba}	3,3±0,4^{Ba}	3,2±0,1^{Ba}
	ST-6	8,1±0,3^{Ya}	8,0±0,2^{YAa}	3,0±0,1^{Ba}	3,3±0,0^{Ba}	3,1±0,1^{Ba}
Ácido Ferúlico derivado II	SC	8,1±0,1^{Ya}	8,3±0,6^{YAa}	3,9±0,3^{Ba}	4,3±0,4^{Ba}	3,9±0,5^{Ba}
	SR-3	8,7±0,8^{Ya}	8,5±0,4^{YAa}	3,5±0,1^{Ca}	3,7±0,0^{BCa}	4,4±0,4^{Ba}
	ST-3	8,1±0,2^{Ya}	8,3±0,2^{YAa}	3,7±0,1^{Ba}	3,7±0,0^{Ba}	4,7±0,1C^{a}
	SR-6	8,1± 0,2^{Ya}	8,2±0,3^{YAa}	3,5±0,2^{Ca}	3,7±0,0^{BCa}	4,3±0,3^{Ba}
	ST-6	8,7 ±0,9^{Ya}	7,9±0,1^{YAa}	3,5±0,2^{Ba}	4,1±0,1^{Ba}	4,1±0,3^{Ba}

Valores expresados en (µg/g base seca). Diferentes letras mayúsculas (Y-Z) en la misma fila expresan diferencias significativas entre las muestras crudas y cocinadas. Diferentes letras mayúsculas (A-D) en la misma fila expresan diferencias significativas entre las muestras cocinada y digeridas. Diferentes letras minúsculas en la misma columna para cada compuesto (poli)fenólico indican diferencias significativas. Las diferencias se consideran cuando $p < 0,05$ según la prueba de rangos múltiples de Tukey. SC: Espagueti control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph.

Tabla 24.- Perfil de compuestos (poli)fenólicos libres de los espaguetis crudos, cocidos y digeridos. Cuantificación del aminoácido aromático L-triptófano.

		Crudos	Cocidos	Oral	Gástrica	Intestinal
Ácido gálico	SC	n.d	n.d	n.d	n.d	n.d
	SR-3	24,9±0,3^{Ya}	16,6±0,6^{ZAa}	n.d	19,4 ± 5,9^{Aa}	n.d
	ST-3	24,8±0,1^{Ya}	15,9±0,1^{ZAa}	n.d	20,4 ± 6,1^{Aa}	n.d
	SR-6	25,6±0,6^{Ya}	16,5±0,3^{ZAa}	n.d	18,1 ± 7,6^{Aa}	n.d
	ST-6	25,4±0,3^{Ya}	16,1±0,1^{ZAa}	n.d	18,0 ± 8,3^{Aa}	n.d
Ácido protocateico	SC	10,7±0,4^{Ya}	7,0±0,1^{ZBa}	2,5±0,1^{Ca}	8,5±2,1^{Ba}	11,5±0,6^{Aab}
	SR-3	10,1±0,2^{Ya}	7,1±0,2^{ZBa}	3,3±1,0^{Ca}	7,4±2,8^{Ba}	10,4±0,4^{Aab}
	ST-3	10,4±0,4^{Ya}	6,9±0,1^{ZBa}	2,5±0,0^{Ca}	7,9±2,9^{Ba}	14,6±3,7^{Aa}
	SR-6	10,6±0,9^{Ya}	7,0±0,1^{ZBa}	3,3±1,0^{Ca}	7,9±2,7^{Ba}	12,7±1,3^{Aab}
	ST-6	11,0±0,4^{Ya}	7,5±0,6^{ZBa}	3,3±1,0^{Ca}	8,0±2,5^{Ba}	13,4±1,3^{Aab}
Vanillina	SC	6,5±0,2^{Ya}	4,2±0,5^{ZAa}	n.d	4,5±0,2^{Ab}	n.d
	SR-3	5,9±0,2^{Yb}	3,9±0,4^{ZBa}	n.d	7,2±0,9^{Aa}	n.d
	ST-3	6,2±0,3^{Yab}	3,8±0,3^{ZBa}	n.d	7,0±0,0^{Aa}	n.d
	SR-6	6,6±0,4^{Yab}	3,8±0,4^{ZBa}	n.d	7,2±0,9^{Aa}	n.d
	ST-6	7,6±0,5^{Ya}	3,7±0,3^{ZBa}	n.d	6,5±1,9^{Aa}	n.d
Ácido Ferúlico	SC	15,4±0,1^{Ya}	9,7±0,1^{ZBa}	tr	tr	15,3±0,2^{Aa}
	SR-3	15,5±0,1^{Ya}	9,6±0,2^{ZBa}	tr	tr	14,3±0,1^{Aa}
	ST-3	15,4±0,0^{Ya}	9,8±0,2^{ZBa}	tr	tr	15,8±0,2^{Aa}
	SR-6	15,3±0,1^{Ya}	9,6±0,3^{ZBa}	tr	tr	14,2±0,1^{Aa}
	ST-6	15,4±0,2^{Ya}	9,7±0,1^{ZBa}	tr	tr	14,3±0,0^{Aa}
Catequina	SC	n.d	n,d	n.d	16,6±1,5^{a}	n.d
	SR-3	n.d	n.d	n.d	13,5±0,1^{a}	n.d
	ST-3	n.d	n.d	n.d	12,9±2,4^{a}	n.d
	SR-6	n.d	n.d	n.d	12,2±0,6^{b}	n.d
	ST-6	n.d	n.d	n.d	11,9±1,2^{b}	n.d
Epicatequina	SC	n.d	n.d	n.d	17,2±1,1^{a}	n.d
	SR-3	n.d	n.d	n.d	16,2±2,3^{a}	n.d
	ST-3	n.d	n.d	n.d	15,5±0,9^{a}	n.d
	SR-6	n.d	n.d	n.d	14,3±2,0^{a}	n.d
	ST-6	n.d	n.d	n.d	14,0±2,2^{a}	n.d
L-Triptófano	SC	54,6±1,7^{Ya}	58,2±3,0^{YCa}	27,5±1,0^{Da}	76,3±4,0^{Ba}	1.394,1±15,9^{Ab}
	SR-3	50,0±4,1^{Ya}	52,1±0,5^{YCa}	20,9±2,7^{Da}	73,3±0,2^{Ba}	1.277,0±53,0^{Ac}
	ST-3	51,3±4,2^{Ya}	51,8±3,0^{YCa}	20,7±3,3^{Da}	93,6±26,0^{Ba}	1.491,6±97,7^{Aa}
	SR-6	50,4±2,4^{Ya}	52,0±3,9^{YCa}	20,8±3,3^{Da}	68,0±3,0^{Ba}	1.253,2±65,0^{Ac}
	ST-6	53,1±5,0^{Ya}	48,2±0,7^{YCa}	17,1±6,1^{Da}	65,1±7,7^{Ba}	1.296,9±41,3^{Ac}

Valores expresados en µg/g base seca. Diferentes letras mayúsculas (Y-Z) en la misma fila expresan diferencias significativas entre las muestras crudas y cocinadas. Diferentes letras mayúsculas (A-D) en la misma fila expresan diferencias significativas entre las muestras cocinada y digeridas. Diferentes letras minúsculas en la misma columna para cada (poli)fenol indican diferencias significativas. Las diferencias se consideran cuando $p < 0,05$ según la prueba de rangos múltiples de Tukey. SC: Espagueti control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph. n.d: no detectado.

Tabla 25.- Valores de la actividad antioxidante determinados por los métodos ABTS y FRAP en ambas fracciones (libre y unida) de las muestras de espagueti estudiadas (crudas, cocidas y digeridas).

			Crudos	Cocidos	Oral	Gástrica	Intestinal
ABTS	**FL**						
(µg Trolox eq. / g base seca)		SC	24,2±1,4^{Yb}	12,4±0,5^{ZBa}	11,4±1,7^{Ba}	15,2±3,6^{Ba}	432,0±138,9^{Aa}
		SR-3	31,1±2,6^{Ya}	16,5±1,8^{ZBa}	8,5±1,4^{Ba}	15,3±3,7^{Ba}	457,1±29,0^{Aa}
		ST-3	26,4±0,8^{Ya}	18,7±1,9^{ZBa}	8,9±0,7^{Ba}	15,4±2,2^{Ba}	492,0±6,5^{Aa}
		SR-6	30,7±3,0^{Ya}	16,2±1,1^{ZBa}	8,2±0,8^{Ba}	15,6±2,3^{Ba}	427,0±9,0^{Aa}
		ST-6	33,6±0,8^{Ya}	16,5±0,6^{ZBa}	7,5±1,5^{Ba}	16,3±4,7^{Ba}	447,2±32,1^{Aa}
	FU	SC	13,7±1,5^{Yc}	16,4±2,2^{AYc}	9,1±0,8^{Ac}	9,1±1,7^{Ac}	6,5±0,5^{Bc}
		SR-3	22,9±1,4^{Yb}	26,0±6,2^{CYbc}	55,2±3,1^{Ab}	51,0±2,5^{Ab}	45,2±1,5^{Bb}
		ST-3	28,9±3,5^{Yb}	33,6±4,9^{CYba}	52,1±7,1^{Ab}	55,5±8,4^{Ab}	47,1±3,1^{Bb}
		SR-6	42,3±3,2^{Ya}	43,9±3,5^{CYa}	72,3±10,1^{Aa}	68,8±11,1^{Aa}	56,9±2,6^{Ba}
		ST-6	40,2±3,6^{Ya}	41,0±7,5^{CYa}	64,2±9,6^{Aa}	63,3±1,1^{Aa}	53,7±7,3^{Ba}
FRAP	**FL**	SC	0,69±0,09^{Yc}	0,20±0,01^{ZCc}	0,27±0,03^{Ca}	0,77±0,01^{Ba}	4,98±0,19^{Ab}
(mg Trolox eq. / g base seca)		SR-3	1,05±0,10^{Ybc}	0,42±0,09^{ZCb}	0,32±0,02^{Ca}	1,24±0,06^{Ba}	5,21±0,74^{Ab}
		ST-3	1,29±0,14^{Yab}	0,37±0,05^{ZCab}	0,36±0,09^{Ca}	1,17±0,08^{Ba}	6,18±0,76^{Aab}
		SR-6	1,52±0,15^{Ya}	0,52±0,07^{ZCa}	0,47±0,07^{Ca}	1,52±0,25^{Ba}	5,83±0,44^{Ab}
		ST-6	1,79±0,13^{Ya}	0,40±0,05^{ZCa}	0,37±0,01^{Ca}	1,49±0,17^{Ba}	7,79±0,95^{Aa}
	FU	SC	0,14±0,02^{Yc}	0,16±0,04^{YBc}	0,25±0,02^{Ab}	0,21±0,06^{ABb}	0,15±0,03^{Bb}
		SR-3	0,49±0,04^{Yb}	0,55±0,14^{YAb}	0,49±0,03^{ABab}	0,49±0,03^{ABa}	0,32±0,05^{Bab}
		ST-3	0,69±0,09^{Yb}	0,75±0,16^{YAab}	0,51±0,03^{ABa}	0,52±0,03^{ABa}	0,46±0,01^{Ba}
		SR-6	1,12±0,09^{Ya}	1,07±0,03^{YAa}	0,49±0,03^{Bab}	0,50±0,02^{Ba}	0,52±0,00^{Ba}
		ST-6	1,10±0,22^{Ya}	1,00±0,09^{YAa}	0,48±0,02^{Bab}	0,54±0,01^{Ba}	0,52±0,01^{Ba}

Diferentes letras mayúsculas (Y-Z) en la misma fila expresan diferencias significativas entre las muestras crudas y cocinadas. Diferentes letras mayúsculas (A-D) en la misma fila expresan diferencias significativas entre las muestras cocinada y digeridas. Diferentes letras minúsculas en la misma columna para cada compuesto (poli)fenólico indican diferencias significativas. Las diferencias se consideran cuando $p < 0,05$ según la prueba de rangos múltiples de Tukey. SC: Espagueti control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph. FU: Fracción unida; FL: Fracción libre.

Tras la digestión gastrointestinal *in vitro*, se produjo una reducción significativa ($p < 0,05$) de los (poli)fenoles unidos, denotando que los procesos digestivos produjeron la liberación de parte de estos compuestos al medio. No obstante, una gran cantidad de los (poli)fenoles permanecieron unidos a la matriz alimentaria como se puede observar en la Figura 20 y 21. Por lo tanto, estos compuestos estarán disponibles para ser metabolizados por la microbiota intestinal. La inclusión de altas cantidades (6%) de harina de caqui a los espaguetis aumentó su fracción colónica disponible de forma significativa ($p < 0,05$). La acción preventiva sobre la aparición de cáncer de colon derivada del consumo de cereales enteros está asociada, en parte a la cantidad de compuestos polifenólicos que llegan al colon (Mileo y col., 2019). En esta línea Costabile y col. (2008) mostró la habilidad de los (poli)fenoles presentes en el trigo para promocionar el crecimiento de *Bifidobacterium* y *Lactobacillus* y reducir el crecimiento de *Escherichia coli* y *Clostridium spp.* Recientemente varios autores han revisado la relación entre los compuestos (poli)fenólicos y su capacidad de modular la microbiota intestinal (Alves-Santos y col., 2020; Peluzio y col., 2021). En el caso concreto del ácido gálico, varios autores han observado que el ácido gálico promueve el crecimiento de bacterias beneficiosas y disminuye el de patógenas, modula la respuesta inmune y reduce la colitis (inflamación del colón) (Li y col., 2019; Yang y col., 2020).

Otro hallazgo relevante fue la detección de catequina y epicatequina libre tras la fase gástrica. Estos dos compuestos no habían sido detectados ni en los espaguetis crudos ni en los espaguetis cocidos. La detección de ambos flavanoles pudo ser debido a la degradación de taninos condensados mediada por el medio ácido de la fase gástrica, como previamente ha apuntado Zhu y col. (2002). Tras la fase intestinal, en la fracción libre, únicamente se detectó ácido ferúlico y ácido protocateico. En consecuencia, la bioaccesibilidad de los compuestos (poli)fenólicos presentes en todas las muestras de espaguetis de sémola de trigo estudiadas fue menor al 20% (Figura 21). El mayor índice de bioaccesibilidad lo presentó el SC, mientras que el menor fue observado en los SR-6. Estos resultados estuvieron en concordancia con los mostrados por Tackás y col. (2018).

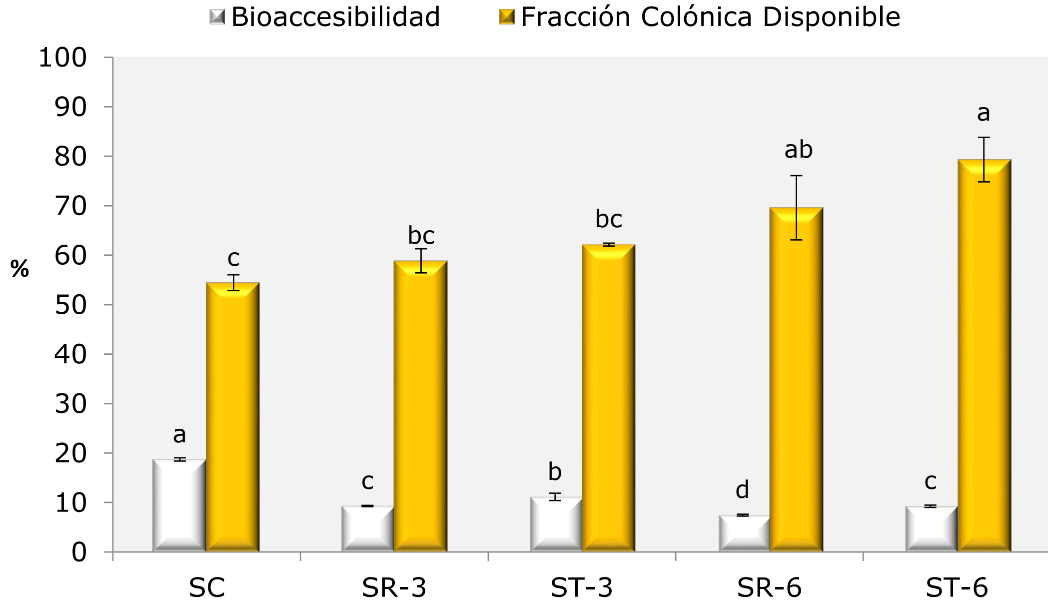


Figura 21.- índice de bioaccesibilidad e índice colónico disponible de las cinco formulaciones de espaguetis estudias.

SC: Espaguetis control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; ST-6: espaguetis con 6% de harina Triumph. Diferentes letras para cada parámetro estudiado indican diferencias significativas ($p < 0,05$) según la prueba de rangos múltiples de Tukey.

4.5.1.2.- Digestibilidad *in vitro* de los espaguetis de sémola de trigo duro

Los alimentos ricos en hidratos de carbono pueden ser clasificados en función de la respuesta glucémica en sangre postprandial que generan. El índice glucémico es el término usado para dicha clasificación. Está definido por la OMS como el incremento del área bajo la curva de la respuesta de glucosa en sangre derivada de una porción de 50 g de carbohidratos de un alimento de prueba expresada como un porcentaje de la respuesta a la misma cantidad de carbohidratos de un alimento estándar ingerido por el mismo sujeto (FAO/OMS, 1998). Los alimentos estándar suele ser el pan blanco o soluciones de sacarosa. En una nueva consulta de expertos realizada por la FAO-OMS (Nishida y Nocito, 2007) se sigue recomendando el índice glicémico (IG) para comparar alimentos. Esto es debido a la correlación entre la respuesta glucémica y la respuesta insulínica para el mantenimiento de la homeostasis de los hidratos de carbono. La insulina es una hormona esencial para la regulación del metabolismo de los hidratos de carbono y grasas. Esta, activa las rutas anabólicas (glucogénesis; síntesis de triglicéridos a partir de glucosa) y paraliza las rutas catabólicas (glucólisis, lipólisis, proteólisis). Los alimentos ricos en hidratos de carbono pueden clasificarse como de bajo IG (< 55), medio ($> 55 < 70$) y alto (> 70). El índice glicémico de los espaguetis suele oscilar entre medio y alto (Foster-Powell y Brand Miller, 2002). No obstante, de entre los distintos alimentos elaborados a base de trigo, pan, cereales de desayuno o masa de pizza, son los que presentan el menor índice glicémico. En consecuencia, conocer la

digestibilidad del almidón y su índice glicémico es de especial interés dada la correlación entre el aumento de glucosa postprandial y la respuesta insulínica. La insulina es una hormona anabólica, cuya principal función es disminuir el nivel de glucosa en sangre tras las comidas. Para ello, facilita la entrada de glucosa a diferentes órganos como el hígado, corazón, músculo y tejido adiposo. El exceso de glucosa es transformado en estos órganos en triglicéridos. Si se mantienen patrones dietéticos continuados que inducen la hiperglucemia, el resultado es un aumento de la grasa visceral, que en los peores de los casos provoca resistencia a la insulina y patologías cardiacas (Gast y col., 2013; Gustafson y col., 2015; de Mutsert y col., 2018).

Los resultados mostraron que la tasa de digestión del almidón disminuyó en los espaguetis enriquecidos con ambas harinas de caqui (Triumph y Rojo Brillante) al 3%, mientras que se produjo un aumento en la velocidad con la que la amilasa rompía el almidón en maltosa, en los espaguetis que presentaban mayor concentración de harina de caqui (SR-6 y ST-6) en comparación con la tasa de digestión del espagueti control ($p < 0,05$) (Figura 22). Se aprecia un cambio de tendencia dependiente de la concentración. Esto podría deberse a los daños que se producen en la matriz del almidón, debido a la presencia de ingredientes diferentes a la sémola de trigo duro como previamente se expone en el Apartado 4.4.2. Sobre todo, estos agujeros pueden deberse a una competencia de agua entre las dos harinas usadas (sémola de trigo duro y harina de caqui) la cual no fue relevante en las concentraciones menores, pero sí lo fue a cantidades de harina de caqui más elevada. Una posible solución para evitar estos colapsos en la matriz es hidratar por separado los dos ingredientes, la sémola de trigo y la harina de caqui. En consecuencia, la disrupción de la red gluten-almidón, hace más accesible los granos de almidón a la amilasa y por consiguiente su ratio de digestión aumenta.

Ambos espaguetis con menor concentración de harina de caqui obtuvieron un índice glicémico estimado, menor que los espaguetis con mayor concentración ($p < 0,05$), no obstante, ninguna de las cuatro formulaciones enriquecidas presentó valores diferentes a la muestra control ($p > 0,05$) (Tabla 26).

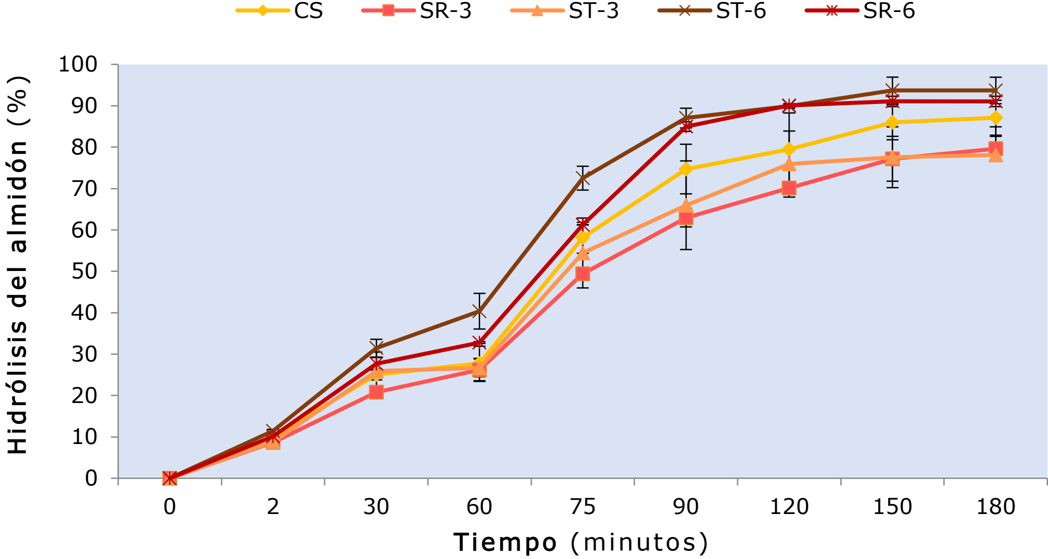


Figura 22.- Cinética de hidrólisis del almidón durante la digestión gastrointestinal *in vitro* de las cinco formulaciones de espaguetis estudiadas.

SC: Espaguetis control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante.

Tabla 26.- Contenido total de almidón (AT), almidón total hidrolizado a los 120 y 180 minutos (AH120 y AH180), constante cinética (k), área debajo de la curva (ADC), índice de hidrólisis (IH) e índice glicémico estimado (IGe).

	AT (g/100 g b.s)	AH_{180} (%)	AH_{120} (%)	k_{120}	AUC_{120}	IH_{120}	IGe_{120}
SC	74,5 ± 1,0^{a}	85,6 ± 4,3^{ab}	78,0 ± 9,0^{ab}	0,036 ± 0,001	7239 ± 889	82,5 ± 10,1	85,0 ± 5,6^{ab}
SR-3	73,4 ± 0,5^{a}	76,6 ± 1,3^{b}	68,7 ± 1,5^{b}	0,035 ± 0,000	6326 ± 146	72,1 ± 1,7	79,3 ± 0,9^{b}
ST-3	72,7 ± 0,7^{ab}	76,6 ± 6,7^{b}	70,1 ± 3,6^{b}	0,035 ± 0,000	6462 ± 354	73,7 ± 4,0	80,2 ± 2,2^{b}
SR-6	68,4 ± 2,7^{bc}	88,5 ± 0,2^{ab}	87,6 ± 0,8^{a}	0,037 ± 0,000	887 ± 78	93,3 ± 0,9	91,0 ± 0,5^{a}
ST-6	65,3 ± 1,1^{c}	91,7 ± 4,1^{a}	89,0 ± 1,3^{a}	0,037 ± 0,000	8324 ± 29	94,9 ± 1,5	91,8 ± 0,8^{a}
PB	65,2 ± 2,1	93,5 ± 2,0	93,5 ± 2,0	0,038 ± 0,000	877 ± 196	100	-

Diferentes letras en la misma columna indican diferencias estadísticamente significativas (p < 0,05) según la prueba de rangos múltiples de Tukey. SC: Espaguetis control; SR-3: espaguetis con 3% de harina Rojo Brillante; ST-3: espaguetis con 3% de harina Triumph; SR-6: espaguetis con 6% de harina Rojo Brillante; PB: Pan blanco. b.s: base seca.

4.5.2.- Evaluación de la funcionalidad de los patés de hígado de cerdo enriquecidos con harina de caqui.

4.5.2.1.- Bioaccesibilidad y biodisponibilidad de los compuestos (poli)fenólicos

La harina de caqui Rojo Brillante aportó 23 compuestos (poli)fenólicos a las dos formulaciones de paté estudiadas (PR-3 y PR-6), de los cuales 21 fueron detectadas en la fracción unida y 2 en la fracción libre (Figura 23). De los 21 compuestos detectados en la fracción unida, únicamente 16 se pudieron identificar, pues el resto correspondía a los cinco compuestos sin identificar observados en la harina de caqui (Tabla 27). El compuesto mayoritario fue el ácido gálico, seguido de la flavanona glucosilada IV y la galocatequina glucosilada. En los patés se detectó glucósido de ácido elágico (Nº 43) (Figura 23 y 24), el cual no fue detectado en la harina de caqui, probablemente debido a la solapación con el glucósido de gallocatequina-3-galalo, cuyo tiempo de retención fue el mismo que para el glucósido de ácido elágico. En la fracción libre únicamente pudieron ser identificados y cuantificados el glucósido de quercetina III y el glucósido de kaempferol II; pese a su baja concentración (quercetina glicosilada III: 2,38±1.39 y 3,70±2.19 µg/g b.s, kaempferol glicosilado II 0,44 ± 0,13 y 0,55 ±0,20 µg/g b.s, para el PR-3 y el PR-6, respectivamente) se pudieron observar sus espectros de absorbancia (Figura 24), lo que permitió su identificación.

Tras la digestión gastrointestinal *in vitro* la mayor parte de los compuestos unidos detectados en el paté continuaron ligados a la matriz alimentaria (Tabla 27). La familia de las flavanonas y de los ácidos fenólicos fueron las que presentaron mayor índice colónico disponible, sobre todo debido a la gran cantidad de ácido gálico y flavanona IV que continuó ligado a la matriz al finalizar ambas fases intestinales simuladas (C1 y C2). No obstante, el ácido elágico y el kaempferol glicosilado II sólo fueron detectados después de la fase oral, lo que indica que estos compuestos probablemente fueron liberados al medio durante la fase gástrica. No obstante, en la fracción libre no se detectó ácido elágico tras la fase gástrica, pues únicamente fueron detectados el kaempferol glicosilado II en cantidades trazas, el cual había sido previamente observado tras la fase oral, y la quercetina glicosilada II. Los resultados de la fracción libre tras la fase intestinal no están disponibles, debido a problemas en la obtención de los extractos, derivados de la gran cantidad de sobrenadante con compuestos en suspensión (ácidos grasos, aminoácidos, etc.). Además, tras la fase oral y gástrica a 280 nm se detectaron aminoácidos (muchos picos compartían el mismo espectro que el aminoácido L-triptófano), lo que dificultó la identificación de posibles compuestos (poli)fenólicos provenientes de la harina de caqui, especialmente los flavanoles (catequina, eqpicatequina, gallocatequina, etc), los cuales se detectan a 280 nm, y comparten las mismas bandas de absorbancia que el L-triptófano.

Gran parte de los compuestos (poli)fenólicos presentes en los patés enriquecidos disponibles para llegar al colon podrán ser metabolizados por la microbiota intestinal, como previamente se ha comentado en el Apartado 4.5.1.1. Este hecho puede ser relevante para reducir las connotaciones negativas de la carne, relacionadas con el desarrollo de cáncer de colon. En esta línea, recientes estudios con animales han mostrado el potencial de los compuestos (poli)fenólicos en modular la microbiota y la respuesta inmune en ratas con inflamación crónica inducida alimentadas con productos cárnicos enriquecidos con extractos antioxidantes (Burri y col., 2021).

La estabilidad de los compuestos polifenólicos y su índice colónico disponible fue diferente en función de la fase intestinal simulada (C1 y C2), salvo excepciones, se observó la tendencia de mayor estabilidad de los compuestos (poli)fenólicos tras la fase intestinal C2 (Tabla 27). Este hecho puede deberse a la mayor presencia de ácidos grasos libres en el medio, los cuales ejercerían un papel protector frente a los (poli)fenoles. Previamente McClements y Xiao (2017) había observado que la bioaccesibilidad de los compuestos hidrófobos se ve mejorada con la inclusión de ácidos grasos en la matriz alimentaria. Mientras que Juániz y col. (2016) observó menor pérdida de los compuestos unidos presentes en pimientos verdes fritos, en comparación con los crudos, después de la digestión gastrointestinal *in vitro*. Además, este hecho también se observa en estudios con humanos, pues Guo y col. (2013) reportaron que la biodisponibilidad *in vivo* de la quercetina aumenta en dietas ricas en grasa. De estos resultados también se puede extrapolar que la estabilidad de los compuestos polifenólicos unidos va a depender del grado de lipólisis que se lleve a cabo durante la digestión gastrointestinal *in vitro*. En consecuencia, es relevante resaltar que los productos cárnicos ricos en grasa como el paté, pueden ser un excelente vehículo de compuestos (poli)fenólicos unidos.

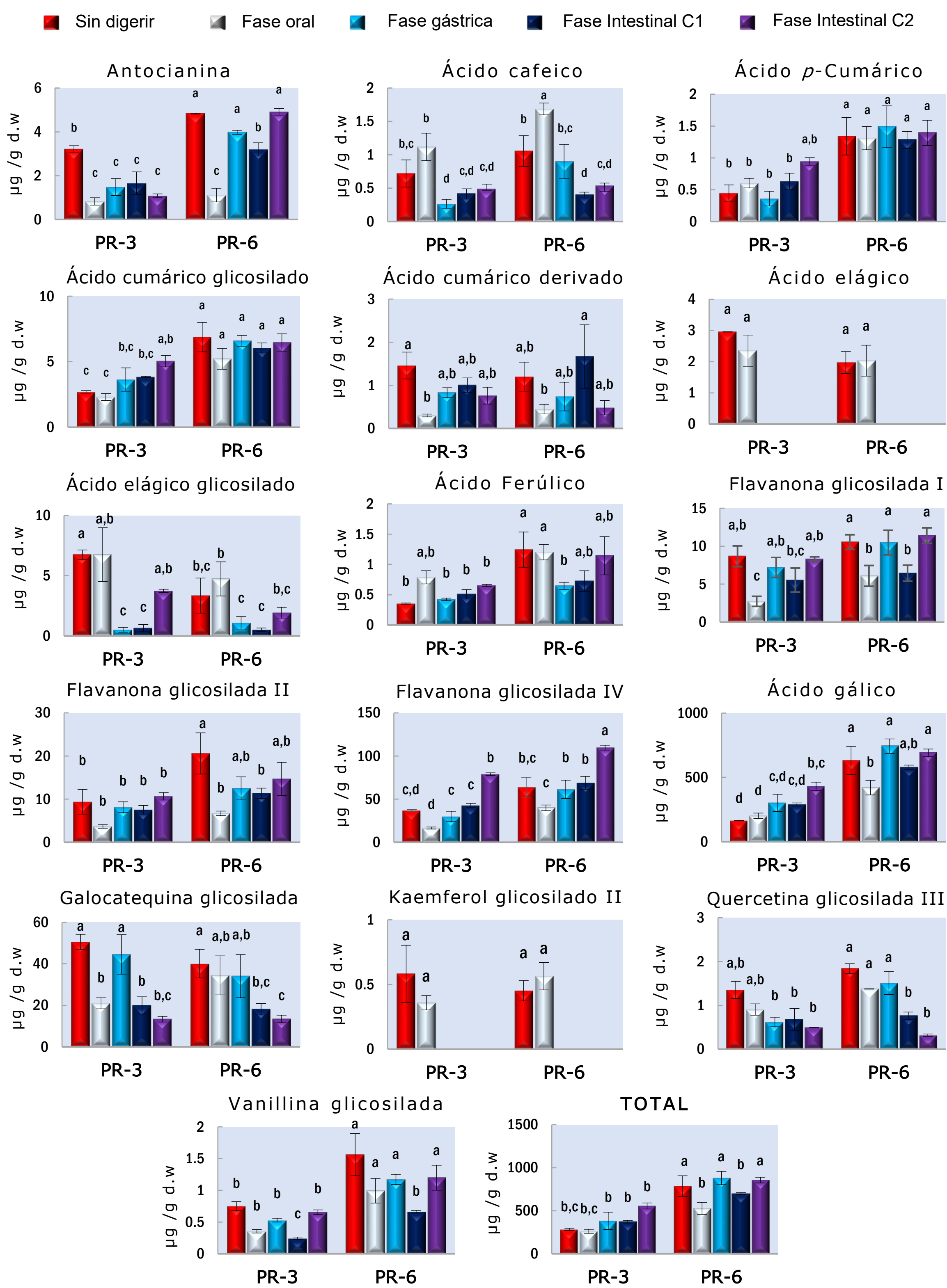


Figura 23.- Compuestos (poli)fenólicos unidos de los patés de hígado de cerdo enriquecidos con harina de caqui en las muestras sin digerir y digeridas.

PR-3: paté con 3% de harina Rojo Brillante; PR-6: paté con 6% de harina Rojo Brillante. Valores con diferente letra indican diferencias estadísticamente significativas ($p < 0,05$) según la prueba de rangos múltiples de Tukey.

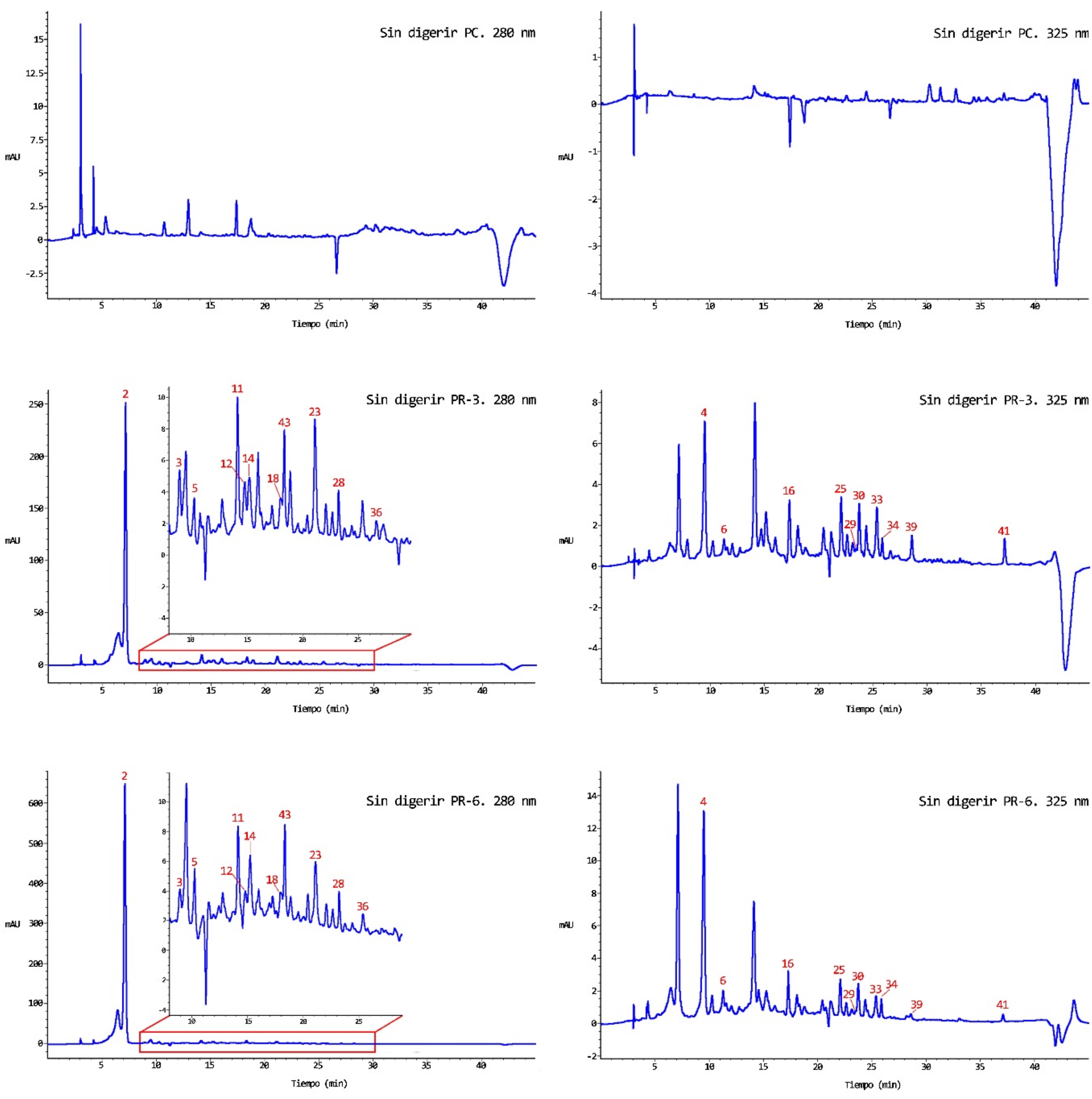


Figura 24.- Cromatogramas de la fracción unida de los patés de hígado de cerdo sin digerir.

Tabla 27.- Índice colónico disponible de los patés enriquecidos tras las dos fases intestinales simuladas.

	PR-3 Fracción colónica disponible (%)		PR-6 Fracción colónica disponible (%)	
(Poli)fenoles	**FI-C1**	**FI-C2**	**FI-C1**	**F2-C2**
Ácido cafeico	57,53±11,25[a]	67,30±2,10[a]	37,58±4,09[b]	44,47±1,52[b]
Ácido *p*-cumárico	140,7±29,0[b]	210,6±13,2[a]	95,74±9,99[b]	104,06±14,73[b]
Ácido cumárico glicosilado	198,75±2,31[b]	268,09±24,94[a]	95,26±6,35[a]	102,20±11,03[a]
Ácido cumárico derivado	68,90±11,43[b]	52,08±13,43[b]	138,36±61,75[a]	40,12±13,88[b]
Ácido elágico	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00
Ácido elágico glicosilado	9,98±4,15[b]	55,44±2,05[a]	15,73±4,08[b]	57,73±13,34[a]
Ácido ferúlico	143,54±22,05[a]	184,50±5,16[a]	58,32±13,66[b]	91,87±25,36[b]
Ácido gálico	176,25±7,29[b]	261,66±20,04[a]	91,56±2,67[c]	109,52±4,58[c]
Vanillina glicosilada	32,54±2,63[b]	87,09±4,90[a]	42,34±1,32[b]	76,67±12,59[a]
Flavanona glicosilada I	63,88±18,17[b]	95,84±3,31[a]	61,15±9,97[b]	108,08±9,65[a]
Flavanona glicosilada II	80,20±29,50[a]	113,51±9,32[a]	55,13±5,73[a]	71,31±18,62[a]
Flavanona glicosilada IV	115,5±7,0[c]	214,1±3,9[a]	108,68±11,70[c]	173,01±4,46[b]
Galocatequina glicosilada	39,53±8,22[a]	26,38±2,67[a]	45,27±6,83[a]	33,59±4,49[a]
Quercetina glicosilada III	50,28±18,45[a]	36,80±0,57[a]	41,51±4,51[a]	17,43±1,49[a]
Kaempferol glicosilado II	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00
Antocianidina	51,8±16,0[bc]	33,7±2,8[c]	66,17±6,26[b]	101,61±3,09[a]
Total Ácidos fenólicos	170,25±6,91[b]	253,98±19,03[a]	91,15±2,35[c]	108,99±4,35[c]
Total Flavonoles	50,28±18,45[a]	36,80±0,57[a]	41,51±4,51[a]	17,43±1,49[a]
Total Flavanonas	105,80±10,04[c]	194,96±1,28[a]	91,70±7,57[c]	139,57±4,82[b]
Total Flavanoles	39,53±8,22[a]	26,38±2,67[a]	45,27±6,83[a]	33,59±4,49[a]
Total Antocianidina	51,8±16,0[bc]	33,7±2,8[c]	66,17±6,26[b]	101,61±3,09[a]
Total (poli)fenoles	131,39±5,49[b]	195,78±12,46[a]	88,73±1,55[d]	108,74±408[c]

PR-3: paté con 3% de harina Rojo Brillante; PR-6: paté con 6% de harina Rojo Brillante. Valores con diferente letra en la misma línea indican diferencias estadísticamente significativas ($p < 0,05$) según la prueba de rangos múltiples de Tukey.

4.5.2.2.- Oxidación lipídica y estabilidad de los ácidos grasos

Los metabolitos derivados de la oxidación de las grasas como el 4-hydroxy-2-nonenal (4-HNE) y el malondialdehido (MDA) son sustancias dañinas para el organismo. Concretamente el MDA se describe como una sustancia cancerígena, aunque el IRAC lo clasifica como sustancia cancerígena no probada (Grupo 3), y el 4-HNE como tóxica (Ayala y col., 2014; IRAC, 2018). Tanto en los procesos de elaboración y almacenamiento de los alimentos ricos en grasas, como durante los procesos digestivos, se producen reacciones de oxidación lipídica. Evitarlos o reducirlos implica que se generaran menor cantidad de sustancias tóxicas para el organismo.

El grado de oxidación de los patés estudiados (PC, PR-3 y PR-6) antes y después de la digestión gastrointestinal *in vitro* expresado como TBARS, se muestra en la Tabla 28. Los resultados mostrados están en concordancia con los reportados por Goethals y col. (2020) en diferentes muestras comerciales de paté de hígado de cerdo sin digerir y digeridas. Con respecto a las muestras sin digerir, se observó que a mayor cantidad de harina de caqui mayor grado de oxidación ($p < 0,05$).

Tabla 28.- Oxidación lipídica (µmoles MDA/kg paté) de las muestras de paté de hígado sin digerir y tras los procesos digestivos (gástrico e intestinales).

	Sin digerir	Fase Gástrica	Fase Intestinal (C1)	Fase Intestinal (C2)
PC	$3,19 \pm 0,33^{d}$	$2,54 \pm 0,48^{d}$	$5,33 \pm 0,68^{b}$	$7,80 \pm 0,50^{a}$
PR-3	$4,20 \pm 0,33^{c,d}$	$3,52 \pm 0,40^{c,d}$	$5,53 \pm 0,35^{b}$	$8,08 \pm 0,88^{a}$
PR-6	$4,63 \pm 0,42^{b,c}$	$4,86 \pm 0,45^{b,c}$	$5,77 \pm 0,58^{b}$	$8,66 \pm 0,85^{a}$

PC: paté control; PR-3: paté con 3% de harina Rojo Brillante; PR-6: paté con 6% de harina Rojo Brillante. Valores con diferente letra en la misma línea indican diferencias estadísticamente significativas ($p < 0,05$) según la prueba de rangos múltiples de Tukey.

Sin embargo, tras la fase intestinal (tanto la C1 como la C2) no se observaron diferencias significativas entre las muestras ($p < 0,05$). Este hecho pone de manifiesto que las harinas de caqui ejercen un efecto protector frente a la oxidación lipídica durante la fase intestinal como se puede observar en la Figura 25, pues el incremento de oxidación lipídica reportado por los patés después de la digestión *in vitro* fue menor para los patés enriquecidos, sobre todo en el PR-6 ($p < 0,05$).

El grado de oxidación fue mayor cuando se simuló la fase intestinal C2 ($p < 0,05$). Esto indicaría que al aumentar la lipólisis aumenta también el grado de oxidación de las grasas. Esta relación ha sido previamente observada por otros autores (Larsson y col., 2012; Tullberg y col., 2019). La razón del incremento podría deberse a que los ácidos grasos presentan mayor susceptibilidad a la oxidación que los triglicéridos (Waraho y col., 2011). Por lo tanto, de nuevo se resalta, que el uso de diferentes pancreatinas con una actividad de la lipasa diversa conlleva resultados diferentes en la misma matriz alimentaria.

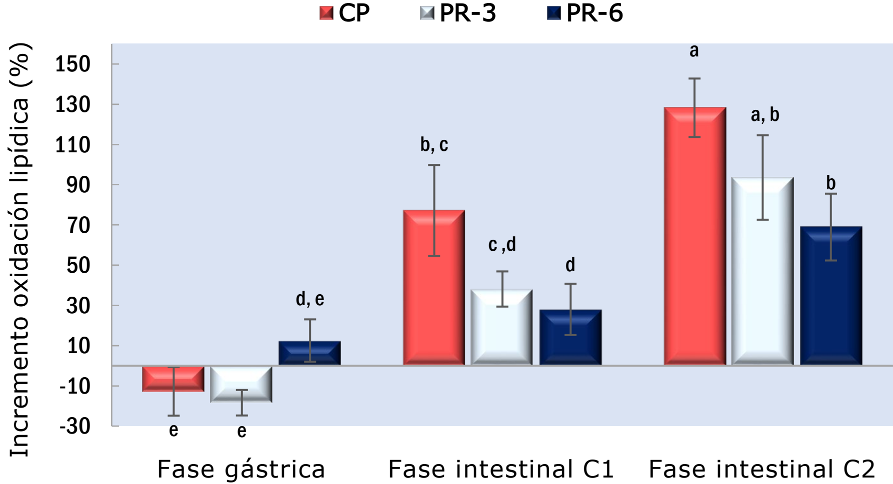


Figura 25.- Incremento de la oxidación lipídica de las muestras de paté estudiadas tras la fase gástrica y las dos fases intestinales.

PC: paté control; PR-3: paté con 3% de harina Rojo Brillante; PR-6: paté con 6% de harina Rojo Brillante. Diferentes letras indican diferencias estadísticamente significativas según la prueba de rangos múltiples de Tukey ($p < 0,05$).

En todas las formulaciones de paté estudiadas los ácidos grasos mayoritarios fueron el ácido oleico (C18:1), el ácido palmítico (C16:0), el ácido linoleico (C18:2) y el ácido esteárico (C18:0). Todos ellos representaron alrededor del 90% del total de ácidos grasos presentes en las muestras de paté de hígado de cerdo (Tabla 29). Estos resultados están en concordancia con lo mostrado previamente por otros autores (Larsson y col., 2012; Zając y col., 2020). Las diferencias más destacables entre la muestra control y las muestras enriquecidas, fue su menor contenido de ácidos grasos insaturados, lo que se podía conectar con su mayor grado de oxidación, pues los ácidos grasos insaturados, al presentar mayor cantidad de dobles enlaces son más susceptibles a la oxidación.

Después de la digestión *in vitro* se reportó un incremento de los ácidos grasos poliinsaturados ($p < 0,05$) (Figura 26), lo cual fue un resultado inesperado, debido a las condiciones prooxidantes del medio digestivo y la susceptibilidad a la oxidación de los ácidos grasos insaturados (Holman y col., 1947; Gorelik y col., 2008; Steppeler y col., 2016). Esta contradicción puede estar relacionada con la naturaleza de los ácidos grasos insaturados (longitud de la cadena, disposición el espacio), además de su localización (suelen formar parte de las membranas celulares), por lo tanto, es más difícil su extracción en comparación con otros ácidos grasos (Zhu y col., 2013; Liu y col., 2021) . En consecuencia, se piensa que los procesos digestivos facilitaron la extracción de estos compuestos. Al mismo tiempo, las diferencias encontradas entre los compuestos C20:3 y las dos condiciones intestinales estudiadas podrían atribuirse a que, a mayor lipólisis mayor extracción, y en consecuencia más tiempo estuvieron

expuestos los ácidos grasos al medio digestivo y por lo tanto su grado de oxidación fue mayor que el mostrado tras la fase intestinal C1.

Por último, la observación microscópica (Figura 27) permitió intuir que las muestras de paté que se sometieron a la fase intestinal C2 presentaron un tamaño de micela más homogéneo que aquellas muestras de paté digeridas con una menor cantidad de lipasa (fase intestinal C1). Sin embargo, se necesitan estudios microscópicos más específicos para sacar conclusiones objetivas acerca del tamaño de las micelas, como la difracción por rayos X.

Tabla 29.- Perfil de ácidos grasos de las muestras de paté de hígado de cerdo sin digerir y digeridas

Ácidos grasos (AG), g/100 g AG	PC			PR-3			PR-6		
	Sin digerir	FI-C1	FI-C2	Sin digerir	FI-C1	FI-C2	Sin digerir	FI-C1	FI-C2
C10:0	0,07±0,00^{b}	0,07±0,00^{b}	0,07±0,00^{b}	0,08±0,00^{a}	0,07±0,00^{a,b}	0,07±0,01^{a}	0,08±0,00^{a}	0,08±0,00^{a}	0,08±0,00^{a}
C12:0	0,10±0,00^{a}	0,10±0,00^{a}	0,10±0,00^{a}	0,09±0,00^{b}	0,09±0,00^{b}	0,09±0,00^{b}	0,09±0,00^{b}	0,09±0,00^{b}	0,09±0,00^{b}
C14:0	1,31±0,00^{a}	1,30±0,00^{a}	1,28±0,00^{b}	1,33±0,01^{a}	1,32±0,00^{a}	1,30±0,02^{b}	1,30±0,04^{a}	1,30±0,00^{a}	1,28±0,02^{b}
C15:0	0,07±0,00^{c}	0,07±0,00^{c}	0,07±0,00^{c}	0,08±0,00^{b}	0,08±0,00^{b}	0,08±0,00^{b}	0,09±0,00^{a}	0,09±0,00^{a}	0,08±0,00^{a,b}
C16:0	22,59±0,50^{a}	22,68±0,04^{a}	22,40±0,15^{a}	23,26±0,39^{a}	22,59±0,03^{a}	22,75±0,07^{a}	22,62±0,72^{a}	22,52±0,10^{a}	22,53±0,06^{a}
C16:1	2,22±0,01^{b}	2,18±0,00^{b}	2,16±0,03^{b}	2,47±0,03^{a}	2,45±0,01^{a}	2,43±0,01^{a}	2,42±0,07^{a}	2,42±0,01^{a}	2,39±0,03^{a}
C17:0	0,39±0,01^{c}	0,39±0,00^{c}	0,38±0,00^{c}	0,43±0,01^{b}	0,42±0,00^{b}	0,42±0,00^{b}	0,46±0,01^{a}	0,46±0,00^{a}	0,46±0,00^{a}
C18:0	11,98±0,44^{a}	12,24±0,03^{a}	11,94±0,13^{a}	12,12±0,47 ^{a}	11,61±0,02 ^{a}	11,71±0,12^{a}	11,57±0,35^{a}	11,58±0,10^{a}	11,78±0,20^{a}
C18:1	41,16±0,43^{b,c}	39,82±0,01^{b,c}	39,68±0,29^{c}	42,45±1,29^{a,b}	40,93±0,18^{b,c}	42,02±0,90^{a,b}	42,16±1,92^{a}	41,06±0,35^{b}	41,51±0,10^{a,b}
C18:2 (n 6,9)	13,43±0,36^{b}	13,99±0,07^{a}	14,14±0,28^{a,b}	11,92±0,15^{d}	12,92±0,04^{c}	12,75±0,24^{c}	11,49±0,31^{d}	12,54±0,09^{c}	12,59±0,14^{c}
C18:3 (n 3,6,9)	0,71±0,03^{b}	0,79±0,01^{a}	0,75±0,02^{b}	0,64±0,01^{c}	0,76±0,01^{a,b}	0,72±0,01^{b}	0,63±0,02^{c}	0,77±0,00^{a,b}	0,75±0,01^{b}
C20:0	0,16±0,00^{b}	0,16±0,00^{b}	0,17±0,00^{b}	0,18±0,01^{a}	0,17±0,00$^{a, b}$	0,18±0,01^{a}	0,19±0,01^{a}	0,18±0,00^{a}	0,20±0,01^{a}
C20:1	0,92±0,01^{a}	0,92±0,00^{a}	0,91±0,00^{a}	0,86±0,01^{b}	0,89±0,00^{a}	0,89±0,01^{a,b}	0,85±0,03^{b}	0,88±0,00^{a,b}	0,90±0,02^{a}
C20:2 (n 11,14)	0,62±0,01^{a}	0,63±0,01^{a}	0,61±0,00^{a}	0,55±0,00^{c}	0,58±0,00^{b}	0,57±0,01^{b,c}	0,52±0,01^{c}	0,56±0,01^{b}	0,56±0,01^{b}
C20:3 (n 8,14,17)	0,57±0,05^{c}	0,82±0,01^{a}	0,74±0,03^{a}	0,52±0,02^{c}	0,81±0,01^{a}	0,71±0,02^{b}	0,51±0,03^{c}	0,83±0,01^{a}	0,76±0,03^{a}
Otros	3,71±0,24^{a}	3,86±0,16^{a}	4,62±0,74^{a}	3,01±0,94^{a}	4,30±0,27^{a}	3,30±0,79^{a}	2,69±0,99^{a}	4,66±0,69^{a}	4,03±8,03^{a}
AGS	36,66±0,97^{a}	37,00±0,07^{a}	36,38±0,30^{a}	37,57±0,88^{a}	36,36±0,06^{a}	36,61±0,22^{a}	36,39±1,14^{a}	36,29±0,21^{a}	36,50±0,29^{a}
AGI	59,62±0,91^{a}	59,15±0,12^{a}	58,99±0,67^{a}	59,42±1,51^{a}	59,34±0,25^{a}	60,09±1,21^{a}	58,60±2,38^{a}	59,05±0,48^{a}	59,47±0,34^{a}
AGM	44,30±0,45^{b}	42,92±0,02^{c}	42,75±0,33^{c}	45,78±1,33^{a}	44,27±0,19^{b}	45,34±0,93^{b}	45,44±2,02^{a}	44,36±0,36^{b}	44,81±0,15^{a}
AGP	15,32±0,46^{b}	16,23±0,10^{a}	16,25±0,34^{a}	13,64±0,18^{c}	15,07±0,06^{b}	14,75±0,28^{b}	13,16±0,37^{c}	14,69±0,11^{b}	14,66±0,19^{b}

PC: paté control; PR-3: paté con 3% de harina Rojo Brillante; PR-6: paté con 6% de harina Rojo Brillante. AGS: ácidos grasos saturados; AGI: ácidos grasos insaturados; AGM ácidos grasos monoinsaturados; AGP: ácidos grasos polinsaturados. Diferentes letras indican diferencias estadísticamente significativas según la prueba de rangos múltiples de Tukey ($p < 0,05$).

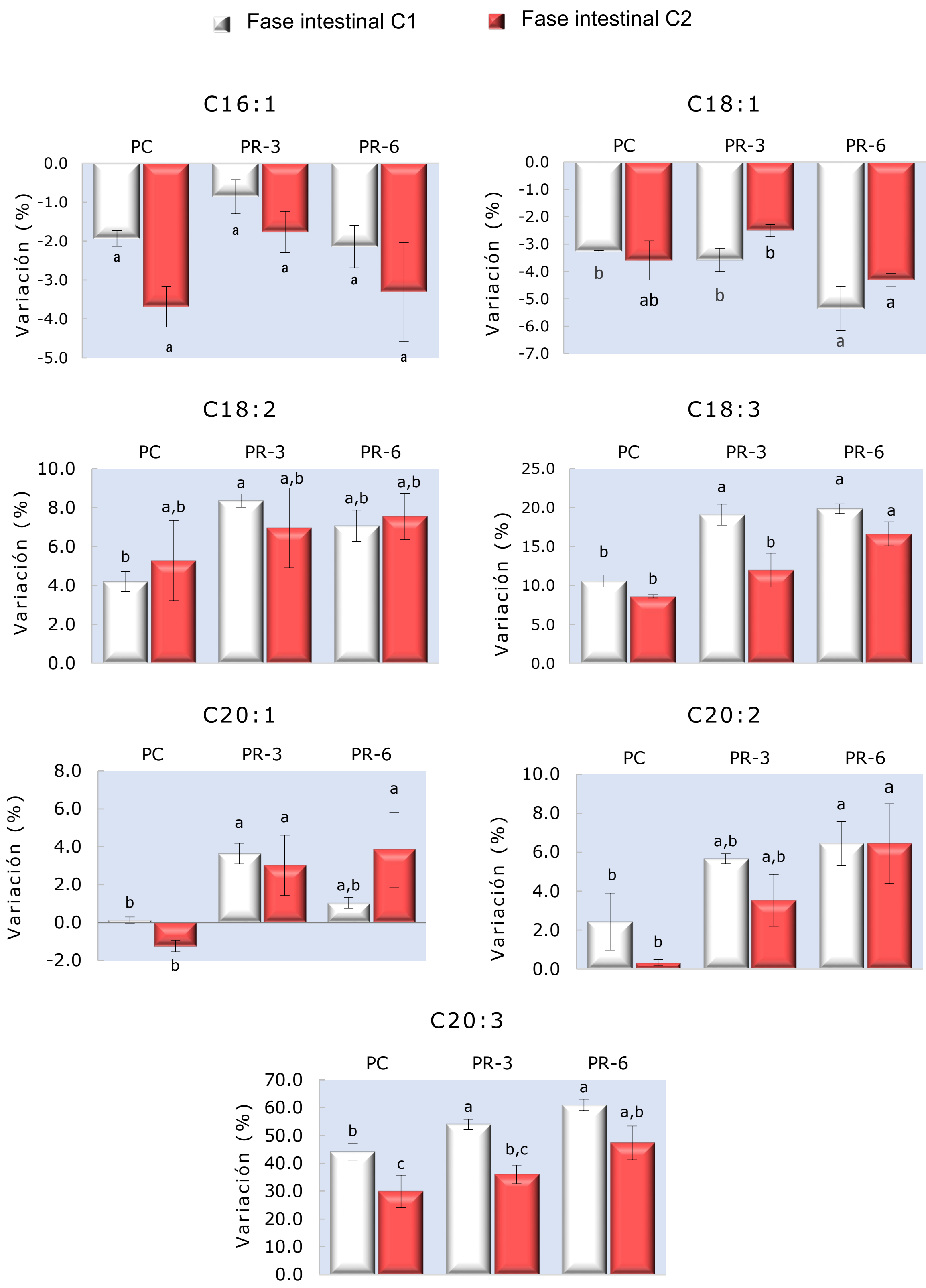


Figura 26.- Variaciones de los ácidos grasos insaturados de los patés de hígado de cerdo.

PC: paté control; PR-3: paté con 3% de harina Rojo Brillante; PR-6: paté con 6% de harina Rojo Brillante. Diferentes letras indican diferencias estadísticamente significativas según la prueba de rangos múltiples de Tukey ($p < 0,05$).

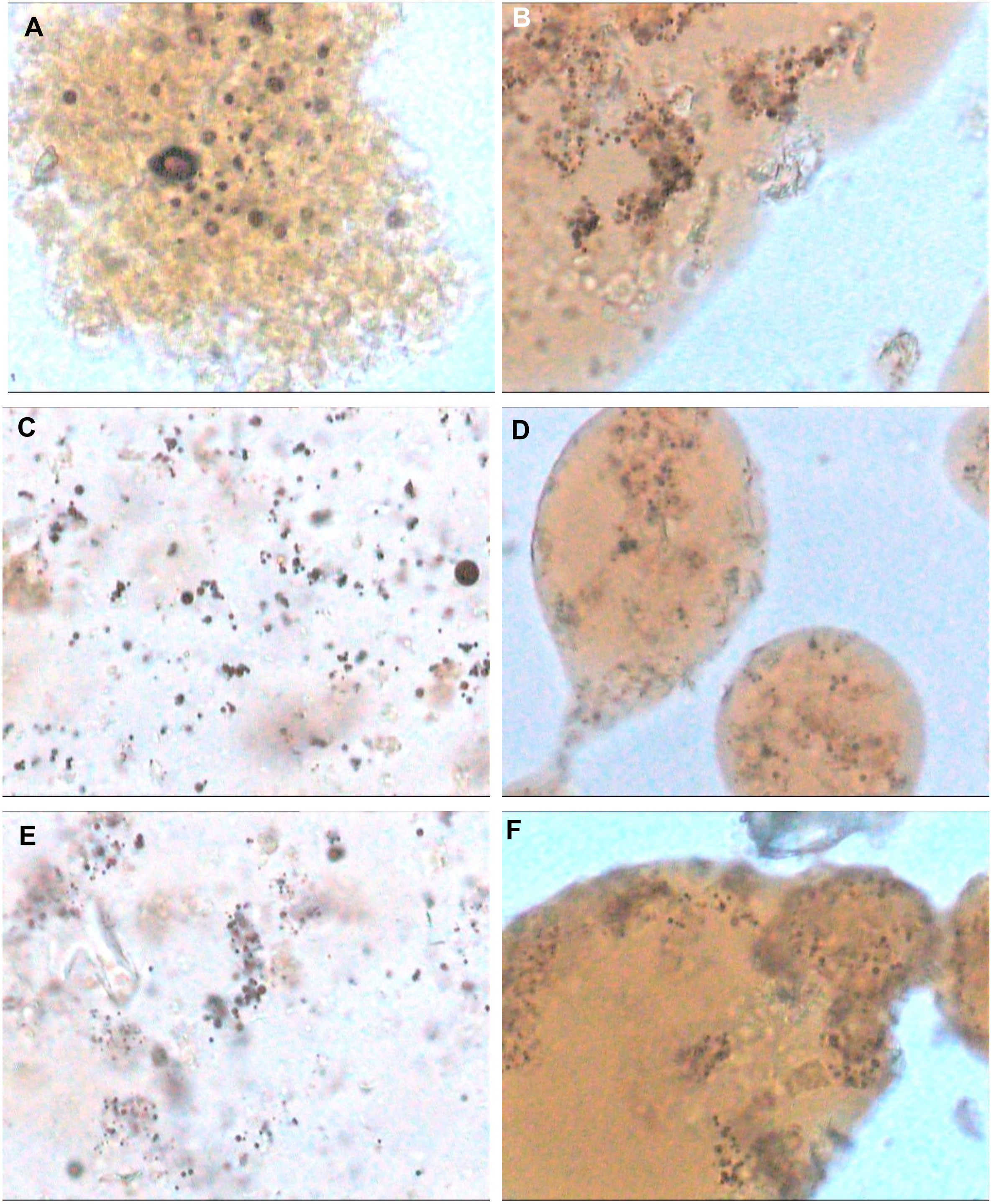


Figura 27.- Observación al microscopio de las muestras de paté sometidas a las dos condiciones diferentes de la fase intestinal. Resolución 40X. **A**. Paté control tras la fase intestinal C1; **B**. Paté control tras la fase intestinal C2; **C**. Paté con 3% de harina Rojo Brillante tras la fase intestinal C1; **D**. Paté con 3% de harina Rojo Brillante tras la fase intestinal C2; **E**. Paté con 6% de harina Rojo Brillante tras la fase intestinal C1; **F**. Paté con 6% de harina Rojo Brillante tras la fase intestinal C2

Capítulo 5.-
CONCLUSIONES
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5.1 CONCLUSIONES

(i) Los coproductos de la elaboración del zumo de caqui (piel y pulpa) obtenidos de los cultivares "Rojo Brillante" y "Triumph" pueden ser tratados para obtener ingredientes intermedios (harinas de caqui) .

(ii) El tamaño de partícula afecta a la composición química, las propiedades fisicoquímicas, tecnofuncionales y fisiofuncionales de la harina de caqui (Rojo Brillante y Triumph).

(iii) Las harinas de caqui (Rojo Brillante y Triumph), independientemente de su tamaño de partícula, pueden ser usadas para fortificar alimentos con fibra dietética, especialmente fibra dietética insoluble, monosacáridos y ácido gálico.

(iv) Las harinas de caqui (Rojo Brillante y Triumph) con menor tamaño de partícula (<210 µm) muestran mayor cantidad de compuestos bioactivos, (poli)fenoles y carotenos totales.

(v) Las harinas de caqui (Rojo Brillante y Triumph) destacan por su capacidad de retención de agua, capacidad de hinchamiento y capacidad de retención de bilis, por lo que presentan aptitudes tecnológicas y fisiofuncionales interesantes para la elaboración de alimentos estables y saludables.

(vi) Las harinas de caqui (Rojo Brillante y Triumph) con menor tamaño de partícula (<210 µm), por su alta capacidad de retener bilis y su alto contenido de compuestos bioactivos presentan mejores aptitudes para ser usadas como ingredientes intermedios en la industria alimentaria.

(vii) La harina de caqui Rojo Brillante presenta mayor cantidad de compuestos (poli)fenólicos unidos (95%) que de compuestos (poli)fenólicos libres.

(viii) La harina de Triumph presenta mayor variedad y contenido de flavonoles que la harina de Rojo Brillante.

(ix) Los compuestos (poli)fenólicos glicosilados presentes en las harinas de caqui (Rojo Brillante y Triumph) son más estables a la digestión gastrointestinal *in vitro* que sus agliconas.

(x) Las harinas de caqui (Rojo Brillante y Triumph) presentan efectos colorantes en las dos matrices alimentarias estudiadas: espaguetis de sémola de trigo duro y paté de hígado de cerdo. El efecto colorante es concentración dependiente.

(xi) Los patés de cerdo enriquecidos con harina de caqui (Rojo Brillante y Triumph) son más aceptados por los consumidores que el paté de hígado de cerdo sin harina de caqui, principalmente debido al incremento de la tonalidad rojiza.

(xii) La harina de caqui (Rojo Brillante y Triumph) reduce el contenido de nitrito residual de los patés, de manera concentración dependiente, siendo más efectiva la harina de Triumph. Ambas harinas podrían usarse como agentes reductores de nitritos en productos cárnicos cocidos.

(xiii) La estabilidad de la emulsión de los patés se ve comprometida por la inclusión de harina de caqui (Rojo Brillante y Triumph) de manera concentración dependiente e influenciada por el tipo de harina: la harina de Triumph, desestabiliza la matriz más que la harina de Rojo Brillante.

(xiv) La inclusión de harina de caqui (Rojo Brillante y Triumph) en espaguetis de sémola de trigo duro aumenta su contenido de fibra y azúcares y reduce su tiempo óptimo de cocción.

(xv) La adición de harina de Triumph en grandes cantidades (6%) en los espaguetis de sémola de trigo duro produce disrupción de su estructura molecular y en consecuencia se obtienen un espagueti crudo más quebradizo y un espagueti cocinado más pegajoso, debido a la liberación del almidón de la red de gluten.

(xvi) La harina de Triumph no es apta para ser incluida en paté o espagueti en grandes cantidades (6%) debido a la desestabilización de la matriz que produce por su gran contenido en fibra dietética insoluble.

(xvii) El enriquecimiento con harinas de caqui (Rojo Brillante y Triumph) a los espaguetis en pequeñas cantidades (3%) reduce la digestibilidad del almidón.

(xviii) La inclusión de harina de caqui (Rojo Brillante y Triumph) en los espaguetis de sémola de trigo duro incrementa, de forma concentración dependiente, el contenido de compuestos (poli)fenólicos unidos, al aportar dos nuevos compuestos, ácido gálico y ácido cumárico-o-hexósido.

(xix) La harina Rojo Brillante es una excelente fuente de compuestos (poli)fenólicos unidos muy útil para enriquecer productos cárnicos ricos en grasas como el paté de hígado de cerdo, al aportar 21 compuestos (poli)fenólicos en los patés, especialmente ácido gálico.

(xx) Las harinas de caqui (Rojo Brillante y Triumph) aportan a las matrices alimentarias estudiadas (espaguetis de sémola de trigo duro y paté de hígado de cerdo) compuestos (poli)fenólicos que podrían alcanzar el colon y ser metabolizados por la microbiota intestinal al resistir y permanecer estos compuestos estables tras la digestión gastrointestinal *in vitro*.

(xxi) Durante la digestión gastrointestinal *in vitro,* el aumento de la lipólisis aumenta la oxidación

lipídica de los patés de hígado de cerdo, con o sin harina de caqui.

(xxii) La oxidación lipídica de los patés de hígado de cerdo enriquecidos con harina de Rojo Brillante se ve reducida durante la fase intestinal de la digestión gastrointestinal *in vitro*.

(xxiii) El incremento de ácidos grasos durante los procesos digestivos simulados (*in vitro*) tienen un papel protector sobre los compuestos (poli)fenólicos unidos. Después de la digestión gastrointestinal, alrededor del 100% del total de los compuestos (poli)fenólicos unidos están disponibles para alcanzar el colón.

(xxiv) Las harinas de caqui (Rojo Brillante y Triumph) pueden usarse como ingredientes intermedios en la industria alimentaria para enriquecer diferentes matrices alimentarias. La incorporación de dichas harinas a un 3% es la más recomendada.

(xxv) La inclusión de las harinas de caqui en la industria alimentaria conlleva la valorización de los coproductos de la industria del caqui, la reducción de residuos y la aparición en el mercado de nuevos alimentos con mayor contenido en fibra, compuestos fenólicos unidos estables a la digestión gastrointestinal, menor contenido de nitrito residual en el caso de productos cárnicos cocidos y menor digestibilidad del almidón en el caso de los espaguetis.

5.2 CONCLUSIONS

(i) Juice persimmon coproducts (peel and pulp) obtained from the cultivars "Rojo Brillante" and "Triumph" can be treated to obtain intermediate ingredients (persimmon flours)

(ii) Particle size of persimmon flours (Rojo Brillante and Triumph) affects their chemical composition, physicochemical, techno-functional, and physio-functional properties.

(iii) Persimmon flours (Rojo Brillante and Triumph), independently of their particle size, can be used to enrich foods with fiber, especially insoluble fiber, monosaccharides, and gallic acid.

(iv) Persimmon flours (Rojo Brillante and Triumph) with the smallest particle size show more amount of bioactive compounds, total (poly)phenols, and carotenes.

(v) Water holding capacity, swelling capacity, and bile holding capacity have been highlighted in both persimmon flours (Rojo Brillante and Triumph). They show attractive techno-functional and physio-functional aptitudes for developing stable and healthy foods.

(vi) Persimmon flours (Rojo Brillante and Triumph) with the samllest particle size (<210 μm) due to their high bile holding capacity and their amount of bioactive compounds present better

aptitudes to be used as intermediate ingredients in the food industry.

(vii) The Rojo Brillante flour has higher bound (poly)phenol amounts (95%) than free poly(phenols).

(viii) The Triumph flour has more variety and quantity of flavonols than Rojo Brillante flour.

(ix) Glycosylated (poly)phenols from persimmon flours (Rojo Brillante and Triumph) are more stable during *in vitro* gastrointestinal digestion than their aglycones.

(x) Persimmon flours (Rojo Brillante and Triumph) exhibit coloring effects in both studied food matrix: durum wheat spaghetti and pork liver pâtè. The coloring effect is dose dependent.

(xi) Due to the redness increase, pork liver pâtès enriched with persimmon flours (Rojo Brillante and Triumph) are more accepted by consumers than pork liver pâtè without persimmon flour.

(xii) Persimmon flours (Rojo Brillante and Triumph) reduce nitrite residue amounts in pork liver pâtè in a dose-dependent way, being Triumph flour the most effective. Both flours could be used as nitrite-reducing agent in cooked meat products.

(xiii) The emulsion stability of pork liver pâtè is negative affected by persimmon flours (Rojo Brillante and Triumph) in a dose-dependent way and influenced by the type of persimmon flour: Triumph flour destabilizes the matrix more than Rojo Brillante flour.

(xiv) The addition of persimmon flours (Rojo Brillante and Triumph) to durum wheat spaghetti increases their fiber and sugar content and reduces their optimum cooking time.

(xv) The addition of Triumph flour at 6% in durum wheat spaghetti disrupts its molecular structure and, in consequence, crude spaghetti more breakable and cooked spaghetti stickier due to the starch release of the gluten network, are obtained.

(xvi) Triumph flour is not suitable for enriching pâté or spaghetti at 6%, since it causes matrix destabilization due to its high insoluble dietary fiber content.

(xvii) The spaghetti enrichment with persimmon flours (Rojo Brillante and Triumph) at 3% reduces the starch digestibility.

(xviii) Theaddition of persimmon flour (Rojo Brillante and Triumph) to durum wheat spaghetti increases, in a concentration-dependent way, the content of bound (poly)phenolic compounds by providing two new compounds, gallic acid and acid coumaric-o-hexoside.

(xix) The Rojo Brillante flour is an excellent source of bound (poly)phenolic compounds. It is useful

to for enriching rich fatty meat products, such as pork liver pâtè, by providing 21 (poly)phenolic compounds, especially gallic acid.

(xx) Persimmon flours (Rojo Brillante and Triumph) provide (to durum wheat semolina spaghetti and pork liver pâtès) (poly)phenolic compounds with the ability to reach the colon and to be metabolized by the intestinal microbiota by resisting and remain stable after their *in vitro* gastrointestinal digestion.

(xxi) During *in vitro* gastrointestinal digestion of pork liver pâtès (with or without Rojo Brillante persimmon flour, at higher lipolysis degree, higher lipid oxidation level.

(xxii) Lipid oxidation of pork liver pâtès enriched with Rojo Brillante flour is reduced during the intestinal phase of *in vitro* gastrointestinal digestion.

(xxiii) The increase in fatty acids during simulated digestive processes (*in vitro*) has a protective role on the bound (poly)phenolic compounds. After gastrointestinal digestion, about 100% of the total bound (poly)phenolic compounds are available to reach the colon.

(xxiv) Persimmon flours (Rojo Brillante and Triumph) can be used as intermediate ingredients in the food industry to enrich different food matrices. The incorporation of these flours at 3% is the most recommended.

(xxv) The use of persimmon flours in the food industry allows the persimmon co-products valorisation, the waste reduction and that new foods can became available at the market, with higher fiber content and bound (poly)phenolic compounds stable to gastrointestinal digestion, lower residual nitrite content, in the case of cooked meat products and lower starch digestibility, in the case of spaghetti.

Capítulo 6.-
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Capítulo 7.-
PUBLICACIONES
Fig. 1
A
in vitro digestion for (Poly)phen
Oral Phase (OP)
Intestinal Phase C1 (IPC1)
Intestinal Phase C2 (IPC2)
Free fraction
Bound Fraction
B
Gastric Phase
Intestinal Phase C2
C
Intestinal phase C2

7.1.- PUBLICACIÓN 1

Título: Evaluation of particle size influence on proximate composition, physicochemical, techno-functional and physio-functional properties of flours obtained from persimmon (Diospyros kaki *Thunb.*) coproducts

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Evaluation of particle size influence on proximate composition, physicochemical, techno-functional and physio-functional properties of flours obtained from persimmon (*Diospyros kaki* Trumb.) coproducts.

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Abstract

The aim of the work was to study the influence of particle size in the composition, physicochemical, techno-functional and physio-functional properties of two flours obtained from persimmon (*Diospyros kaki* Trumb. cvs. 'Rojo Brillante' (RBF) and 'Triump' (THF)) coproducts. The cultivar (RBF and THF) and particle size significantly affected all parameters under study, although depending on the evaluated property, only one of this effect predominated. Carbohydrates (38.07-46.98 g/100 g) and total dietary fiber (32.07-43.57 g/100 g) were the main components in both flours (RBF and THF). Furthermore, insoluble dietary fiber represented more than 68 % of total dietary fiber content. All color properties studied were influenced by cultivar and particle size. For both cultivars, the lower particle size, the higher lightness and hue values. RBF flours showed high values for emulsifying activity (69.33-74.00 mL/mL), while THF presented high values for water holding capacity (WHC: 9.47-12.19 g/g). The bile holding capacity (BHC) and fat/oil binding values were, in general, higher in RBF (19.61-12.19- g/g and 11.98-9.07, respectively) than THF (16.12-12.40 g/g and 9.78-7.96, respectively). The effect of particle size was really evident in both WHC and BHC. Due to their dietary fiber content, techno-functional and physio-functional properties, persimmon flours seem to have a good profile to be used as potential functional ingredient.

Keywords: Persimmon, Particle size, coproduct, techno-functional properties, Dietary fiber.

Abbreviation

BHC	Bile holding capacity
EA	Emulsifying activity
ES	Emulsion stability
FOB	Fat/oil binding
IDF	Insoluble dietary fiber
OHC	Oil holding capacity
RBF	'Rojo Brillante' flour
SDF	Soluble dietary fiber
SWC	Swelling capacity
TDF	Total dietary fiber
THF	'Triumph'flour
WHC	Water holding capacity

Introduction

Persimmon (*Diospyros kaki* Thumb.) is the edible fruit of a number of species of trees belonging to the genus *Diospyros* (*Ebenaceae* family) native of China [1]. In 2013 its global production was 4,637,357 T, being China the biggest producer followed by Republic of Korea and Japan. Additionally, its production increased 360% in the last twenty years (1993-2013) [2]. From a commercial standpoint, persimmons could be divided into astringent cultivars such as 'Rojo Brillante', 'Tomatero', 'Triumph', etc. and non-astringent cultivars like 'Fuyu', 'Hana-Fuyu', 'Jiro', etc. Astringent cultivars, in ripe stage, are sweet, red and too soft. However, its low firmness is a problem to distribute and commercialize. For this reason, actually, different treatments are carrying out in immature fruit to obtain a non-astringent, sweet, orange firm fruit [3]. However, such treatments can damage fruit, and may cause strange colorations. This, together short season fruit, have encouraged industrialization of persimmon cultivation [4]. In Spain, both 'Rojo Brillante' and 'Triumph' are the most important cultivars, in terms of production and commercially [5]. Thus, in the last years, the persimmon fruits have been industrialized and juice, jam and dehydrated persimmon can be found on the market. This industrialization generates high volumes of coproducts like peel, pulp or discarded fruit which are rich in bioactive compounds like fiber and natural antioxidants. These coproducts can be processed to obtain stable rich-fiber flours which could be used as ingredients in new food elaboration process to manufacture ice cream, and dairy or meat products [6]. Furthermore, persimmon has health benefits, such as reducing cholesterol and glucose serum or positive influenced on lipid metabolism [7]. On the other hand, particle size is a decisive parameter that influences if these flours cle size and its influence on functional and structural properties are needed to confirm the suitability of their use in food product development and process engineering [8]. Therefore, the aims of this work were to study the influenced of particle size on the proximate composition, and physicochemical, techno-functional and physio-functional properties of two flours obtained from the coproducts of astringent persimmons (*Diospyros kaki* Thumb) of cvs. 'Rojo brillante' and 'Triumph' to establish whether they could have application in the food industry as potential functional ingredient.

Materials and Methods

Plant material

Astringent persimmons (*Diospyros kaki* Thumb) of cvs. 'Rojo Brillante' and 'Triumph' were purchased from local market (Orihuela, Alicante, Spain) and selected on the basis of uniformity and size. The fruits were in the yellow-orange ripening stage and with 14-16º Brix. The selected fruits were randomly grouped into batches of 50 kg each. The samples were washed, cut and liquefied. The juice was discarded and the coproducts composed by pulp and peels were collected and dried at 45 ºC in a hot air drier for 24 h. Dehydrated samples were ground with a knife mill to obtain a flour. The two fractions obtained were: 'Rojo Brillante' flour (RBF) and 'Triumph' flour (THF). Each sample was separated into different fractions based on the particle size using three sieves with different apertures (0.701, 0.417 and 0.210 mm). Thus, persimmon flours were divided into four particle size ranges: biggest (>0.701 mm), big-intermediate or 0.701-0.417 mm, small-intermediate or 0.417-0.210 mm and finest <0.210 mm. Samples were vacuum packed and stored at darkness until analysis.

Proximate composition

Moisture, ash, lipid, protein, total dietary fiber (TDF), insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) were determined by their corresponding AOAC method [9]. Available carbohydrates were calculated by difference.

Physicochemical analysis

The pH was measured in 10% (w/v) aqueous solutions of the samples while water activity (Wa) was determined using a Sprint TH-500 Novasina Thermoconstanter at 25 ºC. Color was measured with a CM-2600d colorimeter (Minolta Camera Co., Osaka, Japan) with illuminant D_{65}, observer10º, SCI mode, 11 mm aperture for illumination and 8 mm for measurement, based in the CIELab color space. The following color coordinates were determined: lightness (L*), redness (a* ±red-green) and yellowness (b* ±yellow-blue). From these coordinates, hue ($h^* = \tan^{-1} b^*/a^*$) and chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$) were calculated.

Techno-functional properties

Water and oil holding capacity (WHC and OHC, respectively) were determined following the methodology described by Robertson *et al.* [10]. The results were reported as g of water or oil held by g of sample. Emulsifying activity (EA) and emulsion stability (ES) were carried out following the methods described by Chau and Huang [11]. The EA results were calculated as the

volume of the emulsified layer/total sample volume x 100. The ES was calculated as the volume of remaining emulsified layer/original emulsion volume x 100. Swelling capacity (SWC) was measured following the method described by Gómez-Ordoñez *et al.* [12]. Results were expressed as mL water per g of sample.

Physio-functional properties

Bile-holding capacity (BHC) was determined based on Eastwood *et al.* [13] methodology with some modifications. Thus, 5 g of porcine bile (obtained from an official slaughterhouse) were added to a centrifuge tube containing 160 mg of sample. The samples were shaking for 2 min and stored at 25 ºC for 18 h. Then, the samples were centrifuged at 1500 g during 20 min at 25 ºC. The supernatant was discarded and the pellet was weighed. Results were expressed as g of bile held per g of sample. Fat/oil binding (FOB) was determined according to López-Marcos *et al.* [14]. FOB values were expressed as g of oil held by g of sample.

Statistical analysis

For each experiment, three independent samples were examined with three replications per sample. Data obtained for all the properties were analyzed by means of a two-way ANOVA test with two factors: cultivar and particle size. Tukey's *post hoc* test was applied for comparisons of means; differences were considered significant at $p<0.05$. Statistical analyses were carried out using the statistical package SPSS 19.0 (SPSS Inc., Chicago, IL.). Correlation analysis was performed between particle size and chemical, physico-chemical, techno-functional and physio-functional properties of flours using Pearson correlation analysis.

Results and Discussion

Proximate composition

The proximate composition of the flours was discreetly influenced by the particle size and cultivar ($p<0.05$). According to the results (Table 1), the main components in both persimmon flours were carbohydrates and TDF ($p<0.05$). The high carbohydrate contents of both RBF and THF (values comprised between 44.02-46.98 and 38.87-41.25 g/100 g, respectively) was probably due to the large amount of pulp used. The moisture content values obtained ranged from 11.15 and 15.53 g/100 g, which is higher than the 7.07% previously reported by Lee *et al.* [7] for persimmon coproducts. The ash, lipid and protein contents (Table 1) of both RBF and THF were lower than previously reported data for persimmon peel powder [7] and other fruit coproducts, like grapefruit, or pineapple [14,15]. The intrinsic differences between cultivars and fruits, and the seedless nature and high pulp content of the samples studied could explain these results.

Among these last components, only the ash content showed a positive correlation with particle size (r= 0.81).

As regard to the TDF content (Table 1), similar values were reported by Lee *et al.* [7] for persimmon coproducts. In the present study THF fractions showed the highest TDF content (p<0.05). All samples analyzed exhibited a higher (p<0.05) IDF than SDF content, IDF representing more than 68% id TDF in both RBF and THF flours. This fact has been reported by other authors previously [7,16]. Moreover, THF fractions had the highest IDF content (p<0.05). The IDF content of both flours was unaffected (p>0.05) by particle size, while the TDF and SDF contents was significantly affected (p<0.05). It is important to highlight the fact that the lowest SDF content in RBF was found for the biggest particle size, while in the case of THF the lowest SDF content was found for the smallest particle (p<0.05). It is generally accepted that, dietary fiber sources that are suitable as a food ingredient, should have an SDF/IDF ratio close to 1:2 to provide the appropriate physiological effects [17]. Persimmon flours showed higher ratios than 1:2, with the exception to smallest and small-intermediate particles of RBF. As regard the proximate composition, and focusing on the highest compounds (carbohydrates and dietary fiber) the main differences between samples were due to the effect of cultivar and not particle size (although in some cases significant differences were found for the particle size). Therefore, any decision concerning the choice of particle size must be made by taking into account its capacity for incorporation in the food matrix to which the flour is to be added.

Physicochemical properties

Physicochemical parameters studied in RBF and THF (Table 2) were influenced by the particle size and cultivars analyzed (p<0.05). In the case of water activity (Aw) all the samples analyzed had values ranging between 0.351 to 0.438 and, except for the biggest particle, RBF fractions showed, in general, lower values (p<0.05) than the respective THF fractions, which was probably related with their higher carbohydrate content (Table 1). These Aw values were within the values reported for the coproducts of other fruits, like pear (0.415) or date fruit (0.425) [18]. Both RBF and THF flours had pH values ranged from 4.87 to 5.42 (Table 2), although significant differences (p<0.05) were observed between samples. Thus, THF showed the highest (p<0.05) pH values for all the particle sizes analyzed. Due to their pH and Aw values, both RBF and THF seem to have a low risk for deterioration as results of microorganisms and enzymatic or non-enzymatic reactions. As regards the color parameters (Table 2), the values obtained were similar to those reported by Akyildiz *et al.* [19]. In both RBF and THF, lightness (L*) and hue (h*) values decreased with increasing particle size (p<0.05), the maximum L* and h* values belonging to the

finest particles. Furthermore, L* and h* values had a negative correlation with particle size (r= -0.86; r= -0.75, respectively). Ahmed *et al.* [20] postulated that L* behavior is associated with the surface area: the greater the surface area the greater the reflection of light. For the same particle size, THF samples showed higher (p<0.05) L* and h* values than RBF samples. Hue values in all the samples were in the range of orange hues (60-90º), and the lower the particle size, the higher the orange-yellowish h* [21]. On the other hand, redness values (a*) decreased with decreasing particle size from big-intermediate to finest (p<0.05) and positive correlation was observed with particle size (r= 0.64). Furthermore RBF fractions showed the highest (p<0.05) a* values. With respect to yellowness and chroma, the finest particles exhibited the lowest values (p<0.05) in both flours. The significant differences in color values related with particle size (p<0.05), could be due to the loss of pigment during milling [8]. Any such changes in the color parameters need to be taken into consideration before addition to food, since color may be considered unsuitable and influence consumer acceptance [15].

Techno-functional properties

Table 3 shows the results obtained for the techno-functional properties of RBF and THF with different particle sizes. Hydration properties (WHC and SWC) were discreetly influenced by particle size in both RBF and THF (p<0.05). In the case of WHC, this parameter increased in RBF with decreasing particle size from big-intermediate to finest (p<0.05). However, in THF this behavior was not observed, underlining the differences between cultivars (p<0.05). Additionally, the highest WHC was obtained in RBF for the biggest particles size (p<0.05) whilst for THF the highest WHC was obtained for small-intermediate particles (p<0.05). The WHC recorded for both persimmon flours were higher than those mentioned for other fruit coproducts like, pear (4.9 g/g), date (5.7 g/g), grape fiber (6.38 g/g) or lemon fiber (6.96 g/g) [14,18]. The WHC in persimmon flours showed a positive correlation with TDF (r= 0.75) and negative correlation with carbohydrate (r= -0.80) and protein (r= -0.70), which would explain the higher WHC of THF than RBF for all particle sizes, since THF fractions had a higher TDF content, while RBF fractions had a higher carbohydrate content. As regards the SWC (Table 3), in general terms, it was higher in RBF than in THF (p<0.05), and the values obtained for both flours, were similar those previously reported by Akter and Eun [22]. However, particle size affected this property differently in the two cultivars (p<0.05). In the case of RBF the SWC decreased with decreasing particle size, while in THF the SWC increased, as particle size decreased. Nevertheless, in all RBF fractions analyzed, only the finest particle showed significant differences (p<0.05). Due to their hydration properties, both RBF and THF show the potential for use in products that requiring hydration to improve

yield or modify texture and viscosity. In the case of the OHC (Table 3), significant differences were observed between cultivars, THF showing the highest values ($p<0.05$). A reduction in particle size in both persimmon flours caused a moderate increase in their OHC ($p<0.05$), so that a negative correlation was observed between the OHC and particle size ($r= -0.71$). Both flours showed similar OHC to other fruit coproducts including, passion fruit (2.03 g/g) and pineapple and passion fruit (1.57-1.85 g/g) [14,23] but lower than mango (3.76-4.21 g/g) [24]. Thus, due to their OHC values, both RBF and THF show potential as an ingredient in fried products, because they would help prevent an over-greasy sensation [25].

The emulsifying properties (EA and ES) evaluated in RBF and THF (Table 3) showed significant cultivar-based differences ($p<0.05$). However, EA was not affected by particle size in either flours studied ($p>0.05$). By contrast, the ES values reported were influenced by particle size ($p<0.05$). For both emulsifying properties, RBF fractions showed the highest values ($p<0.05$). However, the biggest particle in THF fractions showed the greatest capacity to stabilize emulsions ($p<0.05$). The EA was positively correlated with protein ($r= 0.75$) and carbohydrate ($r= 0.84$) contents, and negatively correlated with pH ($r= -0.94$) and TDF content ($r= -0.82$). These correlations coincide with the fact that polysaccharide-protein complexes present good emulsifying properties and their ability to stabilize interfaces is related with the isoelectric point of proteins [26]. As regards their EA, both RBF and THF showed values considered high compared with those of other fruit coproducts, such as pomegranate (37.3 mL/100 mL) [27] or citrus fruits (53.71-54.67 mL/100 mL) [14]. According to the emulsifying properties analyzed, both persimmon flours, but especially RBF, could be used as emulsifying or stabilizing agents in foods like ice creams, chocolate, butter, or meat products.

Physio-functional properties

Assessment of the bile holding capacity (BHC) is a simple, cheap and fast way of ascertaining the ability of coproducts to retain bile. In this work BHC (Figure 1A) was strongly influenced by the particle size of the persimmon flours ($p<0.05$), showing a negative correlation ($r= -0.90$). The same behaviors in this respect was observed in both flours analyzed (Figure 1A), their BHC increasing as the particle size diminished from biggest to finest particles ($p<0.05$). The values of the BHC obtained for both RBF and THF were considerably higher than those reported by López-Marcos *et al.* [14] for other fruit coproducts such as lemon (8.07 g/g) or grapefruit dietary fiber (9.20 g/g). A high BHC is a very interesting property for fruit coproducts, since the ability to hold bile has been related with the regulation of cholesterol and glucose blood levels [28]. Nevertheless, further studies including *in vitro* digestion or/and human studies are needed

to confirm these properties in persimmon coproducts. For its part, Fat/Oil binding (FOB) is a property that shows the capacity of fiber to adsorb or retain oil/fat in its matrix, simulating the conditions of food digestion. According to the results (Figure 1B), significant differences were observed among particle sizes ($p<0.05$), while RBF had the highest FOB ($p<0.05$). Note that FOB in the persimmon flours analyzed in this work were lower than values reported by López-Marcos *et al.* [14] for other fruit coproducts such as pomegranate (14.00 g/g) or grapefruit (35.43 g/g).

Conclusions

It can be concluded that cultivar and particle size significantly affect the composition, and physicochemical, techno-functional and physio-functional properties of persimmon flours. Depending on the property in question, the predominant effect was related to cultivar or particle size. On the other hand, both persimmon flours of all particle sizes evaluated could be used as potential functional ingredient to develop healthy and sustainable food products, mainly due to, their dietary fiber content, hydration and emulsifying properties and bile holding capacity. However, further studies to confirm their suitability for different food applications and their health benefits are required.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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Table 1.- Effect of different particle sizes on proximate composition of 'Rojo Brillante' (RBF) and 'Triumph' (THF) flours.

Sample	Particle size (mm)	Moisture	Protein	Lipid	Ash	TDF	IDF	SDF	Carbohydrates
RBF	> 0.701	14.16±0.38^{a}	3.14±0.06^{a}	0.56±0.02^{a}	2.07±0.51^{a}	33.44±1.05^{d}	26.85±0.52^{b}	6.59±0.29^{c}	46.63^{a}
	0.701-0.417	13.46±0.66^{c}	3.24±0.02^{a}	0.34±0.00^{c}	1.98±0.11^{a}	36.73±0.84^{c}	26.76±0.35^{b}	10,00±0.50^{b}	44.25^{a}
	0.417-0.210	13.71±0.12^{c}	2.92±0.01^{b}	0.45±0.00^{b}	1.82±0.06^{b}	37.07±0.51^{c}	25.30±0.61^{b}	11.77±0.49^{a}	44.03^{a}
	< 0.210	15.53±0.35^{a}	3.07±0.07^{a}	0.49±0.03^{b}	1.88±0.07^{ab}	32.05±0.62^{d}	22.20±0.29^{b}	9.85±0.41^{b}	46.98^{a}
THF	> 0.701	13.06±0.52^{c}	2.78±0.00^{c}	0.37±0.04^{c}	2.04±0.03^{a}	42.68±1.48^{a}	32.69±0.99^{a}	9.99±0.52^{b}	39.07^{b}
	0.701-0.417	11.15±0.25^{d}	2.89±0.00^{b}	0.46±0.03^{b}	1.86±0.07^{b}	43.26±0.59^{a}	32.08±0.71^{a}	11.18±0.30^{a}	40.38^{b}
	0.417-0.210	12.39±0.46^{d}	2.86±0.04^{b}	0.52±0.01^{a}	1.79±0.07^{b}	43.57±0.48^{a}	33.93±0.44^{a}	6.64±0.33^{c}	38.87^{b}
	< 0.210	14.02±0.22^{b}	2.23±0.03^{d}	0.34±0.01^{c}	1.86±0.01^{b}	40.30±0.48^{b}	34.22±0.49^{a}	6.08±0.22^{c}	41.25^{b}

TDF: Total dietary fiber; IDF: Insoluble dietary fiber; SDF: Soluble dietary fiber

Values expressed as: g/100 g of flour

(a-d)Values in the same column followed with same letter are not significantly different (p>0.05) according to Tukey's Multiple Range Test.

Table 2.- Effect of different particle sizes on physicochemical characteristics of 'Rojo Brillante' (RBF) and 'Triumph' (THF) flours.

Sample	Particle size (mm)	pH	Water activity	Color				
				L*	a*	b*	C*	h*
RBF	> 0.701	4.87±0.01^{c}	0.426±0.00^{ab}	35.09±1.28^{e}	13.61±0.49^{b}	37.94±1.5^{a}	37.09±1.44^{b}	64.00±1.21^{d}
	0.701-0.417	4.90±0.02^{c}	0.381±0.00^{d}	39.28±1.31^{d}	15.51±0.63^{a}	34.16±2.44^{bc}	37.53±2.39^{b}	65.52±1.29^{d}
	0.417-0.210	4.90±0.02^{c}	0.351±0.00^{e}	42.65±2.09^{c}	14.27±0.59^{b}	35.62±1.61^{b}	38.37±1.71^{b}	68.16±0.33^{c}
	< 0.210	4.89±0.05^{c}	0.384±0.00^{d}	49.55±2.35^{b}	12.59±0.35^{c}	33.37±1.09^{c}	35.6±1.13^{c}	69.33±0.27^{b}
THF	> 0.701	5.28±0.06^{b}	0.427±0.00^{ab}	41.34±0.92^{c}	14.04±0.69^{b}	33.66±1.39^{c}	36.47±1.49^{bc}	67.36±0.67^{c}
	0.701-0.417	5.27±0.04^{b}	0.418±0.00^{c}	41.54±1.62^{c}	14.16±0.44^{b}	37.87±1.02^{a}	40.43±1.00^{a}	69.49±0.68^{b}
	0.417-0.210	5.26±0.05^{b}	0.430±0.00^{a}	47.07±2.17^{b}	12.88±0.33^{c}	38.93±0.77^{a}	41.01±0.81^{a}	71.69±0.26^{a}
	< 0.210	5.42±0.00^{a}	0.438±0.00^{a}	54.79±2.21^{a}	11.01±0.32^{d}	33.29±1.05^{c}	35.06±1.09^{c}	71.70±0.18^{a}

(a-e)Values in the same column followed with same letter are not significantly different (p>0.05) according to Tukey's Multiple Range Test.

Table 3.- Effect of different particle sizes on techno-functional properties of 'Rojo Brillante' (RBF) and 'Triumph' (THF) flours.

	Particle size (mm)	WHC (g/g)	OHC (g/g)	SWC (mL/g)	EA (mL/mL)	ES (%)
RBF	> 0.701	8.04±0.09[d]	1.72±0.07[c]	11.52±1.12[a]	69.33±0.47[a]	92.51±0.77[bc]
	0.701-0.417	5.87±0.21[f]	1.67±0.03[c]	11.51±0.86[a]	74.00±4.97[a]	91.84±2.76[c]
	0.417-0.210	6.64±0.41[e]	1.88±0.12[b]	11.41±0.42[a]	69.00±1.41[a]	94.12±0.07[b]
	< 0.210	7.37±0.73[de]	2.15±0.08[a]	10.27±0.36[b]	69.00±0.82[a]	93.93±1.29[b]
THF	> 0.701	10.57±0.25[b]	1.92±0.03[b]	7.95±0.16[d]	60.33±0.47[b]	99.44±0.79[a]
	0.701-0.417	9.47±0.21[c]	2.07±0.09[a]	9.90±0.59[c]	59.00±2.16[b]	71.78±1.35[d]
	0.417-0.210	12.19±0.19[a]	2.18±0.07[a]	11.39±0.40[a]	57.33±1.70[b]	51.85±2.70[f]
	< 0.210	11.23±0.56[ab]	2.26±0.06[a]	11.58±0.33[a]	57.67±1.25[b]	64.08±3.76[e]

WHC: Water holding capacity; OHC: oil holding capacity; SWC: swelling capacity; EA: emulsifying activity; ES: emulsion stability.
[(a-f)]Values in the same column followed with same letter are not significantly different ($p>0.05$) according to Tukey's Multiple Range Test.

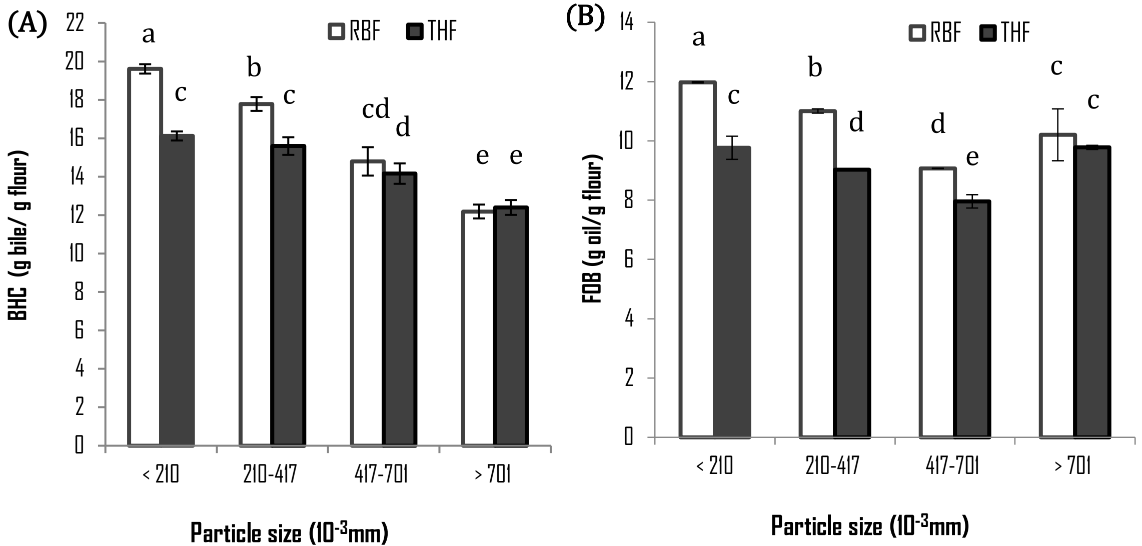

Figure 1.- Effect of different particle sizes on (A) Bile-holding capacity (BHC) and (B) Fat/oil binding (FOB) of 'Rojo Brillante' (RBF) and 'Triumph' (THF) flours.

Different letters denote statistical differences among means values according to Tukey's Multiple Range Test ($p<0.05$).

7.2.- PUBLICACIÓN 2

Título: Effect of particle size on phytochemical composition and antioxidant properties of two persimmon flours from *Diospyros kaki* Thunb. vars. 'Rojo Brillante' and 'Triumph' co-products

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Effect of particle size on phytochemical composition and antioxidant properties of two persimmon flours from *Diospyros kaki* Thunb. vars. 'Rojo Brillante' and 'Triumph' coproducts

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Abstract

BACKGROUND: This contribution studies the influence of the particle size of persimmon flours (from two cultivars, 'Rojo Brillante' and 'Triumph') on their primary (sugars and organic acids) and secondary (polyphenols, flavonoids and carotenoids) metabolite content, and also on their antioxidant activity, to assess whether these flours could find applications in the food industry as a potential functional ingredient.

RESULTS: The main sugars were fructose and glucose and the principal organic acid was malic acid. The highest content of total phenols, flavonoids and carotenoids was found in flour fractions from cv. 'Rojo Brillante'. The phenol profile determined by HPLC identified six phenolic compounds in both persimmon flours, the most abundant being gallic acid. The greatest ABTS and DPPH radical scavenging capacity and ferric reducing power were found in flour fractions from cv. 'Rojo Brillante'. Although the influence of particle size on all these properties was not always evident, in general, the higher bioactive compound content and antioxidant capacity were in the finest particles.

CONCLUSION: Coproducts from cvs. 'Rojo Brillante' and 'Triumph' persimmon juice extraction can be processed to obtain flours rich in sugars, organic acids and bioactive compounds, suggesting their possible use as a functional ingredient (with antioxidant properties) in different food products.

Keywords: persimmon, particle size, HPLC, antioxidant, phytochemicals.

Introduction

Persimmon (*Diospyros kaki* Thunb.) is a fruit that originates in Asia, where China, Japan and Korea remain the main producers worldwide.[1] Although persimmon cultivation in Europe is modest, crops are increasing in countries bordering the Mediterranean, primarily due to the climate, which is well suited to this fruit.[2] A prime example in this respect is Spain, where persimmon production has experienced a significant increase in the last decade.[3] The most important cv. in Spain, both commercially and in terms of production, is 'Rojo Brillante'. Furthermore, it is the only cultivar recognized by the Denomination of Origin Kaki Ribera del Xúquer, in the Valencia region of Spain. Among other Spanish regions, Andalusia is also important for the cultivation of "Sharon fruit", the marketing name for the Israeli-bred cultivar 'Triumph'.[4]

Persimmon cultivars can be classified into four astringency groups: pollination constant non-astringent or astringent ('Rojo Brillante', 'Triumph', 'Fuji') and pollination variant non-astringent or astringent ('Tipo', 'Hana Fuju', 'O'Gosho).[5] The astringent cultivars are subjected to a postharvest treatment to remove the astringency, thereby providing a firm and sweet fruit. If the fruit is allowed to ripen naturally, it becomes very soft, making it difficult to distribute and market. However, the type of postharvest treatment affects the final primary and secondary metabolite content of persimmon fruit. Indeed, some authors have found that astringent cultivars have more bioactive compounds than non-astringent cultivars.[2] Persimmon is an important source of sugars and micronutrients (vitamin C, and bioactive compounds such as carotenoids and polyphenols) and has been widely used in traditional medicine for different purposes.[6] Furthermore, studies in animal models have shown that the consumption of lyophilized persimmon or its coproducts decreases atherosclerotic lesions in the aorta, and has antihyperglycemic effects and a positive influence on lipid metabolism.[7,8]

Although the natural persimmon season is short, limited to autumn months and the start of winter, more specifically from mid-October to the beginning of January, industrialization means that persimmon products are available all year long in the form of juice, dried fruit or jam, for example. The suggestion is that the coproducts generated in these industries (peel, pulp or both) could be used for the development of new foods with improved nutritional properties.

Although several authors have studied primary and secondary metabolites in fresh persimmon fruit, dehydrated persimmon or peel,[9-12] the content of the same has not been studied in coproducts from the persimmon juice industry.

The role that particle size plays in extracting health-promoting compounds has been investigated in several fruits and cereals, including wheat bran, unripe banana flour and black chokeberry coproducts,[13-15] where it has been found that a reduction in particle size significantly affects the concentrations of antioxidant compounds. Factors such as the increase in surface area, the different distributions of particle sizes or the release of bioactive compounds from cells might

be responsible, at least in part, for the significant differences in the concentration of bioactive compounds and antioxidant activities between the various particle sizes. Nevertheless, little information in this respect is available in the scientific literature, although it would seem advisable to determine the best flour particle size for addition to different foods.[16]

Therefore, the aims of this work were to study the influence of the particle size of persimmon flours (from two cultivars, 'Rojo Brillante' and 'Triumph') on the content of primary (sugars and organic acids) and secondary (polyphenols, flavonoids and carotenoids) metabolites and on differences in their antioxidant activity, in order to evaluate whether such flours could have applications in the food industry as potential functional ingredients.

Materials and Methods

Plant material

Astringent persimmons (*Diospyros kaki* Thunb) of cvs. 'Rojo Brillante' and 'Triumph' were purchased from a local market (Orihuela, Alicante, Spain) and were selected on the basis of uniformity and size. The fruits, which were in the yellow-orange ripening stage and with 14-16º Brix, were randomly grouped into batches of 50 kg each. The samples were washed, cut and liquefied. The juice was discarded and the coproducts, composed of pulp and peel, were collected and dried at 45 ºC in a hot air drier for 24 h. Dehydrated samples were ground with a knife mill to obtain a flour. The two flours obtained from cvs. 'Rojo Brillante' and 'Triumph' were separated into different fractions based on the particle size using three sieves of different mesh sizes (0.701, 0.417 and 0.210 mm). As a result, the persimmon flours were divided into four particle size ranges: large (> 0.701 mm), large-intermediate or 0.701-0.417 (< 0.701 > 0.417 mm), small-intermediate or 0.417-0.210 (< 0.417 > 0.210 mm) and small (< 0.210 mm) (Figure 1). Samples were vacuum packed and stored in darkness until analysis.

Organic acid and sugar content

Extraction of organic acids and sugars

One gram of the samples was homogenized with 40 mL of ultrapure water in an Ultra-Turrax at 12000 rpm for 60 s. Then, the samples were centrifuged at 5000 g for 10 min at 4 ºC and the supernatants were filtered through a 0.45 µm Millipore filter (Millipore Corporation, Bedford, USA).

HPLC analysis

Organic acids and sugars were analyzed in a Hewlett-Packard HP-1100 instrument (Woldbronn, Germany) coupled to two detectors: a UV-vis Diode Array Detector G1315A (set at 210 nm) and a refractive index detector G-1362. Ten microliters of sample were injected into a cation exchange column (Supelcogel C-610H, 300×7.8 mm, Supelco, Bellefonte) with a pre-column (Supelguard- H, 50×4.6 mm, Supelco), using phosphoric acid (0.1%) as mobile phase and an operating flow rate of 0.5 mL/min. Samples were run at 30 ºC for a run time of 30 min.[17] Standards of organic acids (L-ascorbic, malic, tartaric, citric, oxalic, fumaric and succinic acids) and monosaccharides (glucose, fructose and sucrose) were obtained from Sigma (Poole, Dorset, UK). Peaks were identified by comparison with the retention times of the standards, and quantified by regression formulas obtained with the standards. The sugar content was expressed as g/100 g of sample while the organic acid content was expressed as mg/100 g of sample.

Total carotenoid content

Extracts were prepared according to the method described by Pfeifhofer[18] with some modification. Briefly, 1 g of the samples was extracted in darkness with 15 mL of ice-cold acetone and 0.01 % of butyl hydroxyl toluene (BHT) and homogenized for 60 s with an external ice bath in an Ultra-Turrax at 8000 rpm for 60 s. Then, the samples were centrifuged at 5000 g for 10 min at 4 ºC and the supernatants were collected in a flask. This process was carried out twice. The resulting mixture was evaporated in a rotary evaporator and the solids were resuspended in 5 mL of ice-cold acetone. The extracts were filtered through 0.45 µm Millipore filter (Millipore Corporation, Bedford, USA) and kept at -20 ^{o}C until analysis. The total carotenoid content (TCC) was determined spectrophotometrically using an HP 8451 spectrophotometer (Hewlett Packard, Cambridge, UK). Absorbance values were measured at 440 nm following the recommendations of Pfeifhofer[18]. The results were expressed as mg β-carotene equivalents (βCE) / g sample.

Total phenol and total flavonoid content

Sample preparation

Three grams of the different persimmon flour samples were homogenized with a mixture (10 mL) of methanol-water (80:20, v/v) in an Ultra-Turrax at 12000 rpm for 60 s. The samples

were then centrifuged at 5000 g for 10 min at 4 ºC and the supernatants were collected in flasks. The pellet was homogenized with a mixture (10 mL) of acetone-water (70:30, v/v) in an Ultra-Turrax at 12 000 rpm for 60 s. Then, the samples were centrifuged at 5000 g for 10 min at 4 ºC and the supernatants were mixed with the other supernatant. This mixture was evaporated in a rotary evaporator and the solids were resuspended in 5 mL of methanol. The extract was filtered through a 0.45 µm Millipore filter (Millipore Corporation, Bedford, USA) and kept at -20 ºC until analysis.

Total phenol content

To determine the total phenol content (TPC) of persimmon flour samples, Folin-Ciocalteu's reagent was used.[19] Absorbance values were measured on a spectrophotometer at 760 nm. The results were expressed as mg gallic acid equivalents (GAE) / g sample.

Total flavonoid content

For the total flavonoid content (TFC), the method described by Blasa and colleagues was used.[20] Absorbance values were measured on a spectrophotometer at 510 nm. The results were expressed in mg rutin equivalents (RE) / g of sample.

Polyphenolic profile

Sample preparation

The extraction method used for HPLC analysis differed from that used for TPC and TFC. Thus, 1 g of the different samples was homogenized with 10 mL of methanol in an Ultra-Turrax at 12 000 rpm for 60 s. The samples were then centrifuged at 5000 g for 10 min at 4 ºC and the supernatants were collected in flasks. The pellet was homogenized with 10 mL of acetone in an Ultra-Turrax at 12 000 rpm for 60 s. Then, the samples were centrifuged at 5000 g for 10 min at 4 ºC and the supernatants were mixed with the other supernatant. This mixture was evaporated in a rotary evaporator and the solids were resuspended in 5 mL of methanol due to the high sugar content present in the samples, which could interfere with the HPLC column. The samples were loaded onto a C18 Sep-Pak cartridge, previously conditioned with 5 mL of methanol, 5 mL of pure water, and then with 5 mL of 0.01 mol/L HCl. The cartridge was washed with 5 mL of pure water and then eluted with acidified methanol (0.1 g/L HCl). The collected fraction was maintained at -4 ºC before HPLC analysis.

HPLC conditions

Methanolic extracts of the different samples were injected into a Hewlett-Packard HPLC series 1200 instrument equipped with C18 column (Mediterranean Sea_{18}, 25 × 0.4 cm, 5 µm particle size) from Teknokroma, (Barcelona, Spain). Phenolic compounds were analyzed in standard and sample solutions, using an elution gradient of 1 mL/min. The mobile phases were

composed of formic acid in water (4.5:95.5, v/v) as solvent A and acetonitrile as solvent B. The chromatograms were recorded at 280, 320 and 360 nm. Polyphenolic compounds were identified by comparing the UV absorption spectra and retention times of each compound with those of pure standards injected in the same conditions.

Antioxidant capacity

To determine the antioxidant properties of persimmons flours, the samples obtained with the procedure described to analyze the TPC and TFC were used.

Determination of antioxidant activity using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method.

Samples were measured in terms of their radical scavenging ability, using the stable radical DPPH.[21] Absorbance values were measured on a spectrophotometer at 517 nm, and the results were expressed in mg Trolox equivalents (TE) / g of sample.

Ferric reducing antioxidant power (FRAP)

The FRAP of samples was determined using the potassium ferricyanide-ferric chloride method.[22] Absorbance values were measured on a spectrophotometer at 700 nm. The FRAP of a sample was estimated in mg Trolox equivalents (TE) / g of sample.

Ferrous ion-chelating ability assay (FIC)

The ferrous ion (Fe^{2+}) chelating activity of samples was determined as described by Carter.[23] Absorbance values were measured on a spectrophotometer at 562 nm and the results were expressed in µg EDTA equivalent/g of sample.

ABTS radical cation (ABTS•+) scavenging activity assay

The $ABTS^{\bullet+}$ scavenging activity assay of the samples was determined as described by Leite and colleagues.[24] Absorbance values were measured on a spectrophotometer at 734 nm, expressing the results as mg Trolox equivalent (TE)/g sample.

Statistical analysis

For each experiment, three independent samples were examined with three replications per sample. The data obtained for all the properties were analyzed by means of a two-way ANOVA test with two factors: cultivar and particle size. Tukey's *post hoc* test was applied for comparisons of means; Differences were considered significant at $p < 0.05$. Statistical analyses were carried out using the statistical package SPSS 19.0 (SPSS Inc., Chicago, IL.).

Results and Discussion

Organic acids and sugars content

In persimmon flour fractions from both cvs. 'Rojo Brillante' and 'Triumph', six organic acids were identified (malic, succinic, fumaric, citric, tartaric and oxalic acids) (Table 1). In the flour fractions from cv. 'Rojo Brillante' malic acid was the predominant acid ($p<0.05$), followed by succinic, citric, fumaric, tartaric and oxalic acid. In the flour fractions from cv. 'Triumph' malic acid was also the main acid ($p<0.05$), but was followed by fumaric, succinic, citric, tartaric and oxalic acid. Note that, with the exception of fumaric acid, which had a 6-7 times higher concentration in all 'Triumph' flour fractions than in 'Rojo Brillante', there were no differences ($p> 0.05$) between the cultivars as regard the organic acids detected,. Particle size only affected the content of malic, succinic and fumaric acids and, furthermore, their distribution seemed to depend on the cultivar and type of organic acid ($p<0.05$). Sójka and colleagues also found significant differences between particle size and organic acid content in dried chokeberry pomace fractions.[15]

Previous studies of the organic acid content in fresh persimmon showed slight variations from the values reported in this work. Thus, Novillo and colleagues[25] reported that the main organic acids in fresh persimmon cv. 'Rojo Brillante' were succinic, malic and citric acid, with values of 9.2, 8.7 and 5.7 µg/mg dry weight, respectively; while Veberic and colleagues[5] found that the predominant organic acids in fresh persimmon cv. 'Triumph' were malic, citric and fumaric acid, with values of 1044, 366 and 68.8 µg/mg fresh weight, respectively. The differences observed between these studies and the current work could be due to the heat treatment applied to the coproducts to obtain the flours. Senica and colleagues[9] reported than in dehydrated firm persimmon, the concentrations of citric, malic and tartaric acids decreased with heat treatment, although the fumaric acid content was less affected. Such differences may also be affected by the maturity stage, the "deastringency" treatment[25] and the type of sample (persimmon fruit vs. coproduct). Compared with other fruit coproducts, persimmon flours showed higher malic and citric acid concentrations than dried chokeberry pomace fractions (2.53-3.01 mg/g and 0.489-0.607 mg/g, respectively), but lower citric acid concentrations than fig coproducts obtained from peel and pulp of cv *Cuello dama* and *Colar* (4.38 and 7.32 mg/g sample, respectively).[26]

The sugars identified in the persimmon flour fractions from both cvs. 'Rojo Brillante' and 'Triumph' were fructose and glucose (Table 2). Both monosaccharides were present in similar proportions ($p> 0.05$) in all the flour fractions, as reflected by the glucose/fructose ratio, which ranged between 0.90-0.99. In this study, sucrose was not found in any sample. However, other authors reported sucrose in fresh persimmon from cv. 'Rojo Brillante'[25] and cv. 'Triumph'.[5] The absence of sucrose in these samples could be attributed to various factors, such as invertase activity (which hydrolyzes sucrose in glucose and fructose), extraction method,[27] the deastringency treatment[25] or type of sample (coproduct vs whole fruit). The results showed that the flour fractions of cv. 'Triumph' had similar levels of glucose and fructose ($p> 0.05$) for all

particle sizes, while the 'Rojo Brillante' fractions showed significant differences ($p<0.05$) in this respect between particle sizes. In this case, large and large-intermediate fractions showed the highest content of both monosaccharides, followed by small and small-intermediate fractions. Wook and colleagues[12] also reported significant differences in the sugar content of different sized particles in Daebong (cv. 'Hachiya') persimmon peel powders. It is important to note that both the large and large-intermediate fractions of cv. 'Rojo Brillante' had higher glucose and fructose contents than all the fractions from cv. 'Triumph'. The fructose and glucose contents in all the persimmon flour fractions were higher than those found in fig powder coproduct from peel and pulp cv. *Cuello dama* and *Colar*.[26]

Bioactive compounds: Total phenol, flavonoid and carotene contents

The total phenol, total flavonoid and total carotenoid contents in persimmon flour fractions obtained from cvs. 'Rojo Brillante' and 'Triumph' are shown in Table 2.

The TPC in all the persimmon flour fractions ranged between 1.00 and 1.75 mg GAE/g sample. For all the particle sizes, samples from cv. 'Rojo Brillante' showed a higher TPC ($p<0.05$) than samples from cv. 'Triumph'. The highest value ($p< 0.05$) was observed in the small fraction of cv. 'Rojo Brillante'. However, the particle size only affected ($p< 0.05$) TPC in samples from cv. 'Rojo Brillante'. The results obtained were similar to those reported by Akyildiz and colleagues[11] in dry persimmon from *Türkay* variety, but higher than the values reported by Park and colleagues[10] in dry persimmon from cv. 'Triumph'. In comparison with other fruit flours studied the persimmon flours in our study showed a lower TPC than pomegranate peel powder coproduct (54.84 mg GAE/g) but higher than that observed in unripe banana flour fractions (0.19-0.31 mg GAE/g). [13, 28]

With respect to flavonoid content, in all the persimmon flour fractions analyzed, the TFC ranged between 1.04 and 1.87 mg RE/g sample. As for TPC, the samples from cv. 'Rojo Brillante' showed higher ($p<0.05$) values than samples from cv. 'Triumph' for all particle sizes. In this case, the particle size only affected ($p< 0.05$) the TFC in samples from cv. 'Rojo Brillante'. The TFC values reported in this work are 10-18 fold lower than those reported for Daebong (cv. 'Hachiya') persimmon peel powders (9.24-21.46 mg RE/g) but similar to or higher than those found in Mexican pomace powder (1.06 mg RE/g).[12, 29]

The TCC in all the persimmon flour fractions ranged between 1.10 and 1.92 mg CE/g sample, and the flours from both cultivars exhibited a similar carotenoid pattern. Both cultivars showed the highest TCC for the small fraction ($p< 0.05$) and a similar TCC for the small and large fractions ($p> 0.05$). These TCC values in the persimmon flour fractions were lower than the values reported by Lee and colleagues[8] in dehydrated persimmon peel powder from an unknown cultivar (3.41 mg βCE/ g).

As mentioned above for the organic acid and sugar content, the differences in TPC, TFC and TCC between our samples and other persimmon samples reported could be due to the type and class of cultivar, type of sample (whole fresh fruit or dehydrated), the type of solvent used and extraction mode or the coproduct composition (peel, flesh or both).[5, 30]

As regard particle size, the distribution of TPC and TFC in the different persimmon fractions agrees with previous works. For example, Brewer and colleagues[14] also found the highest TPC and TFC values in fine particles (the smallest studied particle) in whole wheat bran extracts.

HPLC profile

The HPLC analysis of the persimmon flour fractions (Table 1) pointed to a total of six polyphenolic compounds, which were identified as phenolic acids (gallic acid), flavan-3-ols (catechin and epicathechin), flavonols (quercetin and kaempherol) and ellagitannins (ellagic acid). The most abundant phenolic compound ($p<0.05$) in all the analyzed persimmon flour fractions was gallic acid, with a higher content ($p<0.05$) in the flour fractions from cv. 'Rojo Brillante' than from 'Triumph'. Other authors also reported gallic acid as the main phenolic compound in fresh as well as in dried persimmon from different cultivars.[5, 9, 31] The amount of the other phenolic compounds also depended on the cultivar: in general, flour fractions from cv. 'Rojo Brillante" had higher amounts of epicatechin and catechin but lower amounts of quercetin, kaempherol than flour fractions from cv. 'Triumph'. Only the ellagic acid content seemed not to depend on the cultivar ($P>0.05$). Note that the greatest differences between cultivars were found for quercetin and kaempherol, the flour fractions from cv. 'Triumph' having a 2.6-2.7-fold higher content than the fractions from cv. 'Rojo Brillante'.

However, the effect of particle size was not significant for all the phenolic compounds identified. It was most evident for gallic acid, epicatechin and catechin, although, in general, the highest amount of these compounds was found in the finest particles for both persimmon cultivars. Other authors reported that the particle size did not affect the quercetin and kempherol contents in wine making coproducts, which agrees with our results.[32]

Antioxidant activity

Table 3 shows the results for the antioxidant properties of the persimmon flour fractions from cvs. 'Rojo Brillante' and 'Triumph'. To characterize the different mechanisms naturally involved in the antioxidant activity, four antioxidant assay methods were applied. According to the results, all the flour fractions from 'Rojo Brillante' showed higher antioxidant activities (DPPH, FRAP and ABTS methods) than the flour fractions from cv. 'Triumph' ($p< 0.05$). Furthermore, particle size only affected the antioxidant properties (DPPH and FRAP methods) in the flours from cv. 'Rojo Brillante', and, in this case, the lowest values of antioxidant capacity were observed in

the largest particles. The FIC test results were not affected by cultivar or particle size. Previous studies[12] found that neither the DPPH radical test nor the FRAP assay showed differences between the fractions of Daebong (cv. 'Hachiya') persimmon peel powder. Du and colleagues[33] showed higher antioxidant activity, as measured by DPPH scavenging activity and FRAP, in ultrafine and regular ground fractions of insoluble dietary fiber from qingke. The antioxidant capacity of persimmon flours was lower than that reported for other coproducts (e.g. Mexican apple pomace powder) using DPPH, FRAP and FIC (247.72 µM Trolox equivalent/100 g, 1616.59 µM Trolox equivalent/100 g and 36.53 µM EDTA equivalent/100 g, respectively).

As can be seen, persimmon flours from cv. 'Rojo Brillante' showed the highest TPC, TFC and TCC values (Table 2) and also the highest antioxidant capacity. Although the differences were not so pronounced between particle sizes, in general, the largest particle size showed the lowest TPC, TFC and TCC values and also the lowest antioxidant capacity.

Conclusions

Coproducts resulting from cv. 'Rojo Brillante' and 'Triumph' persimmon juice extraction can be processed to obtain flours rich in sugars, organic acids and bioactive compounds, pointing the way for their use as functional ingredients (with antioxidant properties) in different food products. Six phenolic compounds were identified in the flours analyzed, gallic acid being the most abundant. The antioxidant capacity of these flours is influenced by their total phenolic, total flavonoid and total carotenoid contents. Flours from cv. 'Rojo Brillante' have a higher gallic acid, total phenol, flavonoid and carotenoid contents, and antioxidant capacity than flours from cv. 'Triumph'. Although the influence of particle size in all these properties is not always evident, in general, the highest content of bioactive compounds and the highest antioxidant capacity are found in the finest particles. So, the finest particles from persimmon flours would be best to serve as functional ingredient in food processing, because lower amounts of flour would be needed to achieve the same antioxidant properties, while having a less pronounced impact on their sensory properties.

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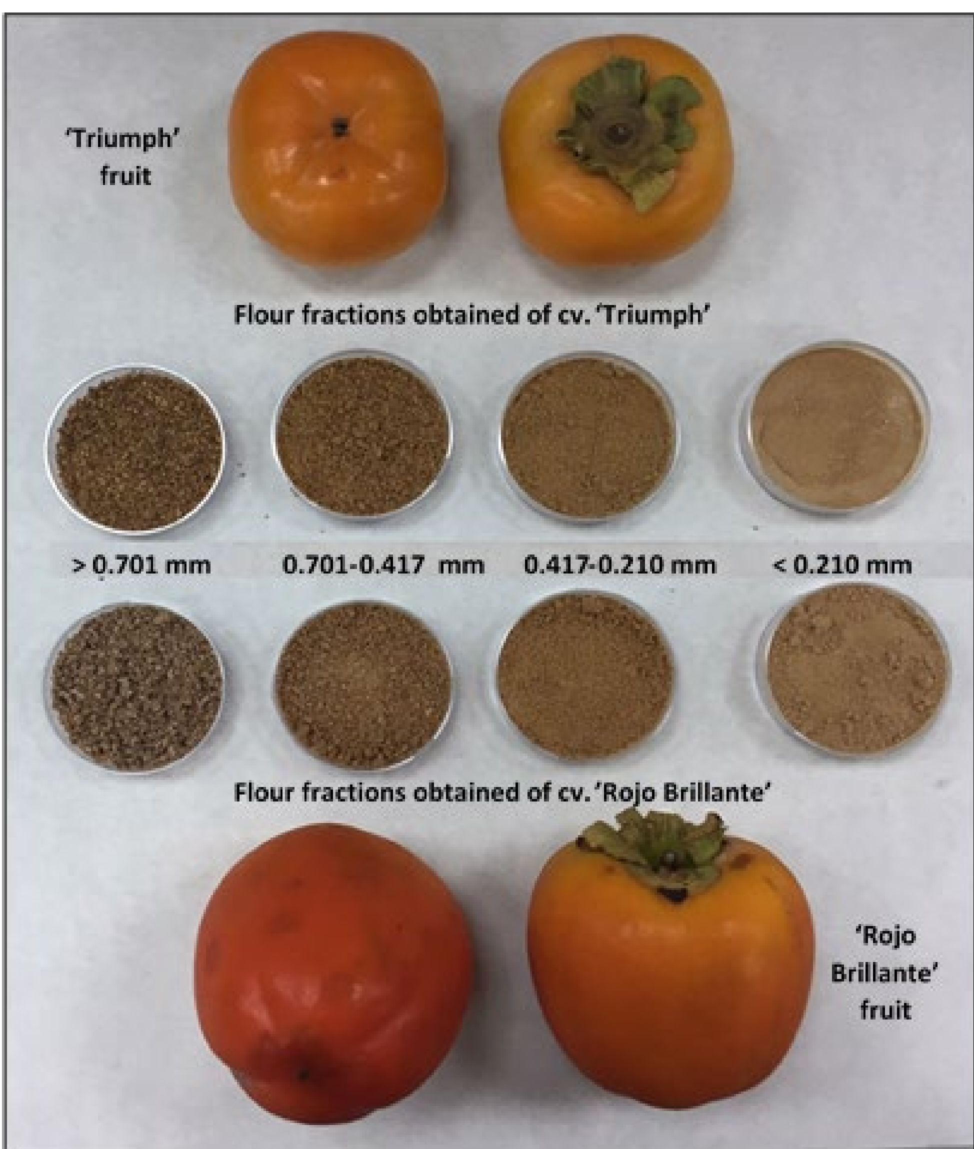

Figure 1.- 'Rojo Brillante' and 'Triumph' persimmon fruit and their corresponding flours with different particle sizes.

Table 1.- Organic acids, sugars content and polyphenolic profile of 'Rojo Brillante' and 'Triumph' persimmon flour fractions (with different particle sizes)

		'Rojo Brillante'				'Triumph'			
	Compound	**> 0.701 mm**	**0.701-0.417 mm**	**0.417-0.210 mm**	**< 0.210 mm**	**> 0.701 mm**	**0.701-0.417 mm**	**0.417-0.210 mm**	**< 0.210 mm**
Sugars (g/100 g)	Fructose	24.40±0.10^{a}	24.45±0.32^{a}	17.86±0.00^{d}	19.57±0.80^{c}	22.45±0.36^{b}	22.09±0.44^{b}	21.94±0.16^{b}	22.57±0.12^{b}
	Glucose	24.06±0.15^{a}	23.93±0.45^{a}	16.39±0.00^{d}	18.45±0.88^{c}	20.66±0.38^{b}	20.36±0.40^{b}	20.16±0.16^{b}	20.25±0.13^{b}
Organic acids (mg/g)	Malic acid	17.65±0.41^{a}	16.01±1.32^{a}	13.18±0.41^{b}	14.15±0.56^{b}	17.04±0.18^{a}	16.08±1.53^{a}	16.96±0.17^{a}	17.39±0.67^{a}
	Succinic acid	6.11±0.18^{a}	5.74±0.01^{a}	4.61±0.14^{b}	4.81±0.15^{b}	4.89±0.13^{b}	4.78±0.05^{b}	5.26±0.23^{ab}	5.48±0.14^{a}
	Fumaric acid	2.03±0.02^{d}	1.92±0.07^{d}	0.96±0.03^{d}	1.59±0.06^{d}	8.28±0.27^{b}	7.22±0.10^{c}	8.23±0.15^{bc}	10.13±0.09^{a}
	Citric acid	2.36±0.08^{a}	2.28±0.05^{a}	1.79±0.06^{a}	1.89±0.10^{a}	2.21±0.00^{a}	2.20±0.06^{a}	2.24±0.13^{a}	2.23±0.11^{a}
	Tartaric acid	0.36±0.02^{a}	0.37±0.03^{a}	0.35±0.00^{a}	0.24±0.01^{b}	0.32±0.03^{a}	0.34±0.01^{a}	0.38±0.00^{a}	0.54±0.03^{a}
	Oxalic acid	0.04±0.00^{a}	0.03±0.00^{a}	0.10±0.00^{a}	0.18±0.01^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
Phenolic acids (μg/g)	Gallic acid	2959±228.95^{b}	3234±99.34^{b}	3582±84.20^{b}	4236±114.12^{a}	1554±117.58^{c}	1718±39.87^{c}	1823±85.57^{c}	2956±258.39^{b}
Flavonoids (μg/g)	Quercetin	1.21±0.08^{b}	1.32±0.05^{b}	1.51±0.00^{b}	1.52±0.00^{b}	3.55±0.28^{a}	3.75±0.22^{a}	3.38±0.27^{a}	3.61±0.08^{a}
	Kaempherol	0.54±0.04^{b}	0.55±0.02^{b}	0.66±0.00^{b}	0.71±0.01^{b}	1.59±0.13^{a}	1.68±0.08^{a}	1.65±0.00^{a}	1.60±0.05^{a}
	Epicatechin	4.08±0.27^{b}	ND	ND	6.81±0.69^{a}	3.23±0.00^{b}	2.83±0.00^{b}	3.18±0.03^{b}	4.35±0.00^{b}
	Catechin	2.73±0.02^{a}	1.89±0.01^{b}	1.21±0.01^{e}	1.50±0.03^{d}	0.45±0.00^{f}	0.34±0.00^{g}	ND	1.62±0.05^{c}
Ellagictannins (μg/g)	Ellagic acid	2.88±0.25^{ab}	2.41±0.11^{bc}	2.38±0.21^{b}	2.98±0.27^{ab}	3.21±0.49^{a}	3.27±0.24^{ac}	2.46±0.15^{b}	3.38±0.25^{a}

Values in the same row followed with same lower case letter (a-d) are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.

Table 2.- Total phenolic content (TPC), total flavonoid content (TFC) and total carotenoid content (TCC) in 'Rojo Brillante' and 'Triumph' persimmon flour fractions (with different particle sizes)

Sample	Particle size (mm)	TPC (mg GAE/g)	TFC (mg RE/g)	TCC (mg CE/g)
'Rojo Brillante'	> 0.701	1.39 ± 0.15^{b}	1.61 ± 0.12^{a}	1.60 ± 0.02^{c}
	0.701-0.417	1.20 ± 0.02^{bc}	1.85 ± 0.07^{a}	1.76 ± 0.03^{b}
	0.417-0.210	1.06 ± 0.02^{c}	1.28 ± 0.10^{b}	1.59 ± 0.04^{c}
	< 0.210	1.75 ± 0.13^{a}	1.87 ± 0.07^{a}	1.85 ± 0.02^{ab}
'Triumph'	> 0.701	1.00 ± 0.09^{c}	1.21 ± 0.08^{b}	1.56 ± 0.01^{c}
	0.701-0.417	1.00 ± 0.05^{c}	1.06 ± 0.03^{b}	1.55 ± 0.00^{c}
	0.417-0.210	1.00 ± 0.04^{c}	1.04 ± 0.06^{b}	1.10 ± 0.07^{d}
	< 0.210	1.06 ± 0.06^{c}	1.19 ± 0.10^{b}	1.92 ± 0.00^{a}

Values in the same column followed with the same letter (a-d) are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test. TPC: mg gallic acid equivalent/g sample; TFC: mg Rutin equivalent/g sample; TCC: mg β-carotene equivalent /g sample.

Table 3.- Antioxidant capacity (measured by DPPH, FIC, FRAP and ABTS methods) in 'Rojo Brillante' and 'Triumph' persimmon flour fractions (with different particle sizes)

Sample	Particle size (mm)	DPPH	FIC	FRAP	ABTS
'Rojo Brillante'	> 0.701	0.79 ± 0.02^{ab}	0.08 ± 0.00^{a}	2.68 ± 0.19^{b}	1.92 ± 0.00^{a}
	0.701-0.417	0.87 ± 0.01^{a}	0.08 ± 0.00^{a}	3.37 ± 0.21^{a}	1.87 ± 0.28^{a}
	0.417-0.210	0.83 ± 0.02^{a}	0.08 ± 0.00^{a}	3.76 ± 0.29^{a}	1.72 ± 0.05^{a}
	< 0.210	0.84 ± 0.01^{a}	0.08 ± 0.00^{a}	3.96 ± 0.24^{a}	2.12 ± 0.16^{a}
'Triumph'	> 0.701	0.73 ± 0.02^{b}	0.08 ± 0.00^{a}	2.23 ± 0.14^{b}	1.31 ± 0.10^{b}
	0.701-0.417	0.73 ± 0.04^{b}	0.08 ± 0.00^{a}	2.18 ± 0.14^{b}	1.16 ± 0.02^{b}
	0.417-0.210	0.70 ± 0.03^{b}	0.08 ± 0.00^{a}	2.34 ± 0.09^{b}	1.16 ± 0.12^{b}
	< 0.210	0.72 ± 0.01^{b}	0.08 ± 0.00^{a}	2.74 ± 0.17^{b}	1.20 ± 0.02^{b}

Values in the same column followed with the same letter (a-d) are not significantly different ($p > 0.05$) according to Tukey's Multiple Range

DPPH: µg Trolox equivalent/g sample; FIC: µg EDTA equivalent/g sample; FRAP: mg Trolox equivalent/g sample; ABTS: mg Trolox equivalent/g sample.

7.3.- PUBLICACIÓN 3

Título: *In vitro* digestion models suitable for foods: Opportunities for new fields of application and challenges

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In vitro digestion models suitable for foods: opportunities for new fields of application and challenges

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Abstract

In vitro digestion assays simulate the physiological conditions of digestion *in vivo* and are useful tools for studying and understanding changes, interactions, as well as the bioaccessibility of nutrients, drugs and non-nutritive compounds. The technique is widely used in fields such as nutrition, pharmacology and food chemistry. Over the last 40 years, more than 2,500 research articles have been published using *in vitro* digestion assays (85% of which have been published in the last two decades) to elucidate multiple aspects such as protein digestibility, nutrient interactions or the viability of encapsulated microorganisms. The most recent trend in the use of this technique involves the determination of the antioxidant activity of bioactive compounds after digestion. However, the inability to reproduce certain *in vivo* digestion events, as well as the multiple models of *in vitro* digestion, point to a need to optimize and validate the method with *in vivo* assays to determine its limitations and uses. The purpose of this paper is to provide an overview of the current state of the art of *in vitro* digestion models through an analysis of how they have evolved in terms of the development of digestion models (parameters, protocols, guidance) and taking into consideration the boom in new fields of application.

Keywords: *In vitro* digestion assay; bioaccessibility; bioactive compounds; food matrix; nano-delivery systems

1.- Introduction

Food digestion is a complex process in which many factors are involved and that has actually aroused the interest of the food industry because of there is a growing relationship between food and health and therefore the reduction of development certain chronic diseases (Bornhorst, Gouseti, Wickham, & Bakalis, 2016). During human digestion, ingested foods are broken down into nutrients which are used by the body for energy, growth and cell repair. Food digestion implies two main processes that occur simultaneously: (*i*) mechanical transformation, whereby larger pieces of food get broken down into smaller pieces, starts in the mouth and continues into the stomach; and (*ii*) enzymatic transformation, whereby several different enzymes break down macromolecules into smaller molecules that can be absorbed into the bloodstream, starts in the mouth and continues into the intestines (Guerra, Etienne-Mesmin, Livrelli, Denis, Blanquet-Diot, & Alric, 2012; Alminger et al., 2014). Several organs, hormones and nervous stimuli are involved in the digestion process. The liver and the pancreas are also important players in the digestive system due to their function of secreting hydrolytic enzymes and biliary salts (Minekus et al., 2014).

Knowledge of the physicochemical changes that occur in foods during the digestion process and the various factors influencing nutrient bioaccessibility (the amount of a compound that is released from the matrix and is solubilized into the water phase (chyme) become available for absorption in the systematic circulation through the gut wall), bioavailability (the total amount of a compound that is released and absorbed, to reach the bloodstream, where it is delivered to the different body tissues) and digestibility (it applies specifically to the fraction of food components that is transformed into potentially accessible matter (present in the complete digesta, soluble and non-soluble fractions) through all physical and chemical processes that take place in the lumen) (Hedren, Diaz, & Svanberg, 2002; Fernández-García, Carvajal-Lérida, Jarén-Galán, Garrido-Fernández, Pérez-Gálvez, & Hornero-Méndez, 2012), would be helpful for designing functional foods since recommended daily nutrient ingestion and the processing conditions that would maximize the health benefits of bioactive compounds (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005; Sengul, Surek, & Nilufer-Erdil, 2014) could be established. It is clear that the digestive system is central to numerous questions raised not only by researchers, but also by commercial companies in various fields such as nutrition, toxicology, pharmacology, and microbiology (Dean and Ma 2007; McAllister 2010).

Testing the efficacy of newly developed foods or delivery systems depends on the availability of digestion models that accurately simulate the complex physicochemical and physiological events that occur in the human gastrointestinal tract (Hur, Lim, Decker, & McClements, 2011). *In vivo* feeding methods, using as models animals or humans, generally offer the most precise results and as mentioned Marcano, Hernando and Fiszman (2015) are still

considered the "gold standard" for determined diet-related questions, but unluckily, analyse the complex multistage process that occurs during the human or animal digestion is technically difficult, costly, and limited by ethical issues when potentially harmful substances are involved (Augustin et al., 2014; Minekus et al., 2014). Consequently, there is a real need for use *in vitro* models that closely mimic the physiological processes occurring during human digestion (Minekus et al., 2014), taking into consideration several factors such as the occurrence and concentration of digestive enzymes, the pH values in gastric and intestinal phases, digestion time and salt concentrations, among other factors (Marcano et al., 2015). Such models should be flexible, accurate, and reproducible. As of now, *in vitro* digestion models provide a useful alternative to animal and human models by rapidly screening food ingredients (Hur, Lee, Kim, Chun, & Lee, 2013).

As, mentioned Coles, Moughab and Darragh (2005) the perfect *in vitro* digestion technique (i) should provide precise results in a short time and (ii) might consequently help as a tool for quickly analysis of foods or food models with different compositions and structures. However, at this moment, any *in vitro* method is inevitably going to fail to match the precision that could be obtained by actually studying a food *in vivo*, basically by the inherent complexity of the digestion process (Fuller 1991; Coles et al., 2005; Hur et al., 2011).

Few years ago, food and animal scientists across the world have used various *in vitro* digestion models to analyse the structural and chemical changes that happen in several food matrices when are submitted to simulated gastrointestinal conditions, enabling the explosion in the number of scientific works published on digestion studies in recent years.

The aims of this paper is to provide an overview of the current status of *in vitro* digestion models through an analysis of the evolution of these methods as regards the development of digestion models (parameters, protocols, guidance) and to study the boom in new fields of application.

2.- Summary of survey

For the survey we have used Scopus® as a data base for the searches because it is the largest searchable citation and abstract source for searching the literature and because it is continually expanded and updated (Chadegani et al., 2013). The following items were introduced in the Scopus web page to refine the search: *Years*, from "all years" to "2016"; *Key words*, "*in vitro* digestion" and "foods"; *Type of document*, "paper" or "review"; The following "subject areas" were excluded: nursing, veterinary, environment sciences, physic and astronomy, materials, neuroscience, computer science, energy, business, psychology, earth and planet and undefined; The following "exact journal titles" were also excluded: Animal, Journal of Animal Science, Poultry Science, British Poultry Science, Journal of Allergy and Clinical Immunology, and Clinical and

Experimental Allergy. With all these restrictions, a total of 2187 document results were obtained. Figure 1 shows a diagram explaining the search criteria and process. Within this final result, different words were independently added to refine the search, depending on the information required.

3.- Interest and evolution of *in vitro* digestion models over time.

A simple analysis of the evolution of the number of publications (Figure 2) is sufficient to appreciate the current interest of the scientific community in the *in vitro* digestion of foods. While the first published paper in this area dates from 1954 (DeBaun and Connors, 1954), which was followed by a trickle of papers every year, the most important efforts to simulated the human stomach and small intestine have been made in the past two decades, a period in which more than 85% of the papers published to date have appeared. In the four years preceding this review, more than 150 papers per year have been published related to *in vitro* digestion of foods, peaking in 2015 when 218 papers were published.

The survey also showed that although *in vitro* digestion models have been applied to all type of foods, the most common foods tested were: vegetables (26%), dairy foods (23%), bakery foods (17%), meat products (13%), marine foods (12%) and egg foods (7%).

3.1 Static/dynamic in vitro digestion models

A huge range of gastrointestinal models have been designed to simulate the food digestion process, ranging from single static systems to multi-compartmental and dynamic systems. In addition, *in vitro* digestion models differed from one another in various parameters. One of these is the number and type of step included in the digestion sequence; depending on the study purpose, simulated digestion models can include the oral, gastric or/and small intestinal phases, and in some cases, large intestinal fermentation (Sek, Porter, & Charman, 2001; Polovic, Pjanovic, Burazer, Velickovic, Jankow, & Velickovic, 2008). Another important variation between models is the chemical composition of the digestive solutions used in each phase - the type and enzyme concentrations, the salts and buffers used, the biological polymers, the surface-active components, and so on (Boise and Eggum, 1991; Kitabatake and Kinewaka, 1998; Chattertona, Rasmussen, Heegaard, Sorensen, & Petersen 2004; Porter et al., 2004; Almaas et al., 2006; Hur, Decker, & McClements, 2009; Hur et al., 2011). Finally, the mechanical stresses as well as the fluid flows used in each phase in the digestion sequence flow geometries and profiles, magnitude and direction of applied stresses, etc. are also an important variation factor (Brandon, Oomen, Rompelberg, Versantwoort, Van Engelen, & Sips, 2006; McClements, Decker, & Park, 2009; Hur et al., 2011). Different reviews have been published addressing these variation factors (Guerra et al., 2012; Lefebvre et al., 2015; Bornhorst, Gouseti, Wickham, & Bakalis 2016). This only gives a brief idea about the sophistication that can be achieved with these systems, which, besides the varying

conditions that can be applied, help understand the difficulties involved in comparing compare results between studies.

A wide number of these works have been accomplished using static models (89%), in which gastric and small intestinal digestion is imitated in three successive phases (oral, gastric and small intestinal). In each phase, the food product is incubated for a specific time and at a specific temperature with simulated artificial saliva and gastric and small intestinal digestive fluids, respectively whilst the pH is generally maintained at a fixed value by using a buffer. While this may seem a simple method, the lack of consensus concerning the physiological conditions applied has led to different models and hence results which cannot be compared across research teams. To minimize this problem, the COST INFOGEST network proposes a general standardized and practical static digestion method based on relevant conditions that can be applied for various ends (Minekus et al., 2014). The objective of this consortium was to harmonize *in vitro* static systems that simulate digestive processes by defining key parameters and conditions. The scientific community has shown great interest in this study, as can be seen from the numerous citations (345, by Scopus®) that it has received since 2014 (year of publication). This harmonized static *in vitro* digestion method for foods should contribute to the production of more comparable data in the future. During the application of this standardized method, several authors have reported some limitations, which much be taken into account. Rodrigues et al. (2016) reported that this general method needs to be adapted in the case of studies applied to lipophilic compounds (carotenoids, plant sterols, among others). They concluded that two steps must be included (micelle separation by centrifugation and carotenoid exhaustive extraction from the micelles with diethyl ether) in the case of analysis on carotenoids from fruits. In spite of all these limitations, a recent review about the correlation between *in vivo* and *in vitro* data on food digestion concludes that although, in vitro static models are oversimplistic and do not reproduce all the dynamic aspects of the GIT, they are increasingly useful in predicting *in vivo* digestion in some cases (Bohn et al., 2017).

The *in vitro* gastrointestinal static models have numerous advantages, the principal purpose to imitate the biochemical processes that happen in the gastrointestinal tract and normally use a single set of initial conditions (pH, concentration of enzymes, bile salts, etc.) for each part of the gastrointestinal tract. Nevertheless, this simplistic method is frequently not an accurate reproduction of the more complex *in vivo* conditions, where the biochemical environment is continually changing and physical parameters such as shear and grinding forces can have a large impact on the breakdown of larger food particles and the release of nutrients (Golding and Wooster 2010). The geometry (vertical alignment, horizontal alignment or beaker) (Campbell, Arcand, & Mainville, 2011; Tompkins, Mainville, & Arcand, 2011), the biochemistry (the different digestive secretions are added to the compartments of the model over time)

(Marciani et al., 2001b) and the physical forces (simulated using Teflon rollers, flexible discs or water jackets) are the three most important factors that have been differently addressed in the design of these dynamic digestion models (Kong and Singh, 2010; Vardakou, Mercuri, Barker, Craig, Faulks, & Wickham, 2011). Some of the more advanced dynamic digestion models have a geometry designed to represent the fundus and antrum of the stomach, and/or the duodenum (Thuenemann, 2015). These designs allow for the simulation of the physical forces exerted on the digesta during transit through the gastro-intestinal tract, which in turn allows simulation of the inhomogeneous nature of the digesta and localized biochemical environments, as *in vivo* (Marciani et al., 2001a; Marciani et al., 2001b; Kong and Singh, 2010; Tompkins et al., 2011; Vardakou and others 2011). Table 1 shows a general comparison between static and dynamic digestion models as a function of the type of study, type of food, major applications, main objectives and advantages and disadvantages.

3.2 Evolution and new fields of application

Of note, too, is how the specific aim of this technique has changed with time, taking into account that it can be applied to several scientific areas such as, nutrition, food chemistry, pharmacology, microbiology and toxicology. The greatest numbers of studies have been into the behavior of macronutrients (mainly proteins) and drugs during digestion (Figure 3). However, in the last decade (2006-2016), new fields of applications have appeared, among which the three most relevant are: (i) the effect of the digestion process on the bioaccessibility of bioactive compounds and on their antioxidant activity (of a total of 562 documents found, 92% were published in last decade), although in most cases the objective was (ii) the specific effect of the food matrix (mainly dietary fiber in vegetable foods) on these properties (of a total of 692 documents found, 70% were published in the last decade); and (iii) the effect of digestion on coating integrity (mainly focused on nanodelivery systems) of bioactive compounds (a total of 481 documents found, 85% were published in last decade). All of these are examples of new applications and the importance given to them in the last decade, because most did not exist in in the 1980s and very few existed in the 1990s (Figure 3).

3.2.1 Effect of in vitro gastrointestinal digestion process on bioaccessibility and antioxidant properties of bioactive compounds

During recent years, several bioactive compounds (vitamins such as A, C, D and E, polyphenolic compounds, carotenoids and dietary fibre) have been studied for their potential health benefits in the development of functional foods, nutraceuticals, pharmaceuticals and other applications (Table 2) (Sotomayor-Gerding et al., 2016). Nevertheless, it should be borne in mind that any healthy effects of above mentioned compounds are determined by their bioavailability due to their chemical, thermal and shelf stability in the face of various processing conditions as

mentioned Sotomayor-Gerding et al., (2016) and Cilla, Bosch, Barberá, & Alegría (2018). Furthermore, their lipophilic nature and insolubility present challenges for their delivery and absorption (Alminger et al., 2014). For these reasons, when the potential functionality of several bioactive compounds are analysed, their bioavailability in food matrix is more significant than the amount of that bioactive compound.

It is well known that fruits and vegetables are an important source of bioactive phytochemicals (mainly polyphenolic compounds) which could exert numerous beneficial effects *in vivo*, mainly related to their high antioxidant potential donating a hydrogen atom or an electron to other compounds, scavenging free radicals and quenching singlet oxygen as reported Oliveira et al., (2009) and Chen, Chen, Zhao, Luo, Li and Gao (2014). This interest in the antioxidant properties of phenolic compounds has led to a high number of papers being published on their characterization (quantity and type of polyphenolic compounds) in all types of foods, and on the effect of processing on their antioxidant properties (Karakaya and Yilmaz, 2007; Colle, Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010; Rosa, Dufour, Lullien-Pellerin, & Micard, 2013). Moreover, the potential availability of antioxidants compounds after digestion is important, and several works have indicated that the bioavailability of individual compounds with antioxidant activity is poor (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011). Because of this, the impact of *in vitro* gastrointestinal digestion on the stability of polyphenolic compounds, and hence on their antioxidant properties, has been one of the more widely examined topics during last decade. Such is the case with a wide variety of fruits including citrus (Chen et al., 2014; Rodrigo, Cilla, Barberá, & Zacarías, 2015; De Ancos, Cilla, Barberá, Sánchez-Moreno, & Cano, 2017), different types of berries (Bermúdez-Soto, Tomás-Barberán, & García-Conesa 2007; Fazzari, Fukumoto, Mazza, Livrea, Tesoriere, & Di Marco, 2008; Liang et al., 2012; Correa-Betanzo, Allen-Vercoe, McDonald, Schroeter, Corredig, & Paliyath, 2014; Huang, Sun, Lou, Li, & Ye, 2014; Lucas-González, Navarro-Coves, Pérez-Álvarez, Fernández-López, Muñoz, & Viuda-Martos 2016), tomato (Svelander, Tibäck, Ahrnè, Langton, Svanberg, & Alminger, 2010; Talens, Mora, Bramley, & Fraser, 2016), grape (Tagliazucchi, Verzelloni, Bertolini & Conte, 2010; Chen et al., 2014), apple (Bouayed, Hoffmann, & Bohn, 2011; Bouayed, Deußer, Hoffmann, & Bohn, 2012) and figs (Kamiloglu and Capanoglu, 2013). But its impact has also been tested in different vegetables (Pugliese et al., 2014; Soriano-Sancho, Pavan & Pastore, 2015), grains and cereals (Gong, Jin, Wu, & Zhang, 2013; Chitindingu, Benhura, & Muchuweti, 2015; Podio et al., 2015), fruit juices (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán 2001; Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002; Cilla, González-Sarrías, Tomás-Barberán, Espín, & Barberá, 2009; Cilla, Perales, Lagarda, Barberá, Clemente, & Farré, 2011; Rodríguez-Roque, Rojas-Grau, Elez-Martínez, & Martín-Belloso, 2013; Aschoff, Kaufmann, Kalkan, Neidhart, Carle, & Schwiggert, 2015; Fawole, Opara, & Chen, 2015), vegetable juices (Wootton-Beard, Moran, & Ryan, 2011) and other types of processed

foods (Dinnella, Minichino, D'Andrea, & Monteleone 2007; Jiwan, Duane, O'Sullivan, O'Brien, & Aherne, 2010; Oliveira and Pintado 2015; Kamiloglu et al., 2015; Dall'Asta et al., 2016; Colantuono, Ferracane, & Vitaglione 2016; Vaghini, Cilla, Garcia-Llatas, & Lagarda 2016). Henning et al., (2014) studied the variability in the antioxidant activity of different dietary supplements commonly used as sources of antioxidant polyphenols (pomegranate, milk thistle, green tea, grape seed, goji and açai). In most of these studies, the stability of phenolic compounds was assessed by determining the total phenolic content (e.g. using the Folin-Ciocalteau method), which does not yield information on the recovery of specific phenolic classes or molecules, and by determining the antioxidant capacity (by methods such as DPPH, FRAP, ABTS or FIC). In this case, controversial results have been reported: in some cases, the total phenol content recovered and hence the antioxidant activities, after the gastric and intestinal phase of *in vitro* digestion was not reduced (compared with the gastric step) (Fazzari et al., 2008; Tagliazucchi et al., 2010; Tagliazucchi, Verzelloni & Conte 2012), but in other cases it was (Bermúdez-Soto et al., 2007). Also in some cases a high and positive correlation between total phenolic content and antioxidant activity was reported, but in some cases no such correlation was found. In conclusion, it seems that digestion may alter antioxidant properties of foods, depending partly on variations in the polyphenol content. Further analyses of specific phenolic compounds, especially their possible degradation products, should be carried out to clarify these conflicting results.

In an interesting work, Chen et al., (2014) analysed 33 fruits, evaluating their total phenolic content and antioxidant capacity before and after *in vitro* digestion. They also reported great variations among fruits; following the gastric phase of the *in vitro* digestion model, there was a significant increase in the total phenolic content of 8 fruits. After the duodenal phase of digestion, the total phenolic content of 25 fruits had increased compared with their initial total values, while the total phenolic content of 8 fruits had decreased. They also found that after the gastric phase of digestion, the DPPH, ABTS and FRAP values of some fruits had significantly increased (by up to 10.74 fold), but others decreased compared with their initial values. Compared with the values observed after the gastric phase of digestion, the DPPH, ABTS and FRAP values of some fruits were significantly lower after the duodenal phase of digestion, but others were higher. As can be seen, there is no overall pattern to the behaviour of these fruits during *in vitro* digestion. Previous studies found that a number of polyphenols increased after the gastric phase of the *in vitro* digestion process since polyphenols are highly sensitive to alkaline conditions. After the pancreatic digestion phase, the antioxidants are degraded by the alkaline pH, leading to an overall loss in the antioxidant capacity after *in vitro* digestion (Bermúdez-Soto et al., 2007). It is possible that when these compounds are exposed to such conditions, a proportion of the polyphenol compounds are transformed into different structural forms with different chemical properties, and different degrees of bioaccessibility, bioavailability and biological

activity, which are undetectable by the individual HPLC and HPLC–MS polyphenol analyses. The presence of polyphenol derivatives has been described in some studies (Aura et al., 2005; Fleschhut, Kratzer, Rechkemmer, & Kulling, 2005).

In the study by Henning et al., (2014) into the effect of *in vitro* digestion on the antioxidant capacity in some commercially available polyphenol-rich antioxidant dietary supplements (including extracts from pomegranate, green tea, grape seed, resveratrol, milk thistle, and acai, and goji berry), differential results were also reported. In some samples, the antioxidant activity after *in vitro* digestion remained unchanged, but in other samples it was increased by 50%, compared with non-digested controls. Such modifications were attributed to the hydrolysis of some of these compounds during digestion and the formation of other metabolites with higher or lower antioxidant activity (Janisch, Ölschläger, Treutter, & Elstner, 2006; Bialonska, Kasimsetty, Khan, & Ferreira, 2009).

In a study to assess the polyphenolic profile stability and changes in the antioxidant potential of maqui berry during *in vitro* digestion, Lucas-González et al., (2016) demonstrated that polyphenolic compounds present in maqui are released, mainly in the early phases of gastrointestinal digestion, where they might exert bioactivity as antioxidant compounds after their absorption in gastric digestion. However, their stability, especially that of anthocyanin compounds, is profoundly affected in the last phase of digestion, probably modifying their physico-chemical properties which are reflected in their antioxidant properties and bioaccessibility.

In vitro digestion studies have been applied not only in fruits and vegetables but also their respective processed co-products (Table 2). Although the co-products resulting from plant food processing represent a major disposal problem for the industry concerned, they also represent a promising source of bioactive compounds. In this case, the bioaccessibility of some of these bioactive compounds found in fruit-processing (grape, mango, pomegranate, apple, etc) (Wang, Williams, Ferruzzi, & D'Arcy, 2013; Blancas-Benitez, Mercado-Mercado, Quiros-Sauceda, Montalvo-Gonzalez, Gonzalez-Aguilar, & Sayago-Ayerdi, 2015; Gullón, Pintado, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2015a; Gullón, Pintado, Barber, Fernández-López., Pérez-Álvarez, & Viuda-Martos, 2015b; Mosele, Macia, Romero, Motilva, & Rubio, 2015; Colantuono et al., 2016) and vegetable-processing co-products (cauliflower, black carrot, etc.) (Gonzales et al., 2015; Kamiloglu et al. 2016) is also being investigated. For example, Gullón et al., (2015a) concluded that although the digestion process of pomegranate peel flour reduces the polyphenolic concentration and the antioxidant properties, this co-product could be used in the food industry as potential ingredient to develop functional foods that promote health benefits. Kamiloglu et al. (2016) reported a significant decrease (23-83%) in the total phenolic content, total monomeric anthocyanin content and total antioxidant capacity in black carrot, peel and pomace as a result of

in vitro gastrointestinal digestion. Nevertheless, the amount of pomace anthocyanins released at all stages of *in vitro* gastrointestinal digestion was higher than that of black carrot anthocyanins, suggesting that pomace may be a better source of bioaccessible anthocyanins.

It is important to highlight that although fruits, in general, are rich source of polyphenolic compounds, the quantity of compounds that are available for absorption under the environments of the small intestine, is possibly quite small. As mentioned Kamiloglu and Capanoglu, (2013) this does not mean that the ingested insoluble compounds have no role in health protection, as these compounds, if they are not absorbed in the small intestine, can reach the large intestine, where they can be transformed and/or degraded by the colon microflora. Recent researches have focused on studying the metabolites obtained, which might have a beneficial effect on the large intestine cells and/or bacteria and also be absorbed to exert a biological action (Gullón et al., 2015b). In this way, Cilla, Alegría, Barberá , & Lagarda (2013) reported that the applications of combined systems, that include the fractions obtained from simulated human digestion (gastrointestinal and/or colonic fermentation) and the incorporation of cell culture-based models, allow to evaluate bioaccessibility and to conduct bioactivity studies, in order to gain better insight from a nutritional/functional point of view of the chemopreventive action derived from foods and bioactive compounds in cell models of disease.

Bohn et al. (2015) published a review about gaps of knowledge on the bioaccessibility of bioactive compounds in which the effect of food matrix and food processing was also commented; but this review was mainly focused on factors effecting micelle formation, co-constituents influencing influx and efflux via transporter systems or altering phase I/II metabolism, as these have often been overlooked or excluded from consideration.

3.2.2. Effect of food matrix on bioaccessibility of bioactive compounds during the in vitro digestion process

The bioaccessibility of bioactive compounds from solid matrices must also be taken into account since only the compounds released from the food matrix and/or absorbed in the small intestine are potentially bioavailable and able to exert their beneficial effects (Tagliazucchi et al., 2010; Tagliazucchi et al., 2012). The main food components are proteins, carbohydrates, fiber and fats, and their interactions with phytochemicals must also been considered. It is clear that the stability of bioactive compounds during gastrointestinal digestion depends on their structure and the food matrix. For this reason, different studies to investigate the effects of the food matrix and food components on the bioaccessibility of bioactive compounds from different sources, using *in vitro* gastrointestinal digestion models, have been proposed (Table 3).

Fruits and vegetables possess matrices rich in dietary fiber, whose association with phytochemicals modulates their relative bioaccessibility (Alminger et al., 2014). In a study comparing the stability and bioaccessibility of carotenoids in pure forms or from whole food,

Courraud Berger, Cristol and Avallone (2013) demonstrated that vitamin A and carotenoid standards were unstable, whereas food carotenoids were generally better protected by the food matrix (30-100% recovery compared with 7-30% for standards). Podsędek, Redzynia, Klewicka and Koziołkiewicz, (2014) studied the stability and antioxidant capacity of anthocyanins present in raw red cabbage and in its anthocyanin-rich extract, to evaluate the effect of the cabbage composition. The results also demonstrated that the food matrix is an important factor influencing the stability of red cabbage acylated anthocyanins subjected to *in vitro* gastrointestinal digestion. The authors suggested that vegetable constituents (mainly dietary fiber) protect the labile anthocyanins from degradation under the physiological conditions simulated.

Although, it is clear that the susceptibility of phytochemicals to degradation increases after their release from the food matrix, other interactions with compounds released form the food matrix (including soluble fibers) and overall viscosity may also affect their bioaccessibility (Schwiggert, Mezger, Schimpf, Steingass, & Carle, 2012; Alminger et al., 2014). Several studies have reported that molecular interactions between dietary fibers and phenolic compounds could negatively affect their bioaccessibility (Bouayed et al., 2011; Palafox-Carlos et al., 2011; Alminger et al., 2014), as fiber-entrapped polyphenols are both poorly extractable and barely soluble in gastrointestinal fluids. Some of these studies have even shown that this interaction may not only limit their absorption, but also prevent the hydroxyl groups from polyphenols from stabilizing free radicals. This effect limits the bioaccessibility and consequently lowers the antioxidant activity due to the fewer hydroxyl groups available to stabilize radicals (Palafox-Carlos et al., 2011). Velderrain-Rodríguez et al., (2016) also reported that this decrease in antioxidant properties could be related to its instability due to changes in pH during digestion. So, foods with a high amount of insoluble fiber and/or phytochemicals bound to dietary fiber or entrapped in the food structures had lower levels of bioaccessible phenolic compounds and so a lower antioxidant capacity after *in vitro* digestion. It should be noted that the greatest loss of these compounds takes place in the intestine and colon, not during gastric digestion (Podsędek, et al., 2014). Moreover, recent studies have demonstrated that in this type of food, the action of bacterial enzymes dramatically increases the antioxidant potential of the food residues in the lower gastrointestinal tract (Napolitano, et al., 2008; Azurra-Papillo, Vitaglione, Graziani, Gokmen, & Fogliano, 2014).These findings suggest the need to reconsider the correlations performed and it would be advisable to include such measurements after the enzymatic digestion procedure, including microbiota-like bacterial enzymes, to obtain a more reliable picture, particularly when the health effects of the foods within the gastrointestinal tract are being considered (Azurra-Papillo et al., 2014).

However, not only dietary fibers affect the release of bioactive compounds during the *in vitro* digestion process, and several studies have highlighted the role of proteins and fats in this

process. Mullen, Edward, Crozier, & Serafini, (2008) reported a positive effect of the fat content on the bioavailability of pelargonidin 3-O-glucoside from strawberries; their bioavailability was higher when strawberries were consumed with cream due to positive effect of the fat in the cream on the absorption of strawberries in metabolism. Ortega, Reguant, Romero, Macia and Motilva (2009) reported that during the *in vitro* digestion of cocoa food products, the extractability of phenolic acids, flavonoids and proanthocyanidins appeared to be improved in the presence of fat, increasing by a factor of1.2 to 3 in cocoa liquour (50% fat content) compared to cocoa powder (15% fat content). Other authors have also reported the preserving effect of fat/oil on the total phenolic content during digestion, an effect that could be related to the delay in absorption and metabolism of fatty foods that resulted in greater polyphenol absorption (Sengul et al., 2014).

The affinity of milk and egg proteins as well as gelatins for polyphenols depends on both the protein and phenolic structures (Bohin, Vincken, Van der Hijden, & Gruppen, 2012). For example, chlorogenic acid associated with milk caseins rather than with β-lactoglobulin, and this complexation was relatively stable in simulated gastric and intestinal steps (Dupas, Marset-Baglieri, Ordonaud, Ducept, & Maillard, 2006). Keogh Mclnemey and Clifton, (2007) determined that the absorption of flavonoids in milk chocolate decreased due to double bonds formed between flavonoids and milk proteins.

Sengul et al., (2014) proposed an interesting study to understand how the food matrix and individual food components affect the bioavailability of phenolics, especially the anthocyanins found in pomegranate fruit (one of the richest sources of phenolics). Model systems composed of both commonly consumed foods - liquid foods (sunflower oil, skim milk, soy milk, honey, cream, skim plain yogurt, probiotic yogurt and lemon juice) and solid foods (minced lean meat, bread, read apple, soybean and wheat starch) - and food components (soy protein, casein, meat protein, gluten, stearic acid, linoleic acid, starch, lactose, galactose, fructose, glucose, pectin, cellulose, ascorbic acid, tocopherol, citric acid and salt) were developed. The results showed that the phenolic compounds from pomegranate were more stable to gastrointestinal digestion than anthocyanins and that they were mostly lost during pancreatic digestion rather than gastric digestion. By and large, the consumption of pomegranate with foodstuffs or food components exerted an inhibiting effect or no effect on the total phenolic content. The preserving effect of oil and carbohydrates on the total phenolic content during digestion was also observed in this study. By contrast, significant decreases during gastric digestion were evident in both the total phenolic and anthocyanin content of pomegranate when it was consumed with foods rich in protein, such as milk, bread, yogurt, soy protein, casein and meat protein.

In short, the application of *in vitro* simulated gastrointestinal digestion has demonstrated that food components or food matrices have different effects on bioactive compounds, and, in some cases, only a minor fraction of the total quantity of these compounds in foods is potentially

bioaccessible. So, while developing or consuming a functional food product, the interaction of the bioactive compound with a food component in a given food matrix needs to be taken into consideration to increase their bioaccessibility.

Another interesting application that has emerged in the recent years is the use of *in vitro* digestion models in nanotechnology, more specifically for assessing the digestion and absorption of engineered nanomaterials (metal/mineral-based, soft lipid and solid biochemical macromolecules, including new proteins, polysaccharides and nucleic acids) released from food matrices. A recent review published on this aspect also highlighted the need to further adapt and standardize the available models and the corresponding analytical methods to allow quantification of the digestive fate of engineered nanomaterials, including their uptake across the gastrointestinal barrier (Lefebvre et al., 2015).

That the interest of these studies remains very high is clear; an example of this is the recent review published about their contribution for a better and extensive understanding of *in vivo* digestion conditions in different groups of the population (infants, elderly and patients of cystic fibrosis or gastric bypass surgery) which would offer better opportunities to develop relevant products with high bioefficacy (Levi, et al., 2017).*Improved in vitro digestion stability of bioactive compounds by nano-delivery systems*

As can be seen above, the bioavailability of bioactive compounds contained in foods is affected by their solubility and stability, the food matrix in which they are included and the location in the human gastrointestinal tract where they are released, often in response to an environmental trigger, such as pH, ionic strength or enzyme activity. Therefore several approaches to increase the solubility, stability (mainly protection against oxidation) and bioaccessibility (biosorption) of these bioactive compounds, whether naturally present in foods or intentionally added, have been exploited in the last decade (Saenz Tapia, Chavez, & Robert, 2009; Bakowska-Barczak, & Kolodziejczyk, 2011; Shin, Chung, Kim, Joung, & Park, 2013; Çam, Içyer, & Erdogan, 2014; Wang, Hu, Yin, Yang, Lai, & Wang, 2015; Li et al., 2015a; Li et al., 2015b; Jilani, Cilla, Barberá, & Hamdi, 2015, 2016; Li, Shin, Lee, Chen, & Park, 2016).

In order to better integrate some of these bioactive compounds into a food matrix or beverage system, nano-delivery systems made of food grade materials have attracted much attention (Fathi, Mozafari, & Mohebbi, 2012; Li, et al., 2016). A delivery system is defined as one in which a bioactive material is entrapped in a carrier to control the rate of bioactive release. A nano-delivery system (smaller than 100 nm) may confer bioactive compounds with a rapid dissolution speed, higher stability, a tailored release pattern, higher permeation rates, higher bioavailability and other advantages compared with other similar delivery system (micro-sized or larger) (Weiss, Gaysinksy, Davidson, & McClements, 2009; McClements 2015). Typically, food applicable nano-delivery systems can be carbohydrate, protein or lipid-based, although the

possibility of industrial production and the greater encapsulation efficiency and lower toxicity attributed to lipid-based systems have tended to attract more attention (Fathi, et al., 2012; Aditya, Shim, Lee, Lee, Im, & Ko 2013; Livney, 2015). As a consequence, various kinds of lipid-based nano-delivery systems have been evaluated to encapsulate bioactive compounds in order to better incorporate them into food and beverage systems, including nanoemulsions, nanoliposomes, nanoparticles and nanospheres (Table 4) However, the stability and the absorption efficiency of these carriers within the gastrointestinal tract is still a major barrier, which has led to growing interest in understanding the digestion process of food colloids.

3.2.3.1 Nanoemulsions

Oil in water (O/W) nanoemulsions are colloidal dispersion systems composed of small lipid droplets (50-100 nm) dispersed within an aqueous medium (McClements 2012). This structure is particularly attractive for encapsulating, protecting and transporting lipophilic nutraceuticals for food and related applications. The relatively small size of the droplets in nanoemulsions provides them with a number of potential advantages over conventional emulsions: high optical clarity, good stability for gravitational separation and particle aggregation, and increased bioavailability (Liu, Sun, Li, Liu, & Xu, 2006; Shakeel and Ramadan, 2010; McClements and Rao, 2011). On the other hand, there is a limit to the amount of a bioactive component that can be successfully incorporated into a nanoemulsion before crystals are formed, which may lead to physical instability of the delivery system as well as a reduction in the bioaccessibility of the encapsulated component (Li et al., 2012). These nanoemulsions can been prepared using different methods, including high-pressure homogenization, ultrasonication, emulsification-evaporation, etc. Kim, Hyun, Yun, Lee, & Byun, (2012) used high pressure homogenization to prepare nanoemulsions of supercritical CO_2 extracted astaxanthin which were unaffected during storage under light and thermal conditions. Acevedo et al., (2014) found that these nanoemulsions also exerted a higher antioxidant protective effect against cellular oxidative stress and oxidative stability than free astaxanthin. Lycopene nanoemulsions (with linseed oil) were prepared by Kim, Ha, Choi and Ko (2014) using an emulsification-evaporation method. Ha et al. (2015) and Sotomayor-Gerding et al., (2016) reported that such nanoemulsions protected the antioxidant activity and improved the bioaccessibility of lycopene-enriched tomato extract. Nanoemulsions are thermodynamically unstable and need certain emulsifiers or encapsulating methods on the oil-water interface to stabilize the colloidal system (Gonnet, Lethuaut, & Boury, 2010). Biopolymer is usually used for coating nanoemulsions to increase the stability and, absorption rate, as well as to modulate the payload release pattern (Abbas, Bashari, Akhtar, Li, & Zhang, 2014; Ozturk, Argin, Ozilgen, & McClements, 2015). Chitosan is a natural polysaccharide widely applied used in functional foods and recently studied as a coating for the encapsulation of several bioactive compounds (Shin, et al., 2013). Li, Shin, Lee, Chen, & Park, (2016) reported that

chitosan coatings inhibit the degradation of curcumin during thermal and UV irradiation treatment but may interfere with the lipolysis of nanoemulsions during *in vitro* digestion, which also slightly decreases its bio-accessibility. Liang, Shoemaker, Yang, Zhong, and Huang (2013) reported that, through *in vitro* digestion, the bioaccessibility of β-carotene was significantly improved after encapsulation in nanoemulsions stabilized by modified starches. Pool, Mendoza, Xiao, and McClements (2013) reported that quercetin dissolved in nanoemulsions stabilized by a globular protein (β-lactoglobulin) had higher bioaccessibility than quercetin dissolved in bulk oils or in bulk water.

3.2.3.2. Nanoliposomes

Nanoliposome is a new technology for the encapsulation and delivery of bioactive compounds (Zou, et al., 2014). Liposomes (spherical bilayer vesicles resulting from the dispersion of polar lipids in aqueous solvents) have been widely studied for their ability to act as drug delivery vehicles by shielding reactive or sensitive compounds prior to release (Schroeder, Kost, & Barenholz, 2009; Liu et al., 2011). Nanoliposomes, measuring less than 100 nm, can be prepared using different methodologies such as extrusion, sonication and dynamic high pressure microfluidization among others (Zou, et al., 2014). Compared with other delivery systems, liposomes have several benefits like (*i*) the option of large scale production, (*ii*) target ability, and (*iii*) the ability to transport water-soluble, water-insoluble and amphiphilic compounds (Laye, McClements, & Weiss, 2008; Chen, Han, Cai, & Tang, 2010; Fathi, et al., 2012). Nevertheless, the applied of nanoliposomes have been limited by different factors such as their insufficient physical and digested stability (short release time) in the gastrointestinal tract, the disruption of liposome integrity and leakage of the encapsulated molecule (Reza, Johnson, Hatziantoniou, & Demetzos, 2008; Liu, Jianhua, Wei, Ti, & Chengmei, 2013). To improve nanoliposomal stability a variety of surface modified systems have been developed, including poly-surface-conjugated nanoliposomes (Ramez and Palmer 2011), chitosan coated nanoliposomes (Liu, et al., 2013), silica external-layered nanoliposomes (Mohanraj, Barnes, & Prestidge, 2010) and protein site-specific modified nanoliposomes (Guo, Wu, & Guo, 2012).

Zou et al., (2014) successfully applied nanoliposome encapsulation to epigallocatechin gallate (from green tea) and they found that the degradation rate of their antioxidant activities during *in vitro* digestion was slowed by this method. Liu, et al., (2013) developed an alginate-chitosan-coated nanoliposome to make better lipid membrane stability and avoid depletion of encapsulated food components (mainly medium chain fatty acids). These authors found that this delivery system might better resist lipolytic degradation and facilitate a lower level of encapsulated component release in simulated gastrointestinal conditions. Rashidinejad, Birch and Everett (2016) encapsulated green tea catechins in nanoliposomes using soy lecithin and added them into a full-fat cheese; the authors reported a significant increase in the total phenolic content

and antioxidant activity of the full-fat cheese and no effect on the pH or proximate composition. In the same way, these authors (Rashidinejad et al., 2016) found that individual catechins were recovered in different quantities comprised between 15 and 52% from cheese digesta after 6 h of gastrointestinal digestion; the authors also provided suggestion for the association of nanoliposomes with the surface of milk fat globules inside the cheese matrix.

3.2.3.3. Solid nanoparticles

Couvreur, Dubernet and Puisieux, (1995) defined nanoparticles as sub-micron solid particles, which may be used for the nano-encapsulation of compounds that showed bioactivity. However, it should be borne in mind that its possible obtained different nano-compunds like as nanoparticles, nanospheres or nanocapsules according on the method of preparation as mentioned Faridi-Esfanjaniand and Jafari, (2016). Thus, Nanospheres can be considered matrix systems in which the bioactive compounds are physically and uniformly dispersed, whilst nanocapsules can be considered vesicular systems in which the bioactive compounds are confined in a cavity consisting of an internal liquid core enclosed in a polymeric membrane (Couvreur et al., 1995). To obtain the biodegradable polymeric nanoparticles, different compounds could be used for example (*i*) proteins like gelatin, whey or milk proteins, (ii) polysaccharides as chitosan, sodium alginate and starch, or (*iii*) synthetic polymers as reported Faridi-Esfanjaniand and Jafari, (2016). At present, the use of biodegradable polymeric nanoparticles has attracted the attention of several scientific research groups mainly in food fields thanks to their favourable properties which include as mentioned Bae and Kataoka, (2009) a good biocompatibility, easy design and preparation, structure variations and very interesting bio-mimetic characters. Especially in the field of smart bioactive carriers, polymer nanoparticles can deliver bioactive compounds directly to the intended site of action (Faridi-Esfanjaniand and Jafari, 2016). Conversely, natural nano-carriers have appeared as very attractive choices for controlled systems with bioactive compounds due to their resemblance to the extracellular matrix in the human body and various other favourable physicochemical properties. In this way, casein nanoparticles could be used to bind phenolic compounds throughout hydrophobic interactions between the phenolic rings and prolines, wrapping the casein around the phenolic compounds as reported Jöbstl, O'Connell, Fairclough and Williamson (2004).

There are some reviews about the nano-encapsulation of bioactive compounds, most of them focused in the methods used for their preparation, and characterization, improving stability, and the suitability requirements for the bioactive compounds selected (Fathi, et al., 2012; Livney, 2015; Faridi-Esfanjaniand & Jafari, 2016). However, the number of studies on the application of *in vitro* gastrointestinal digestion methods to evaluate the effect of these nanoencapsulation methods on the stability, bioavailability and bioaccessibility of the bioactive compounds is limited.

Whatever the case, it is true that a greater number of such studies deal with nanoemulsions, followed by nanoliposomes while very few look at nanoparticles.

Some of these studies have compared the effectiveness of each encapsulation method in protecting the bioactive compound during the *in vitro* digestion process. Chuacharoen and Sabliov (2016) carried out a work with two different types of delivery system, such as nanoparticles and nanoemulsions and they analyzed the capacity of these compounds to improve the physicochemical stability and the antioxidant properties of β-carotene in the presence of milk under *in vitro* gastrointestinal environments. They reported that nanoparticles enhanced the physic-chemical stability and antioxidant properties of entrapped β-carotene compared with emulsions in the presence of milk under *in vitro* gastrointestinal environments. Zou et al., (2016) investigated the potential of three nanoparticle-based delivery systems to improve curcumin bioavailability. These authors found that the loading capacity of curcumin, the capacity to protect this compound from chemical degradation and its solubilisation inside intestinal fluids depended mainly on the nanosystem composition. These authors conclude that, in general terms, lipid nanosystems mainly nanoemulsions appeared to be the most effective at increasing the amount of curcumin available for absorption (Zou et al., 2016).

In view of the results of some of these papers, we conclude that each type of delivery nanosystem (nanoemulsions, nanoliposomes or nanoparticles) has their strengths and weaknesses to encapsulate, to protect and to release bioactive compounds and those specific studies need to be considered to improve the bioavailability and bioaccessibility of the same.

4.- Conclusions

In vitro gastrointestinal digestion systems are a valuable tool for understanding the behaviour of food and food components during human digestion, as demonstrated by the large number of publications that they have generated. Their application has changed with time, not only as regards the process conditions (parameters, length scale, protocols and guidance) but also in the selection of new fields of application. In the last decade, for example, the greatest changes have tried to: (*i*) standardize and harmonize *in vitro* static and dynamic systems which gastrointestinal processes are simulate by defining critical parameters and setting that could be applied for various ends and that allow results to be compared across research teams; (*ii*) study the effect of the food matrix on the release, bioaccessibility and antioxidant properties of different bioactive compounds present in foods; and (*iii*) develop nano-delivery systems to increase the stability of these bioactive compounds and to evaluate their behavior in each phase of gastrointestinal digestion.

The results obtained have contributed to understanding the behavior of these compounds with bioactivity and hence to improve the development of new healthy foods.

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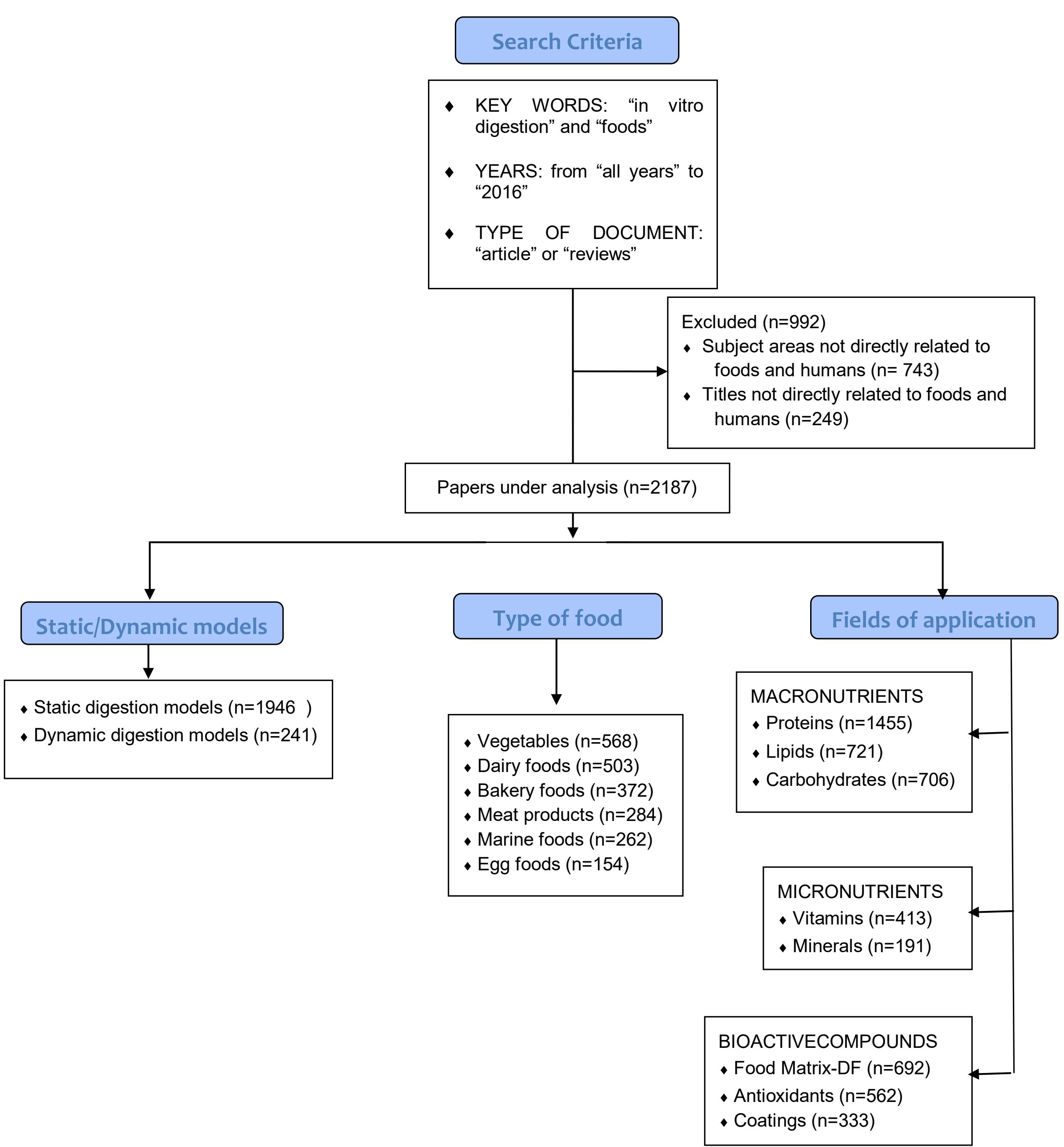

Figure 1.- Flow chart of the search criteria applied to select the papers used in this review (based on the CONSORT diagram for clinical research).

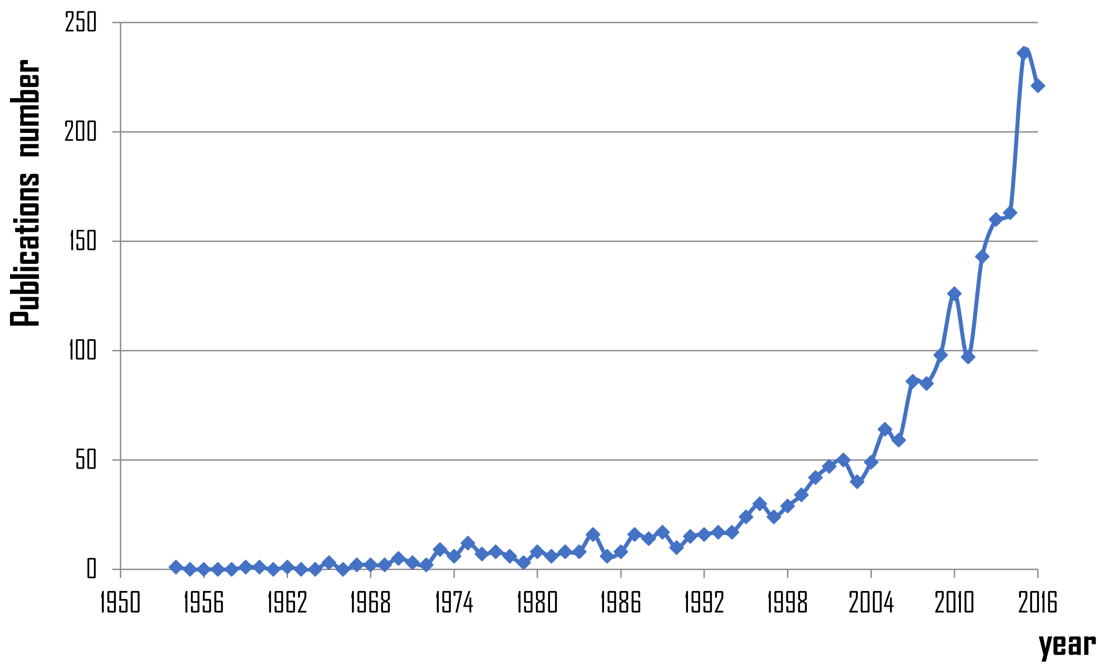

Figure 2.- Evolution of the number of publications on *in vitro* digestion studies.

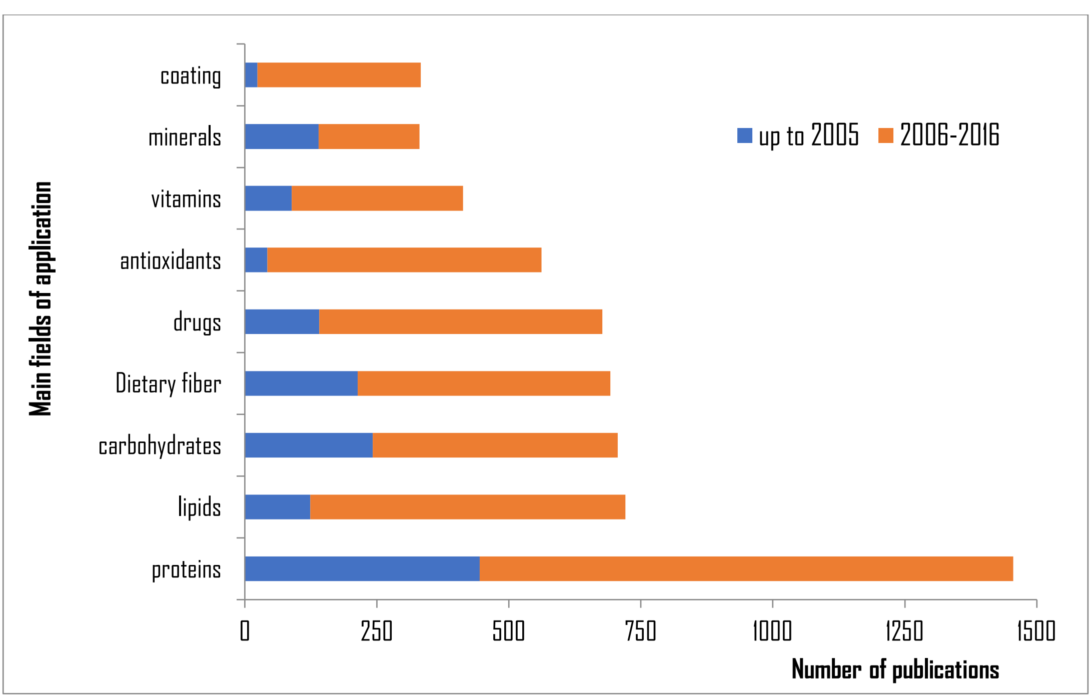

Figure 3.- Change in main fields of application of *in vitro* digestion studies with time.

Table 1.- Static *vs* dynamic *in vitro* human digestion models for food applications

STATIC	DYNAMIC
Type of study	
Useful for limited digestions (gastric and/or intestinal step)	Applicable to total digestion studies
Type of food	
Homogenized/simple foods Isolated or purified food compounds	Complex foods
Major applications	
Macronutrients *Protein hydrolysis *Lipid hydrolysis *Starch resistance Bioactive molecules *Release from simple food matrices *Solubility and bioaccessibility	Foods & Pharmaceutical *Release and bioaccessibility of nutrients from complex food matrices *Protein digestion *Lipid separation *Peptide production
Main objectives	
Improve food properties Preliminary trials to justify possible nutrition and health claims	Effect of food structure on nutrient delivery, nutrient interactions, probiotic survival, prebiotic delivery , etc.
Main advantages	
Rapid and simple Cost-effective Need to be validated only in light of their intended use	Better accuracy the dynamic environment of the intestine: peristaltic movements, physical forces, shear forces, etc. Allow direct comparison with the results *of in vivo*/clinical studies
Main disadvantages	
Lack the mechanical forces that contribute to *in vivo* digestion and the constant changes in biochemical environment; excessive metabolite accumulation, which can interfere with digestion	Should be validated for their ability to reproduce the conditions of the gastrointestinal tract.

Table 2.- Recent studies about the effect of *in vitro* digestion process on antioxidant capacity and bioaccesssibility of bioactive compounds in foods.

Type of Food	Bioactive compound	Properties evaluated*	Reference
Whole Foods			
Citrus fruits	Polyphenols	Bioaccesibility and antioxidant properties	Chen et al., (2014)
Mullberries	Anthocyanins	Bioaccesibility and antioxidant properties	Liang et al., (2012)
Maqui berries	Polyphenols	Bioaccessibility and antioxidant properties	Lucas-González et al., (2016)
Wild blueberries	Polyphenols	Bioaccessibility and antioxidant properties	Correa-Betanzo et al., (2014)
Chinese bayberries	Polyphenols	Antioxidant properties	Huang et al., (2014)
Raspberries	Polyphenols	Antioxidant properties	McDougall et al., (2005)
Chockeberries	Polyphenols	Antioxidant properties	Bermudez-Soto et al., (2007)
Figs	Polyphenols	Bioaccessibility	Kamiloglu and Capanoglu, (2013)
Grapes	Polyphenols	Bioaccessibility and antioxidant properties	Tagliazucchi et al., (2010); Chen et al., (2014)
Apples	Polyphenols	Bioaccessibility and antioxidant properties	Bouayed et al., (2011), Bouayed et al., (2012); Tenore et al., (2013); Chen et al., (2014)
Chaenomeles fruits	Polyphenols	Stability and antioxidant properties	Miao et al., (2016)
Tomato	Polyphenols	Bioaccessibility	Svelander et al., (2010); Talens et al., (2016)
Sweet cherry	Polyphenols	Antioxidant properties	Fazzari et al., (2008); Chen et al., (2014)
Chilli peppers	Carotenoids	Bioaccessibility	Pugliese et al., (2014)
Bean seeds	Polyphenols	Bioaccessibility and antioxidant properties	Soriano-Sancho et al., (2015)
Cereals	Polyphenols	Antioxidant properties	Gong et al., (2013); Chitindingu et al., (2015); Masisi et al., (2016)

Pomegranate juice	Polyphenols	Bioaccessibility and antioxidant properties	Pérez-Vicente et al., (2002); Fawole et al., (2015)
Orange juice	Carotenoids, flavonoids and Vitamin C	Bioaccessibility	Gil-Izquierdo et al., (2001); Aschoff et al., (2015)
Others fruit juices	Polyphenols	Antioxidant properties	Ryan and Prescott (2010); Rodríguez-Roque et al., (2013)
Vegetable juices	Polyphenols	Antioxidant properties	Wootton-Beard et al., (2011); Helal et al., (2014)
Extra virgin olive oil	Polyphenols	Antioxidant properties	Dinnella et al., (2007)
Black carrot jams and marmalades	Polyphenols	Bioaccessibility	Kamiloglu et al., (2015)
Soluble coffee	Polyphenols	Bioaccessibility and antioxidant properties	Podio et al., (2015)
Dietary supplements	Polyphenols	Antioxidant activity	Henning et al., (2014)
Strawberry and peach yoghurt	Polyphenols	Bioaccessibility and antioxidant properties	Oliveira and Pintado (2015)
Different fortified foods	Carotenoids and retinoids	Bioaccesibility and antioxidant properties	Courraud et al., (2013)
Bread	Phenolic acids	Bioaccessibility	Dall'Asta et al., (2016)
Durum wheat pasta + barley flour enriched with β-glucan	Polyphenols	Antioxidant properties	Montalbano et al., (2016)
Pomegranate peels enriched cookies	Polyphenols	Bioaccessibility and antioxidant properties	Colantuono et al., (2016)
Spice enriched starchy foods	Thymol and carvacrol	Bioaccessibility and antioxidant properties	Aravena et al., (2016)
Plant sterols enriched fermented milk beverages	Plant sterols	Bioaccessibility	Vaghini et al., (2016)

Organic and non-organic baby foods	Carotenoids	Bioaccessibility	Jiwan et al., (2010)
Coproducts from agrofood industries			
Grape coproducts	Polyphenols	Bioaccessibility	Wang et al., (2013)
Mango coproducts	Polyphenols	Bioaccessibility	Blancas-Benitez et al., (2015)
Pomegranate coproducts	Polyphenols	Bioaccessibility and antioxidant properties	Mosele et al., (2015); Gullón et al., (2015a); Colantuono et al., (2016)
Apples coproducts	Polyphenols	Bioaccessibility and antioxidant properties	Gullón et al., (2015b)
Date palm coproducts	Polyphenols	Bioaccessibility and antioxidant properties	Gullón et al., (2015b)
Cauliflower coproducts	Polyphenols	Bioaccessibility and antioxidant properties	Gonzales et al., (2015)
Black carrot coproducts	Polyphenols	Bioaccessibility	Kamiloglu et al., (2015)

* Bioaccessibility is evaluated as bioaccessibility index, representing the proportion of the amount of the bioactive compound in the soluble fraction of the digested sample respect to its amount in the total digested sample (soluble + non-soluble fraction). Antioxidant properties are evaluated comparing the antioxidant activity in the undigested sample (using antioxidant methods such as DPPH, ABTS, FRAP, FIC, etc) respect to the same values after each phase of *in vitro* gastrointestinal digestion. Stability of each bioactive compound is estimated as its amount after each digestion phase respect to the amount in the undigested sample.

Table 3.- Recent studies about the effect of food matrix on antioxidant capacity and bioaccesssibility of bioactive compounds during the *in vitro* digestion process

Type of food matrix	Bioactive compound	Properties evaluated*	References
Plant foods matrix	Polyphenols	Antioxidant properties	Azurra-Papillo et al., (2014)
Dietary fiber	Polyphenols	Bioaccessibility	Carlos et al., (2011)
	Vitamin A and carotenoids	Bioaccessibility	Courraud et al., (2013)
	Phenolic compounds	Bioaccessibility	Velderrain-Rodríguez et al., (2016)
	Phenolics and anthocyanins	Bioaccessibility	Sengul et al., (2014)
	Polyphenols	Bioaccessibility	Palafox-Carlos et al., (2011)
	Polyphenols	Bioaccessibility and antioxidant properties	Bouayed et al., (2011)
	Carotenoid	Bioaccessibility	Schwiggert et al., (2012)
Soluble dietary fiber	Polyphenols	Bioaccessibility	Ortega et al., (2011)
Solid food matrices (peach, plums, prunes, walnuts and tomatoes)	Polyphenols, flavonoids, anthocyanins and carotenoids	Bioaccessibility	Tagliazucchi et al., (2012)
Red Cabbage	Anthocyanins	Stability and antioxidant properties	Podsędek et al., (2014)
Chocolate matrix (fats, proteins and dietary fiber)	Polyphenols	Digestibility and bioaccessibility	Serafini et al., (2003); Keogh et al., (2006); Ortega et al., (2009); Neilson et al., (2009); Fogliano et al., (2011)
Coffe and milk	Polyphenols	Antioxidant properties	Dupas et al., (2006)
Fat matrix	Polyphenolic compounds	Bioaccessibility	Mullen et al., (2008)

Vegetable oil (hazelnut oil)	Polyphenols	Bioaccessibility	Ortega et al., (2011)
Blueberry, oat meal and milk	Polyphenols	Antioxidant properties and bioaccessibility	Cebeci &Sahin-Yesilcubuk (2014)
Carob flour	Polyphenols	Bioaccessibility	Ortega et al., (2011)
Dairy and egg	Polyphenols (grape extracts)	Bioaccessibility and antioxidant properties	Pineda-Vadillo et al., (2016)

* Bioaccessibility is evaluated as bioaccessibility index, representing the proportion of the amount of the bioactive compound in the soluble fraction of the digested sample respect to its amount in the total digested sample (soluble + non-soluble fraction). Antioxidant properties are evaluated comparing the antioxidant activity in the undigested sample (using antioxidant methods such as DPPH, ABTS, FRAP, FIC, etc) respect to the same values after each phase of *in vitro* gastrointestinal digestion. Stability and digestibility of each bioactive compound is estimated as its amount after each digestion phase respect to the amount in the undigested sample.

Table 4.- Recent studies about the use of nanodelivery systems for coating bioactive compounds and their behaviour in the *in vitro* digestion process.

Type of nanosystem	Stabilization	Bioactive compounds	References
Nanoemulsions		Astaxanthin	Kim et al., (2012) Acevedo et al., (2014)
Nanoemulsions	β-lactoglobulin	Quercetin	Pool et al., (2013)
Nanoemulsions	Modified starches	B-carotene	Liang et al., (2013)
Nanoemulsions		Lycopene	Kim et al., (2014)
Nanoemulsions	Soy protein	Beetroot pomace extract	Tumbas-Šaponjac et al., (2016)
Nanoemulsions	Chitosan	Curcumin	Shin et al., (2013); Li et al., (2016)
Nanoemulsions		Curcumin	Ahmed et al., (2012)
	Lactoferrin and lactoferrin/alginate	Curcumin	Pinheiro et al., (2016)
Nanoemulsions	Carotenoids (asthaxantin and lycopene)		Acevedo et al., (2014); Ha et al., (2015); Sotomayor-Gerdin et al., (2016)
Nanoemulsions	Modified starch	β-Carotene	Liang et al., (2013)
Nanoemulsions	Binary emulsifiers and β-cyclodextrin complexes	Tocopherols and phenolic compounds	Cheong et al., (2016a,b)
Nanoemulsions	Different carrier oils	β-Carotene	Quian et al., (2012)
Nanoliposomes		Epigallocatechin gallate from green tea	Zou et al., (2014)
Nanoliposomes	Alginate-Chitosan	Medium-chain fatty acids	Liu et al., (2013)
Nanoliposomes	Soy lecithin	Green tea catechins	Rashidinejad et al., (2016)
Nanoliposomes		Curcumine	Zou et al., (2016)
Nanoparticles	Witepsol and camauba	Rosmarinic acid	Madureira et al., (2016)
Nanoparticles	Pluronic F127 and lecithin	B-Carotene	Chuacharoen & Sabliov, (2016)
Nanoparticles	β-Lactoglobulin-dextran	B-Carotene	Yi et al., (2014)
Nanoparticles		Curcumin	Noak et al., (2012)
Nanoparticles		Polyphenol compounds	Pool et al., (2012)

7.4.- PUBLICACIÓN 4

Título: Changes in bioaccessibility, polyphenol profile and antioxidant potential of flours obtained from persimmon fruit (*Diospyros kaki*) co-products during in vitro gastrointestinal digestion

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Changes in bioaccessibility, polyphenol profile and antioxidant potential of flours obtained from persimmon fruit (*Diospyros kaki*) co-products during *in vitro* gastrointestinal digestion

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Abstract

The aim was to evaluate (i) the phenol and flavonoid recovery and bioaccessibility indexes, (ii) the stability of individual polyphenolic compounds and (iii) the antioxidant activity of persimmon flours (cultivars 'Rojo Brillante' and 'Triumph') during the *in vitro* digestion. The recovery index for phenolic and flavonoid content was dependent on flour type and digestion phase. After the dialysis phase, the bioaccessibility for phenolic compounds from both flours was similar; for flavonoids it was higher in 'Triumph' than 'Rojo Brillante' flour. After *in vitro* digestion, 13 polyphenolic compounds were detected in both flours, of which only six were detected in the intestinal phase. Their antioxidant activity (ABTS•+, FRAP and DPPH) decreased after intestinal phase, while their chelating activity (FIC assay) increased in both flours. So, persimmon flours could be included in the formulation of foods to improve either their scarcity of bioactive compounds or an unbalanced nutritional composition.

Keywords: *in vitro* digestion, persimmon, antioxidant, polyphenol compounds, bioaccessibility.

Abbreviations list:

RB: Rojo Brillante; TH: Triumph; GID: gastrointestinal digestion; TFC: total flavonoid content; TPC: total phenolic content; ABTS•+: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical; DPPH: 2, 2 - diphenyl, 1- picryl hydrazyl; FIC: ferrous ion chelating; FRAP: ferric reducing antioxidant property; PF: pellet fraction SCF: soluble chime fraction; IN: intestinal absorbed fraction; OUT: intestinal unabsorbed fraction

1.- Introduction

The incidence of chronic diseases derived of an unbalanced dietary pattern, characterized by eating meals rich in saturated fats and refined carbohydrates and poor in fiber and bioactive compounds, is increased worldwide. This is one of the reasons why the past year the United Nations General Assembly proclaimed a Decade of Action on Nutrition that will run from 2016 to 2025, with the aim to reduce hunger and improving nutrition around the world (ONU, 2016). Taking it into consideration, the search of new based-vegetable ingredients rich in bioactive compounds and other dietary components like fiber, protein or complex carbohydrates is necessary for the development of new foods with high nutritional value and antioxidant properties. In this way, agro-industrial co-products could be used as potential functional ingredients for the development of different foods such as pâté, pasta, ice creams or biscuits, due to their important amounts of dietary fiber and antioxidant compounds (Viuda-Martos et al., 2012; López-Marcos, Bailina, Viuda-Martos, Pérez-Alvarez, & Fernández-López, 2015).

Nevertheless, the bioaccessibility and bioavailability of these bioactive compounds after human digestion determine their biological action in the body (Etcheverry, Grusak, & Fleige, 2012). In order to make a first screening of their behavior after human digestion, *in vitro* gastrointestinal digestion (GID) is being used. These tests simulate the physical (agitation, temperature and pH) and chemical (enzymatic and salinity) processes that occur during GID and provide information about the changes that occur in bioactive compounds, the release of food matrix, the interactions with other compounds and their bioaccesibility (Minekus et al., 2014); relevant information for designing and formulating foods with antioxidant potential.

Several researches have studied the bioaccesibility and recovery index of phytochemicals present in fruits, vegetables, seeds and agro-industrial co-products (Chandrasekara, & Shahidi, 2011; Gawlik-Dziki, 2012; Neto et al. 2017), showing that dietary matrix, pH changes, enzymatic activity, interactions with dietary compounds, as well as the nature of each compound, are the factors that have the greatest impact on the stability and release of antioxidant compounds after GID (Kroll, Rawel, & Rohn, 2003;. Alminger et al., 2014).

Persimmon fruit is rich in fiber and has important amounts of minerals, carotenes and polyphenols (De Ancos, González, & Cano, 2000; Park et al., 2006; Lucas-González, Viuda-Martos, Pérez-Álvarez, & Fernández-López, 2017). Additionally, several studied have showed antidiabetics, antiaetrogenic and antiobesity effects of persimmon leaves and fruits (Lee, Chung, & Lee, 2006; Son et al., 2013; Kim et al., 2016). These evidences make very attractive the use of persimmon co-products as potential functional ingredients.

For these reasons, the aim of the current work was to evaluate (i) the phenol and flavonoid recovery and bioaccessibility indexes, (ii) the stability of individual polyphenolic compounds and

(iii) the antioxidant activity of both persimmon flours (obtained from juice co-products of cultivars 'Rojo Brillante' and 'Triumph') during the *in vitro* gastrointestinal digestion.

2.- Material and Methods

2.1.- Plant material

Persimmon flours co-products of cultivars 'Rojo Brillante' and 'Triumph' were obtained as described by Lucas-González et al. (2017). The particle size of both persimmon flours were <0.210 mm.

2.2.- Simulated in vitro gastrointestinal digestion (GID) & Extraction method

In vitro GID of three independent samples were made following the methodology described by Gullón, Pintado, Fernández-López, Pérez-Álvarez, & Viuda-Martos (2015a) (Figure 1). Three phases were simulated: oral, gastric and intestinal. The intestinal phase was divided in two steps: agitation step and dialysis process. At the end of the dialysis process, the solution left inside the dialysis tubing was taken as the IN sample, representing the material that remained in the gastrointestinal tract (colon-available), and the solution that managed to diffuse into the dialysis tubing was taken as the OUT sample (serum-available). Finally, all digestion mixtures were lyophilized and stored at -20 ºC until further use.

Polyphenol compounds of undigested sample and digested samples were extracted following the methodology described by Pellegrini, Lucas-Gonzalez, Fernández-López, Ricci, Pérez-Álvarez, Lo Sterzo, & Viuda-Martos (2017).

2.3.- Total phenol and total flavonoid content

To determine the total phenol content (TPC) of digested samples, the Folin-Ciocalteu's reagent (Singleton, & Rossi, 1965) was used. The method described by Blasa, Candiracci, Accorsi, Piacentini, Albertini, and Piatti (2005) was used for determining total flavonoid content (TFC). Both methods are based on spectrophotometric measures (760 nm for TPC and 510 nm for TFC) which were made with a HP 8451 spectrophotometer (Hewleck Packard, Cambridge, UK). The results of TPC were expressed as mg gallic acid equivalents (GAE) /g sample, while in the case of TFC the results were expressed as mg rutin equivalents (RE)/g of sample.

2.4.- Recovery and bioaccessibility index

To analyze the effect of *in vitro* GID on TPC and TFC two different indexes were applied, following the indications of Ortega, Macia, Romero, Reguant, and Motilva (2011): the recovery percentage and bioaccessibility percentage. The recovery percentage allows to know the amount of phenolic and flavonoid compounds recuperated after the oral, gastric and intestinal digestion, by comparison with the amount in the undigested sample. The recovery index was measured according to the equation *i*. Bioaccesibility index was calculated comparing the total amount of

bioactive compounds in the intestinal phase (IN+OUT) with the amount in the OUT fraction (serum-available), following the equation *ii*.

$$(i)\ Recovery\ index\ (\%) = \frac{PC_{DF}}{PC_{TM}} \times 100$$

Where PC_{DF} is the TPC or TFC (mg) in the digested fraction (CSF + PF) after each digestion phase (oral, gastric and intestinal) and PC_{TM} is the TPC or TFC (mg) quantified in the test matrix.

$$(ii)\ Bioaccessibility\ index\ (\%) = \frac{PC_{S}}{PC_{DF}} \times 100$$

Where PC_S is the TPC or TFC (mg) in the OUT sample after the dialysis phase and PC_{DF} is the TPC or TFC (mg) in the total digested sample (IN + OUT) after the dialysis phase.

2.5.- Determination of polyphenolic compounds

Polyphenolic profiles of all samples obtained in each phase of *in vitro* GID were determined by High Performance Liquid Chromatography (HPLC) following the methodology described by Genskowsky, Puente, Pérez-Álvarez, Fernández-López, Muñoz and Viuda-Martos (2016). A Hewlett-Packard HPLC series 1200 instrument, equipped with C_{18} column (Mediterranea sea_{18}, 25 × 0.4 cm, 5 µm particle size) from Teknokroma, (Barcelona, Spain) was used. Phenolic compounds were analyzed, in standard and sample solutions, with a gradient elution of 1 mL/min. The mobile phases used were formic acid in water (1:99, v/v) as solvent A, and acetonitrile as solvent B. The chromatograms were recorded at 280, 320 and 360 nm. The identification of polyphenolic compounds was carried out by comparing UV absorption spectra and retention times of each compound with those of pure standards injected in the same conditions. When standards were unavailable, the compounds were tentatively identified by comparing their UV/Vis spectra with previously published data (Medina-Medrano, Almaraz-Abarca, González-Elizondo, Uribe-Soto, González-Valdez, & Herrera-Arrieta, 2015; Martínez-Las Heras, Quifer-Rada Andrés, & Lamuela-Raventós, 2016). Quantification of phenolic acids and flavonoids were executed based on linear curves of authentic standards.

2.6.- Antioxidant activity

To assess the antioxidant activity, four methods were used:

DPPH radical scavenging assay: The free radical scavenging activity was measured according to the methodology described by Brand-Williams, Cuvelier, and Berset (1995) using the stable radical DPPH. Absorbance values were measured on a spectrophotometer at 517 nm. Results were expressed as mg Trolox equivalent (TE)/g sample.

ABTS radical cation (ABTS•+) scavenging activity assay: This method was determined as described by Leite, Malta, Riccio, Eberlin, Pastore, & Marostica (2011). Absorbance values were measured on a spectrophotometer at 734 nm. The results were expressed as mg Trolox equivalent (TE)/g of sample.

Ferric reducing antioxidant power (FRAP): This method was determined using the methodology described by Oyaizu (1986). The FRAP values were measured on a spectrophotometer at 700 nm and the results estimated in mg Trolox equivalents (TE)/g of sample.

Ferrous ion-chelating ability assay (FIC): Ferrous ions chelating activity was measured by inhibiting the formation of Fe2+-ferrozine complex, following the method of Carter (1971). Absorbance values were measured on a spectrophotometer at 532 nm. Results were expressed as mg EDTA equivalent/g sample.

2.7.- Statistical Analysis

The results were expressed as the mean ± SD of 2 parallel trials (n=4). Data obtained for each digestion phase and persimmon flour were analyzed by means of a two-way ANOVA test with two factors: cultivar and digestion phase. Tukey's post hoc test was applied for comparisons of means; differences were considered significant at $p<0.05$. Statistical analyses were carried out using the statistical package SPSS 19.0 (SPSS Inc., Chicago, IL.).

3.- Results and Discussions

3.1.- Recovery and bioaccessibility indexes

Recovery index for the TPC and TFC after each digestion phase (oral, gastric and intestinal) can be observed in Figure 2.

As regard the recovery index for the TPC, no statistical differences ($p>0.05$) were found between 'Rojo Brillante' (RB) and 'Triumph' (TH) flours after each digestion phase, except for the oral phase. In this phase, TPC recovery index for the RB flour didn't change ($p<0.05$) with respect to undigested sample, whereas in the case of TH flour the recovery index value was lower than undigested sample ($p<0.05$). The highest recovery index values were showed after the gastric phase ($p<0.05$), whereas the lowest recovery index values were found in the last phase of gastrointestinal digestion ($p<0.05$). A similar situation was detected for the TFC, although in this case, after the oral phase, the recovery index was significantly higher for RB than in undigested sample ($p<0.05$).

These results showed that oral digestion phase affected the persimmon flours and bioactive compounds in different ways, since the release of polyphenol compounds from persimmon flour matrix was dependent on the cultivar and type of bioactive compounds ($p<0.05$). These facts could be due to: (*i*) the different composition of persimmon flours, mainly related to dietary fiber content (total dietary fiber (TDF) is higher in TH than RB flour, and also their content in insoluble dietary fiber (Lucas-González et al., 2017); (*ii*) the interaction of polyphenolic compounds with α-amylase, thus, some authors have reported antidiabetic effects in persimmon leaves and fruits, due to their interactions (Lee et al., 2006; Kawakami, Aketa,

Nakanami, Iizuka, & Hirayama, 2010). In the literature, Martínez-Las Heras, Pinazo, Heredia, & Andrés (2017) reported a similar recovery index for phenolic and flavonoids compounds in peel-fiber from RB persimmon after the oral phase, whereas the decreases of phenolic compounds after the oral phase have been previously reported by Pellegrini et al. (2017) in different quinoa seeds.

The differences showed in TH flour between digestion phases could be explained by their higher TDF content and also due to the different pH of the digestion phases; the acid medium in the gastric phase promotes the break of bonds between bioactive compounds and nutrients, like fiber, protein and carbohydrates, helping to the remain of polyphenolic compounds in the food matrix (Alminger et al., 2014). Other authors have reported increases in polyphenol content in different vegetable products after gastric digestion (Chandrasekara et al, 2011; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013).

The decrease in the amount of polyphenols after intestinal digestion has been widely reported by the scientific community (Ortega et al., 2011; Rodríguez-Roque et al., 2013; Lucas-González, Navarro-Coves, Pérez-Álvarez, Fernández-López, Muñoz, & Viuda-Martos, 2016). The drastic losses in bioactive compounds after intestinal digestion, probably were due to different factors: (*i*) interactions with other dietary compounds, like fiber, protein, carbohydrate and minerals, (*ii*) chemical reactions, mainly oxidation and polymerization, affording the formation of other phenolic derivatives, such as chalcones (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001) or, (*iii*) changes in molecular structures due to enzymatic action and consequently in its solubility (Kroll et al., 2003).

The intestinal digestion is the largest phase in the GID, where the majority of nutrients are absorbed in the intestinal epithelium. The bioaccesibility can be defined as the amount of an ingested chemical compound that is available for absorption (Etcheverry et al., 2012). The bioaccesibility index for the TPC (Figure 3) was similar ($p>0.05$) in both flours. Additionally, the bioaccesibility for flavonoids was dependent on persimmon cultivar (Figure 3), since significant differences were found in their bioaccesibility index value ($p<0.05$), showing TH flour higher values (45.31 %) than RB flour (21.54 %) ($p<0.05$). This fact is probably due to the different flavonoid profile of both persimmon flours, as can be observed in Table 1. According with these results, a substantial amount of phenolic and flavonoid compounds remained into the IN fraction (colon-available) and could be metabolized by colonic bacteria, transforming dietary polyphenols in simple phenolic compounds, which could result in metabolites more biologically active than the original compounds (Selma, Espín, & Tomás-Barberá, 2009). This fact could be due to the complex matrix of persimmon flours, which are rich in sugars and insoluble fiber, with presence of protein and minerals (Lucas-González et al., 2017). Because of this composition, multiple interactions can occur between dietary components and bioactive compounds, which could produce a reduction in their bioaccessibility. In the scientific literature Gullón et al. (2105a) reported a value of 64.02

% for the bioaccessibility index of flavonoids in pomegranate peel flour, while the bioaccessibility index of polyphenolic compounds present in date pits flour and apple bagasse flour, at the end of intestinal digestion, were 78.54 and 91.58%, respectively (Gullón, Pintado, Barber, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2015b).

3.2.- Stability of polyphenolic compounds during simulated in vitro gastrointestinal digestion

Polyphenols detected after *in vitro* GID are showed in Table 1. In both persimmon flours, RB and TH, the following compounds have been identified: gallic acid, 4-hydroxibenzoic acid, epicatechin, quercetin-*o*-pentoside I, quercetin, kaempferol-*o*-rhamnoside and ellagic acid. Furthermore, in TH flour, the following flavonoid compounds have been detected: quercetin-*o*-hexoside, quercetin-*o*-pentoside II, kaempferol-*o*-hexoside I and kaempferol); and in RB flour: coumaric acid-*o*-hexoside and kaempferol-3-*o*-glucoside. The majority compounds in both studied persimmon flours were gallic acid and 4-hydroxybenzoic acid, furthermore the results showed that RB had more phenolic acids than TH flour ($p<0.05$) and TH flour had more presence of flavonoids compounds than RB flours ($p<0.05$). The polyphenols identified have been previously detected in persimmon fruit and leaves by different authors (Martínez-Las Heras et al., 2016; Lucas-González, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2018).

The GID had different repercussions in the stability of the polyphenols detected in RB and TH flours. After the oral and gastric digestion all polyphenols identified in both persimmon flours were detected. However, both factors, digestion phases and flour type, affected, in different way, their stability and release from food matrix. Thus, after the oral digestion the phenolic acids concentration increased 160.6% and 176.7% in RB and TH flours, respectively; begin gallic acid, which presented the highest release from food matrix. The stability of flavonoid compounds was compromised in this phase and a significant decrease was observed in epicatechin, quercetin (only in TH flour) and in all glycoside flavonoids ($p<0.05$), with the exception of kaempferol-3-*o*-glucoside, detected in RB flour, whose concentration didn't change compared to undigested sample ($p>0.05$). As regard to ellagic acid, their concentration didn't change in RB flour, whereas in TH flour significant losses were observed ($p<0.05$). Similar behavior was observed in polyphenols stability after the gastric digestion; phenolic acids increased their concentration, showing significant differences with oral phase ($p<0.05$); with exception of coumaric acid-*o*-hexoside, which didn't show variation in their concentration in oral and gastric phases with respect to undigested sample. The most remarkable fact was that the concentration of glycoside flavonoids present in TH flour increased their concentration with respect to oral phase ($p<0.05$), whereas in RB flour not significant differences ($p>0.05$) were observed. The increase in polyphenolic compounds after gastric phase could be due to the breakage of the bonds with the dietary components of the persimmon flours, proteins and fiber, induced by the acid medium. These facts, highlight the different behavior of flavonoids after GID, and confirm the TFC recovery

index showed in both persimmon flours. From the 17 polyphenols detected in persimmon flours only six appeared after intestinal phase: 4-hydroxybenzoic acid, ellagic acid and the glycosylated flavonoids (with exception of quercentin-*o*-pentoside II and kaempferol-3-*o*-glucoside). All these compounds showed a decrease in their concentration with respect to other phases and to undigested sample ($p<0.05$); with the exception of 4-hydroxybenzoic acid, which reported the biggest concentration in this phase ($p<0.05$). Rodríguez-Roque et al. 2013 also reported an increase of 4-hydroxybenzoic acid from soymilk after the intestinal phase respect to gastric and undigested sample. On the other hand, the distribution of these compounds in the intestinal fractions, IN and OUT, were dependent on the individual compound and persimmon flour type ($p<0.05$).

Other authors have detected glycoside flavonoids, like rutin, quercetin-3-galactoside, cyanidin-3-glucoside, quercetin-*o*-(rhamnosyl)rutinoside or kaempferol-*o*-rutinoside and ellagic acid and 4-hydroxybenzoic acid after *in vitro* digestion in different vegetable products (Lucas-Gonzalez et al., 2016; Pellegrini et al., 2017; Pinto, Spínola, Llorent-Martínez, Fernández-de Córdova, Molina-García, & Castilho, 2017). These results were in agreement with the results showed in the current work and would indicate that glycoside compounds are more stable to gastrointestinal digestion than aglycone compounds. Furthermore, the instability of gallic acid and kaempferol after intestinal digestion has been previously reported in different vegetable extracts (Schulz et al., 2017).

The losses of polyphenol compounds after intestinal digestion could be due to alkaline pH values (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010), interaction with other dietary compounds and/or interaction with bile salts, since Kida, Suzuki, Matsumoto, Nanjo, and Hara (2000) have identified biliary metabolites of (-)-epigallocatechin gallate in rats after oral administration.

3.3.- Antioxidant properties

Bioactive compounds have different mechanism for developing their antioxidant activity, such as hydrogen atom, single electron transfer or metal chelation (Leopoldini, Russo, & Toscano, 2011). There is not an unique method to evaluate all antioxidant mechanism, so in this work, different methods have been used to evaluate the antioxidant activity of the undigested and digested samples of persimmon flours (Table 2).

From the four methods used (DPPH, ABTS•+, FRAP and FIC) only the ABTS•+ values showed in the undigested samples of both flours were significantly different ($p<0.05$). However, the antioxidant activity of both persimmon flours was affected by the GID in different way, highlighting the dependence on cultivar and gastrointestinal phase in their antioxidant activity. After *in vitro* GID, the DPPH radical scavenging activity of RB flour decreased gradually from the oral phase to intestinal phase ($p<0.05$). A similar behavior was observed in TH flour, however the

highest loss in their DPPH radical scavenging activity was observed after gastric digestion (25.8 %). Martínez-Las Heras et al. (2017) reported similar results in peel and pulp fiber obtained from persimmon cv. RB undergoing an *in vitro* GID. Other authors have informed an increase in DPPH radical scavenging activity after gastric and intestinal digestion in different vegetable samples (Burgos-Edwards, Jiménez-Aspee, Thomas-Valdés, Schmeda-Hirschmann, & Theoduloz , 2017). As regards to ABTS•+ after oral digestion, the ABTS•+ radical scavenging activity of both persimmon flours didn't change with respect to undigested flours ($p>0.05$). After gastric digestion, RB and TH flours showed an increase of 25.37 and 32.73%, respectively ($p<0.05$). However, the intestinal digestion compromises their ABTS•+ radical scavenging activity in both persimmon flours ($p<0.05$). The losses in the antioxidant activity measured by ABTS•+ assay after intestinal digestion and dialysis phase have been previously reported in onion, lettuce and tomato (Gawlik-Dziki, 2012).

The ability of RB flours to reduce Fe^{+3} to Fe^{+2} didn't change after oral and gastric digestion, however, after intestinal digestion, a significant decrease ($p<0.05$) in their metal reduction activity (60.1 %) was observed. As regards to TH flour, the oral digestion didn't affect their metal reduction ability ($p<0.05$), however, after gastric digestion, a significant increase in their antioxidant activity ($p>0.05$) was observed, followed by a drastic decrease (66.6 %) in their metal reduction activity after the intestinal phase ($p>0.05$). Lucas-González et al. (2016) also reported an increase (24.8 %) in the ferric reduction activity power after gastric phase in lyophilized maqui. The negative influence of the intestinal digestion on the reducing power activity has been previously reported by Neto et al. (2017).

As regard to the ability to chelate metals, determined by FIC method, both persimmon flours, experimented the same behavior after GID: after oral phase, their chelate metals ability was not affected ($p>0.05$), while after gastric digestion, significant losses were observed ($p<0.05$). Whereas, after intestinal phase their antioxidant activity increased with respect to gastric phase ($p>0.05$) and was similar to undigested samples ($p>0.05$). This increase could be due to the fact that several compounds with chelating activity, like reducing carbohydrates, tocopherols and carotenoids, could have been solubilized and probably, the antioxidant activity showed were mediated by these bioactive compounds (Gullón et al., 2015a). Other authors have reported some increase in FIC values in different antioxidant products after intestinal phase (You, Zhao, Regenstein, & Ren, 2010; Pellegrini et al., 2017). These results showed that a high part of the antioxidant activity of these persimmon flours could arrive to the large intestine and to play a protective role against oxidation reactions.

The distribution of antioxidant activity between both intestinal fractions, IN and OUT, was dependent on cultivar and antioxidant method, as can be observed in Table 2. Thus, the antioxidant activity determined by DPPH assay in RB flour showed the same activity in both

fractions ($p>0.05$), whereas TH flour reported the highest antioxidant activity in IN fraction ($p<0.05$). In the case of ABTS•+ radical scavenging activity, RB and TH flour showed higher activity in IN fraction and OUT fraction, respectively. The same behavior was observed in the distribution of their antioxidant activity determined by FRAP and FIC methods ($p>0.05$)

In the scientific literature, there are contradictory works about the antioxidant activity obtained in the serum and colon available fractions. Thus, in the case of lyophilized maqui, the antioxidant activity determined with different methods (DPPH, FRAP, FIC and ABTS•+) after dialysis phase, was higher in the serum-available fraction than in colon-available fraction (Lucas-González et al., 2016). However, in pomegranate peel flour, the antioxidant activity measured by FRAP, DPPH, ABTS•+ and ORAC, was higher in the colon-available fraction than in the serum-available fraction (Gullón et al., 2015a). Probably, the content of total dietary fiber and their nature in the raw material, determines the release of polyphenol compounds from food matrix.

4.- Conclusion

The best of our knowledge is the first time that polyphenols profile in persimmon flours from juice co-products of cultivars 'Rojo Brillante' and 'Triumph', has been studied after *in vitro* gastrointestinal digestion. Recovery index for total phenolic and flavonoids content was dependent on persimmon flour type and digestion phase, being intestinal phase which showed the highest effect on their recuperation. The bioaccesibility index for total phenol and flavonoid content, as well as, individual polyphenols detected, were strongly affected after gastrointestinal digestion; however, some polyphenol compounds remained as such at the end of the digestion, especially the glycosylated flavonoids and 4-hydroxibenzoic acid, which increased their concentration after gastrointestinal digestion. Their antioxidant activity also was affected, showing that their protective action against free radicals and their ability to reduce metals increased after the gastric phase, whereas after the intestinal phase, their metal chelating activity improved. For these reasons, persimmon flours of cvs. 'Rojo Brillante' and 'Triumph' could be included in the formulation of foods to improve either their scarcity of bioactive compounds or an unbalanced nutritional composition.

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Table 1. Polyphenolic profile of undigested and digested samples (oral, gastric and intestinal phase) of persimmon flours: 'Rojo Brillante' (RB) and 'Triumph' (TH).

		Undigested sample	Oral phase	Gastric phase	Intestinal phase		
					IN	OUT	Total
Phenolic acids							
Gallic acid (mg/g)	RB	3±1^{c}	6±0^{b}	7±1^{a}	ND.	ND.	-
	TH	2±0^{b}	4±0^{c}	4±0^{c}	ND.	ND.	-
4-Hydroxybenzoic acid (µg/g)	RB	200±20^{c}	220±10^{c}	300±10^{b}	270±20^{x}	240±10^{x}	510±40^{a}
	TH	110±10^{d}	89±15^{d}	200±7^{c}	110±6^{y}	230±40^{x}	340±30^{b}
Coumaric acid-*o*-hexoside (µg/g)	RB	24±5^{a}	21±1^{a}	20±3^{a}	ND.	ND.	-
	TH	ND.	ND.	ND.	ND.	ND.	-
Flavonoids							
Epicatechin (µg/g)	RB	36±4^{a}	22±2^{b}	19±2^{b}	ND.	ND.	-
	TH	33±3^{a}	18±4^{b}	23±3^{b}	ND.	ND.	-
Quercetin-*o*-hexoside (µg/g)	RB	ND.	ND.	ND.	ND.	ND.	-
	TH	110±20^{a}	45±2^{c}	67±1^{b}	11±0^{x}	14±2^{x}	25±2^{c}
Quercetin-*o*-pentoside I (µg/g)	RB	54±1^{c}	44±1^{d}	36±2^{f}	15±4^{x}	16±3^{x}	31±1^{g}
	TH	110±20^{a}	45±4^{d}	71±2^{b}	14±0^{y}	19±3^{x}	33±2^{g}
Kaempferol-3-*o*-glucoside (µg/g)	RB	5±1^{a}	5±0^{a}	4±1^{a}	ND.	ND.	-
	TH	ND.	ND.	ND.	ND.	ND.	-
Quercetin-*o*-pentoside II (µg/g)	RB	ND.	ND.	ND.	ND.	ND.	-
	TH	23±3^{a}	16±1^{b}	17±1^{b}	ND.	ND.	-
Kaempferol-*o*-hexoside I (µg/g)	RB	ND.	ND.	ND.	ND.	ND.	-
	TH	19±3^{a}	8±1^{bc}	12±0^{b}	3±1^{x}	3±0^{x}	7±1^{c}
Kaempferol-*o*-rhamnoside (µg/g)	RB	12±1^{a}	5±0^{c}	4±1^{c}	3±0^{x}	1±0^{y}	5±0^{c}
	TH	14±2^{a}	8±1^{b}	9±0^{b}	3±0^{x}	2±0^{y}	5±0^{c}
Quercetin (µg/g)	RB	1±0^{c}	1±0^{c}	2±0^{c}	ND.	ND.	-
	TH	17±3^{a}	9±0^{b}	12±0^{b}	ND.	ND.	-
Kaempherol (µg/g)	RB	ND.	ND.	ND.	ND.	ND.	-
	TH	2±0^{a}	2±0^{a}	2±0^{a}	ND.	ND.	-
Ellagictannins							
Ellagic acid (µg/g)	RB	8±0^{b}	6±1^{b}	16±2^{a}	4±1^{x}	2±0^{y}	6±1^{b}
	TH	8±2^{b}	4±1^{c}	4±0^{c}	1±0^{x}	1±0^{x}	3±0^{c}

For the same polyphenol, values with same lower case letter (a–g) are not significantly different among digestion phases and cultivar ($p > 0.05$) according to Tukey's Multiple Range Test.

For the same polyphenol and different intestinal fraction (IN and OUT) values with same lower case letter (x–y) are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.

Table 2.- Antioxidant properties of the two fractions (pellet fraction and soluble chime fraction) obtained after each phase (oral, gastric and intestinal) of *in vitro* gastrointestinal digestion of persimmon flours cultivars 'Rojo Brillante' (RB) and 'Triumph' (TH) measured with DPPH, FRAP, FIC and ABTS•+ assays.

		DPPH (mg TE /g)		**FRAP (mg TE /g)**		**FIC (mg EDTA eq. /g)**		**ABTS (mg TE / g flour)**	
		RB	TH	RB	TH	RB	TH	RB	TH
Test matrix		3.2 ± 0.1^{a}	3.4 ± 0.0^{a}	5.3 ± 0.4^{b}	5.6 ± 0.1^{b}	0.1 ± 0.0^{a}	0.1 ± 0.00^{a}	2.0 ± 0.1^{c}	3.0 ± 0.0^{b}
Oral phase	Total	3.1 ± 0.1^{ab}	2.7 ± 0.1^{b}	5.4 ± 0.6^{b}	3.1 ± 0.2^{c}	0.1 ± 0.0^{a}	0.1 ± 0.01^{a}	2.0 ± 0.2^{c}	3.0 ± 0.5^{b}
	% Var.	-2.4	-21.2	2.5	-44.9	-0.6	-14.6	2.9	-2.1
Gastric phase	Total	2.7 ± 0.2^{b}	2.5 ± 0.1^{bc}	5.1 ± 0.7^{b}	7.3 ± 0.3^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	2.4 ± 0.1^{bc}	4.0 ± 0.2^{a}
	% Var.	-13.1	-25.8	-2.8	30.8	-82.2	-85.1	25.4	32.7
Intestinal phase	IN	1.2 ± 0.1^{B}	2.0 ± 0.4^{A}	1.1 ± 0.2^{A}	0.9 ± 0.2^{A}	0.2 ± 0.0^{A}	0.1 ± 0.0^{B}	0.8 ± 0.0^{A}	0.3 ± 0.0^{B}
	OUT	1.0 ± 0.0^{B}	0.7 ± 0.1^{B}	1.0 ± 0.1^{A}	0.9 ± 0.2^{A}	0.1 ± 0.0^{C}	0.0 ± 0.0^{C}	0.5 ± 0.0^{B}	0.7 ± 0.1^{A}
	Total	2.2 ± 0.1^{c}	2.7 ± 0.2^{b}	2.1 ± 0.5^{d}	1.9 ± 0.4^{d}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	1.2 ± 0.1^{d}	1.1 ± 0.1^{d}
	% Var.	-30.8	-7.2	-60.1	-66.6	17.3	8.7	-35.9	-61.8

% Var.: Percentage of variation between the initial values and the values obtained after digestion.

For the same antioxidant assay, values with same lower case letter (a–f) are not significantly different among digestion phases and cultivar ($p > 0.05$) according to Tukey's Multiple Range Test.

For the same antioxidant assay, values followed with same upper case letter (A–B) are not significantly different among the same digestion phase and cultivar ($p > 0.05$) according to Tukey's Multiple Range Test

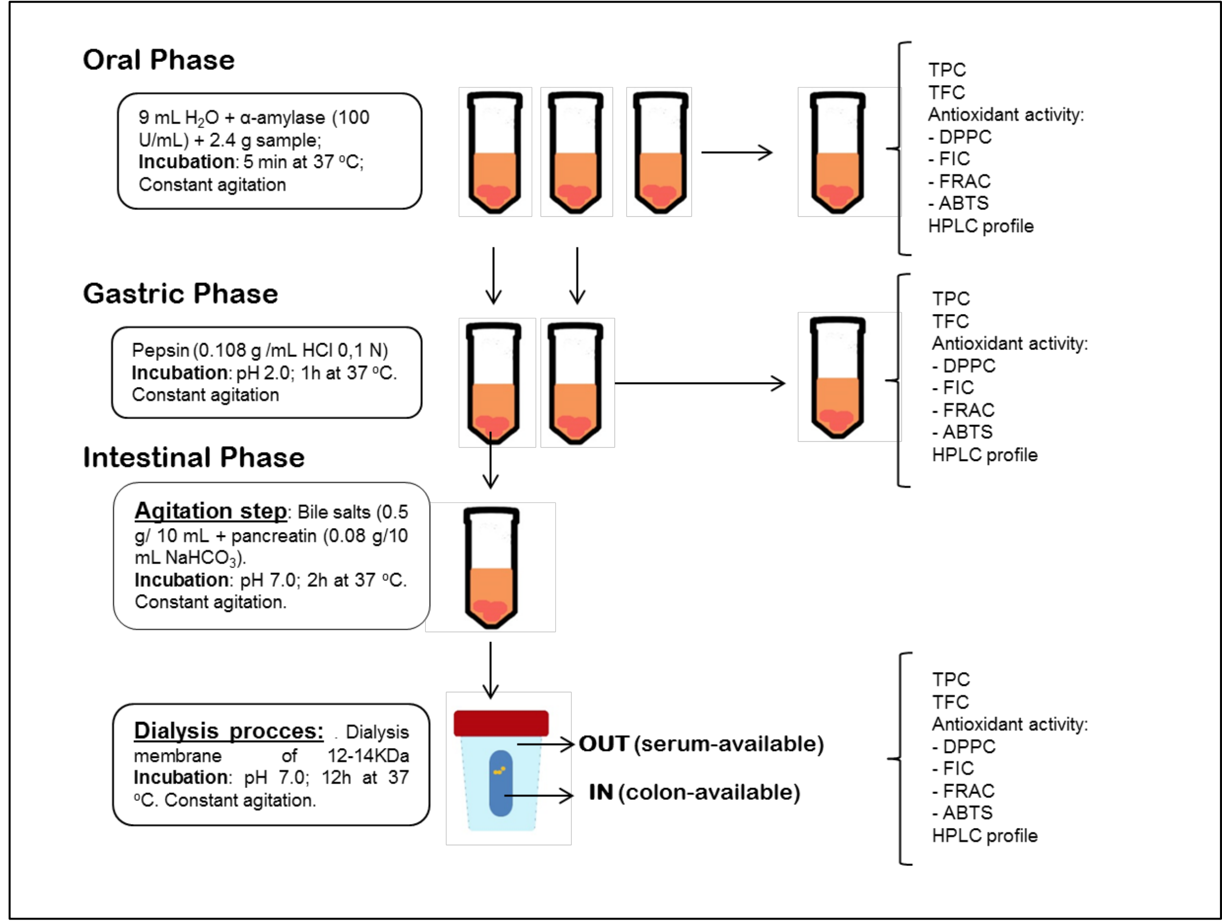

Figure 1.- Schematic representation of *in vitro* gastrointestinal digestion method carried out on persimmon flours.

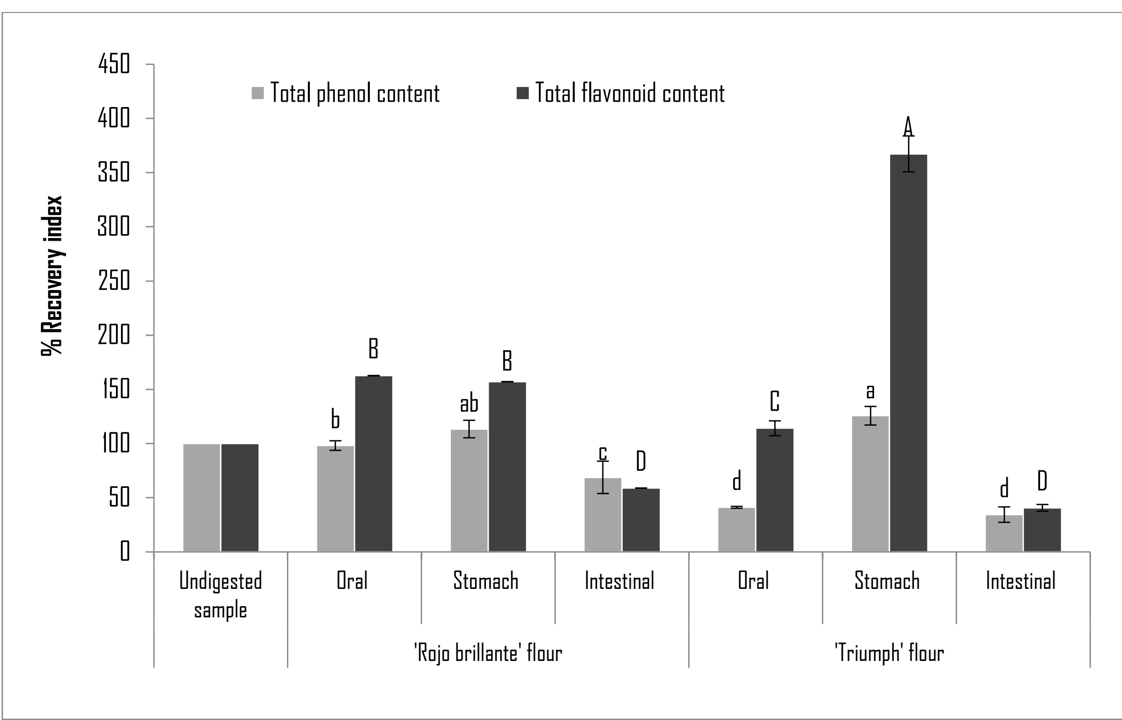

Figure 2.- Recovery index of total phenolic and total flavonoid content, obtained after each phase (oral, gastric and intestinal), of *in vitro* gastrointestinal digestion from persimmon flours (cv. 'Rojo Brillante' and 'Triumph').

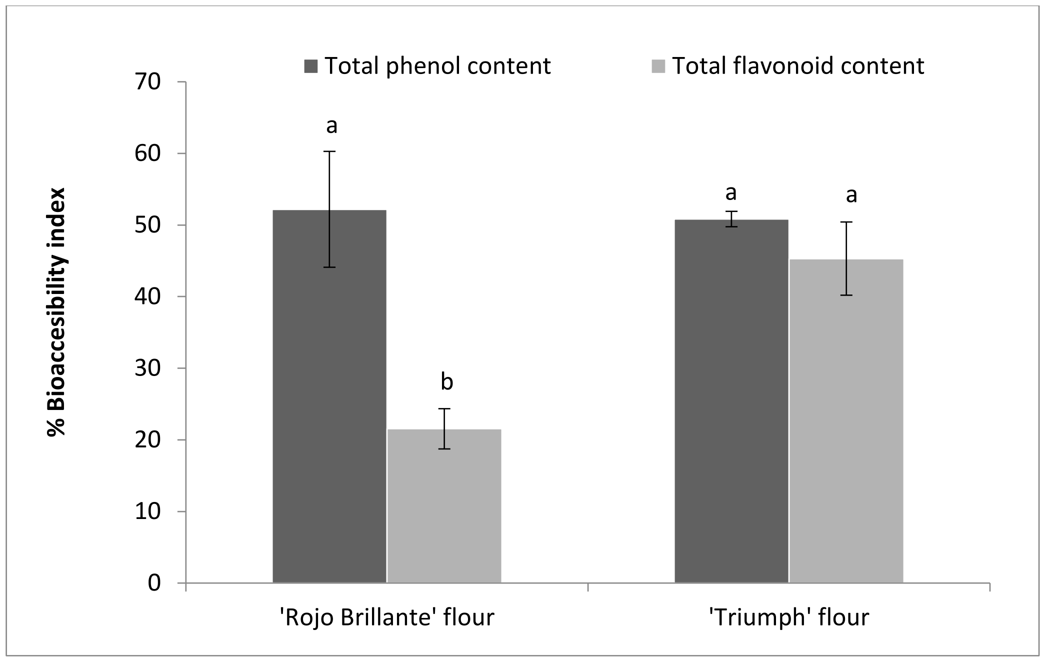

Figure 3.- Bioaccessibility index of total phenolic content and total flavonoids content after intestinal phase of *in vitro* gastrointestinal digestion from persimmon flours (cultivars 'Rojo Brillante' and 'Triumph').

7.5.- PUBLICACIÓN 5

Título: Persimmon (*Diospyros kaki* Thunb.) coproducts as a new ingredient in pork liver pâté: influence on quality properties

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Persimmon (*Diospyros kaki* Thunb.) coproducts as a new ingredient in pork liver pâté: Influence on quality properties

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Running title: Persimmon Flours Added To Pâté

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Abstract

Persimmon (*Diospyros kaki* Thunb.) coproducts are rich in fiber, sugar, minerals and bioactive compounds, make it a good candidate for use in the meat industry. The aim was to evaluate the effect of adding (3% and 6%) two different types of persimmon flour (from cultivars 'Rojo Brillante' and 'Triumph') on the chemical composition (proximate composition, heme iron and nitrite residual level), physicochemical properties (pH, water activity and color), lipid oxidation, emulsion stability, texture and sensory acceptance of pork liver pâté. The addition of persimmon flours decreased residual nitrite levels in pâté to an extent that depended on the concentration and type of flour. Lipid oxidation in pâtés was also reduced by persimmon flour addition, an effect made evident by the increase in pâté redness, which could allow its application as natural antioxidant. In a sensory analysis, all the pâtés with persimmon flour added were better accepted than the control.

Key words: meat industry; persimmon; coproducts; natural antioxidant; nitrite residual.

INTRODUCTION

Liver pâté is a meat paste product, whose ingredients are mainly liver and fat. It is highly appreciated by consumers and has a long gastronomic tradition in Europe. Furthermore, liver pâtés are a good source of biologic value proteins, vitamins like B_1, B_{12} and folic acid, and heme iron (Brito *et al.,* 2006), making them an even more attractive product, especially for children and women, who may suffer anaemia from iron deficiency, a common disease throughout the world (WHO, 2015). Because of the manufacturing process involved (chopping, cooking), and their chemical composition (high amounts of fat and heme iron, and the low level of antioxidants), they are highly susceptible to oxidation (Russell *et al.,* 2003). For this reason, different preservatives, like sodium nitrite and/or synthetic antioxidants (butylated hydroxytoluene, butylated hydroxyanisole) have been widely used in pâtés (Pateiro *et al.,* 2015). However, the potential health risks related to residual nitrite or its derivative products (N-nitroso compounds), the fact that some lipid and protein oxidation products have been linked with the development of cancer (Joosen *et al.*, 2009), and even the potential health hazards associated with the use of synthetic antioxidants, have led the meat industry to look for alternatives to reduce residual nitrite levels and for natural antioxidants or naturally occurring compounds with antioxidant activity. For this reason, and taking into account the high nutritional value of pâté, it would clearly be of interest to identify natural ingredients able to prevent pâté oxidation, maintain heme iron levels, reduce residual nitrite levels and, ultimately, to improve its overall quality.

Coproducts from the agrifood industry are regarded as a good source of natural antioxidants, which therefore have a double function: (i) improving meat products by preventing oxidation and providing nutrients such as minerals, vitamins and fiber; and (ii) valorising the coproduct in question, which entails the reduction of waste and, possibly, attaining the price of synthetic antioxidants. In this context, persimmon coproducts (from the persimmon juice industry) are rich in fiber, sugar, minerals and bioactive compounds like carotenoids and phenolic acids (Akter *et al.,* 2010; Hwang *et al.,* 2011; Lucas-González *et al.,* 2017, 2018), making them good candidates for use as natural antioxidants. Furthermore, several studies in mice and rats have revealed interesting effects (hypocholesterolemic, hypolipidemic and antioxidant) associated with the intake of persimmon fruit, (Gorinstein *et al.,* 1998; Matsumoto *et al.,* 2010). These physiological properties suggest that the consumption of persimmon as an accompaniment to high fat foods could be a very interesting option, because the persimmon components would help counteract the negative connotations associated with the intake of high fat foods.

Taking all this into consideration, the aim of the current work was to evaluate the effect of adding different concentrations (3 % and 6 %) of two different types of persimmon flour (obtained from the juice coproducts of the cultivars 'Rojo Brillante' and 'Triumph') on the chemical composition, physicochemical properties, lipid oxidation, and sensory acceptance of

liver pork pâté, and to compare the effects observed with a control pâté (without persimmon flours).

MATERIALS AND METHODS

Manufacture of liver pâtés

For this study, five batches (1 kg each) of liver pâté were prepared: control pâté (CP), pâté with 3% 'Rojo Brillante' flour (PRB-3), pâté with 6% of 'Rojo Brillante' flour (PRB-6), pâté with 3% 'Triumph' flour (PT-3) and pâté with 6% 'Triumph' flour (PT-6). The pâtés were prepared in the food pilot plant of the Orihuela Campus (Miguel Hernández University). The formula was the same for all batches, except for the addition of different types and amounts of persimmon flour (obtained from persimmon industrialization coproducts). All the ingredients (Table 1) were obtained from a local market (Orihuela, Alicante, Spain). The persimmon flours derived from the cultivar 'Rojo Brillante' and 'Triumph' were obtained as described by Lucas-González *et al.* (2017). The particle size of both persimmon flours was <0.210 mm.

The pâté manufacturing process was as follows: the pork liver was chopped and soaked in cold water for 10 min and the dewlap was left in boiling water for 15 min. Then the dewlap, backfat and salt were mixed in a bowl mixer with a spiral dough hook (Tecator 1094 Homogeneizer, Tekator, Höganäs, Sweden) until the particle size was less than 2 mm. Then the additives were added and mixed for 1 minute, before adding the spices and persimmon flour and mixing for another minute. To generate the emulsion, hot water (80ºC) was added. The emulsion formed was then stuffed into artificial casings Fibran-Pack (Fibran, Girona, Spain) of 5 cm diameter and 10 to 15 cm length, which were clipped at both ends (Polyclip system/ Niedecker, Germany) and heated in hot water (95 °C) until the core attained 72 °C (approximately 40 min). When the end point temperature was achieved, the samples were chilled in ice/water until analysis. The batches were made in triplicate.

Chemical composition

Moisture, fat, protein and ash content were determined according to AOAC methods (AOAC, 2007).

Residual nitrite level was determined by following the standard ISO/DIS 2918.26 (ISO/DIS*, 1975*). The results were expressed as ppm $NaNO_2$.

Physicochemical properties

The pH of samples was measured after homogenizing 5 g of sample with 25 mL of distilled water, using a pH-meter (Model 507, Crison Instruments S.A., Barcelona, Spain) at 25 ºC.

Color parameters on the surface of the pâté formulations were measured at room temperature. CIELAB coordinates, lightness (L* ± white-black), redness (a* ± red-green) and yellowness (b*

± yellow-blue), were determined using a CM-2600d colorimeter (Minolta Camera Co., Osaka, Japan) with the following settings (illuminant D65, observer 10°, SCI mode, 11 mm aperture for illumination and 8 mm for measurement).
Water activity was determined at 25 °C in a Novasina thermoconstanter humidity meter (TH-500, Axair Ltd., Pfaeffikon, Switzerland).

Lipid oxidation

Lipid oxidation was evaluated by the 2-thiobarbituric acid reactive substances (TBARS) test according to Rosmini *et al.* (1996). The results were expressed as mg malonaldehyde (MDA)/kg pâté.

Emulsion stability

Emulsion stability was determined following the method described by Hughes *et al.* (1997). Results were expressed as the percentage of total expressible fluid (% TEF), which was calculated as follows:

$$\%TEF = \frac{Weight\ centrifuge\ tube\ and\ sample - Weight\ centrifuge\ tube\ and\ pellet}{Weight\ sample} \times 100$$

Texture test

Texture profile analysis (TPA) was performed with a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, England). Samples from each batch were cut and placed in cylinders (1×1×1 cm) and subjected to a 2-cycle compression to 75% original height with a speed of 5 mm/s and at 20–25 °C. Cohesiveness, hardness, adhesiveness, springiness and gumminess were calculated (Bourne, 1978).

Sensory assay

Thirty experienced panelists (12 men and 18 women) with an age range of 20 to 55 selected from staff and students of the Miguel Hernández University (Orihuela, Alicante, Spain) carried out the sensory evaluation of the five formulated pork liver pâtés. All panelists were regular consumers of pâté. The test took place in the tasting room of Miguel Hernández University (Orihuela, Alicante, Spain). Rectangular pieces of approximately 1.5 x 2 cm were cut from the center of the slices and served at room temperature. A Quantitative descriptive analysis was carried out (IFT 1981). The panelists evaluated ten sensorial attributes: color intensity, brightness, rancidity, hardness, juiciness, particle detection, cohesiveness, greasiness, saltiness and sweetness. Fig. 1 shows the attribute scale of the sensory analysis. At the end of the test, panelists were asked to give a score for general acceptability of the product from 0 to 10.

Statistical analysis

All assays in the current work were carried out in triplicate, and the results are shown as mean ±standard deviation. For the statistical analysis of the results, one way analysis of variance (ANOVA) using SPSS 19.0 (SPSS Inc., Chicago, IL.) was performed for all the variables considered in the study. Tukey's post hoc test was applied for a comparison of means among the formulations at a significance level $p<0.05$.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the five pâtés is given in Table 2. The addition of 3% persimmon flours to the pork liver pâtés (PRB-3 and PT-3) lowered ($p<0.05$) the moisture content compared with control values, whereas no differences were observed in this respect when they were added at 6% (PRB-6 and PT-6). Controversial results on the moisture content of meat products due to the addition of fibrous ingredients (have also been reported by other authors. For example, López-Vargas *et al.* (2014) reported a decrease in moisture values of pork burger with added albedo-fiber powder obtained from yellow passion fruit coproducts. Ham *et al.* (2017) found no change in the moisture content of cooked sausage with lotus rhizome. Yogesh *et al.* (2015) reported lower moisture content in cooked meat batter incorporating various levels of cold milled flaxseed powder, than in a control without the flaxseed powder. The behavior of the moisture content in our pâté samples could be due to two main factors: the low moisture content of persimmon flours and their hydration properties (Lucas-González *et al.,* 2017); According that, our hypothesis would be that when persimmon flours were added at low concentrations (3%) the effect of their low moisture content was stronger than that of their hydration properties, but when persimmon flours were added at a high concentrations (6%), the hydration properties would make up for the effect of the low moisture content.

As regards the protein content, pâté samples with persimmon flour had lower values than the control pâté, especially in the case of 'Rojo Brillante' flour. However, only the PRB-6 samples presented significant differences with respect to the control pâté ($p<0.05$).

The ash content increased proportionally with the addition of persimmon flour ($p< 0.05$). The pâté samples with 'Triumph' flour showed higher values in both formulations than the corresponding pâté samples with 'Rojo Brillante' flour ($p<0.05$). Similar behavior was reported by others authors when fibrous ingredients were added to meat products (Sánchez-Zapata *et al.,* 2010; López-Vargas *et al.,* 2014).

Nitrate/nitrite is an additive widely used in the manufacture of meat products due to its antimicrobial activity, influence on meat color, the prevention of fat oxidation and to the development of the typical flavor in cured meats (Viuda-Martos *et al.,* 2009). However, their use needs to be controlled, due to the negative impact on human health (Joosen *et al.,* 2009; Viuda-

Martos *et al.,* 2009). While most of the nitrate/nitrite added to meat products is reduced to NO, which reacts with myoglobin to form nitric-oxymyoglobin (Viuda-Martos *et al.,* 2009), a fraction (residual nitrite level) does not and remains available to react with other compounds, generating, among other compounds, metahemoglobine and nitrosamine, which are harmful for human health (Joosen *et al.,* 2009). For this reason, the replacement or reduction of residual nitrite levels in meat products is an interesting strategy for developing healthier meat products. In this context, persimmon flours have the potential to be used as a nitrite reducing agent, since their addition to pork liver pâté produced a significant decrease in residual nitrite level ($p<0.05$). These results agree with those of other studies that focused on the ability of vegetable ingredients (Fernández-Ginés *et al.*, 2004; Viuda-Martos *et al.*, 2009; Wang *et al.*, 2015) to reduce residual nitrite levels in different meat products. In all these studies the bioactive compounds present in these vegetable ingredients seemed to be responsible for reducing residual nitrite levels through their ability to scavenge nitrite. It is important to remark that this reduction was concentration-dependent and was also influenced by the type of persimmon flour ('Triumph' flour lowered the residual nitrite level more than 'Rojo Brillante' flour) ($p<0.05$).

Physicochemical parameters

Pâté samples with added 'Triumph' flour (at both concentrations, 3 and 6%) showed lower ($p<0.05$) water activity values than the control samples (Table 3). However, the addition of 'Rojo Brillante' flour did not modify the water activity values. Although 'Rojo Brillante' flour has lower water activity values (0.384) than 'Triumph' flour (0.438), its water holding capacity (7.37 $\pm$0.73 g/g) is significantly lower than that of 'Triumph' flour (11.23 $\pm$0.56 g/g) (Lucas-González *et al.,* 2017). This fact could explain the lower availability of water in pâtés with added 'Triumph' flour. Other authors reported no changes in the water activity of meat products with added vegetable ingredients (Sánchez-Zapata *et al.*, 2010; López-Vargas *et al.*, 2014)

With respect to the pH values of the pâté samples (Table 3), this parameter seemed to be affected by the pH of persimmon flours, since a slight pH reduction was observed in pâtés with both flours at 3%, whereas a significant decrease ($p<0.05$) was observed when they were added at higher concentrations (6%). This reduction in pH was also influenced by the type of persimmon flour added since the pâté samples with 'Rojo Brillante' flour had lower pH values ($p<0.05$). Therefore, the reduction in pH values was dependent on both, the concentration and type of persimmon flour.

Table 3 details the surface color parameters (L*, a* and b*) of pâtés containing different concentrations of persimmon flours. As can be seen, the addition of the persimmon flours strongly affected the final product color. L* values decreased significantly in all the pâtés made with the persimmon flours ($p<0.05$), the lowest values being obtained with added 'Triumph' flour ($p<0.05$). This decrease in L* following the addition of persimmon flours, could be due to the hydration properties of this type of ingredient, leading to a corresponding decrease in surface water and so in light reflection. As regards redness, this parameter was significantly increased ($p<0.05$) in all pork the liver pâtés with added persimmon flours. In this case, the highest a* values ($p<0.05$) were observed in pâtés containing 'Triumph' flour. In fatty meat products (such as pâté), some authors have reported a negative correlation between fat content and redness (Terrasa *et al.*, 2016), which also seems to be the case in our study. This correlation was particularly evident: in PT-6 pâté, which had the lowest fat content (Table 2) and the highest a* value. Concerning yellowness, its behavior depended on the type of persimmon flour added. The b* values increased in pâtés with 'Triumph' flour ($p<0.05$) and decreased when 'Rojo Brillante' flour was added ($p<0.05$). In the case of all the color coordinates (L*,a* and b*), the variations due to the addition of 'Rojo Brillante' flour were concentration-dependent, whereas there were no significant differences between the values due to the concentrations of Triumph flour ($p>0.05$). The composition of these persimmon flours, mainly as regard their content of highly colored compounds (such as carotenes) could also be a contributory factor to the changes observed in the a* and b* coordinates.

Lipid oxidation

The oxidative stability of liver pâtés was measured based on the TBARs index (Fig. 2), which is frequently used as a marker of lipid oxidation. Lipid oxidation has negative connotations in meat products due to the generation of undesirable rancid off-flavors and to a decrease in the nutritional value of products. Several factors can affect the lipid oxidation of pâtés, such as the complex matrix, the manufacturing process and a high fat content (Russell *et al.,* 2003). Persimmon flour added to pâtés had a protective effect against lipid oxidation, since, all the pâtés containing the flours showed TBARs values lower than that of the control pâté ($p<0.05$). The presence of bioactive compounds, like polyphenols and carotenoids (Lucas-González *et al.,* 2017), in persimmon flours could be responsible for the decrease in the oxidative process in the studied pâtés. These bioactive compounds have antioxidant activity due to their capacity for hydrogen atom or single electron transfer and/or to chelate metal (Leopoldini *et al.,* 2011). The protective role of persimmon flours in the pork liver pâtés was influenced by the type of persimmon flour added, but not by the concentration. The 'Rojo Brillante' flour showed greater antioxidant activity than the 'Triumph' flour ($p<0.05$), while there were no differences between the concentrations used ($p>0.05$).

The differences seen in the antioxidant activity between the pâtés with added flours could be due to the differences in the phenolic and carotenoid content and composition. 'Rojo Brillante' flour had a high lycopene content, whereas only traces of lycopene were detected in 'Triumph' flour (Ancos *et al.,* 2000). This difference may determine their respective antioxidant activities, bearing in mind that lycopene is a more efficient antioxidant than β-carotene, α-carotene or α-tocopherol (Mascio *et al.,* 1989). Other authors have reported antioxidant effects of different vegetal ingredients in meat products (Martín-Sánchez *et al.,* 2013; Longato *et al.,* 2017). Liver pâtés contain high amounts of fat and iron, so that oxidative deterioration is the major deterioration reaction expected and one that is very significant for the product's shelf-life. Georgantelis *et al.* (2007) reported that a rancid flavor can be detected in meat products with TBARs values higher than 0.6 mg MDA/kg. In the present work, all pâtés with added persimmon flours had TBARs values below 0.6 mg MDA/kg. Based on these findings, persimmon flours have an important effect as pâté antioxidants and could be used as natural antioxidants, with a corresponding improvement in quality.

Emulsion stability

The presence of persimmon flour in the liver pâtés significantly decreased their emulsion stability compared to the control (Fig. 3), an effect that was concentration-dependent and influenced by the type of persimmon flour added. The addition of 'Triumph' flour led to the highest fluid losses ($p<0.05$), which was not unexpected because 'Rojo Brillante' flour has a higher

emulsifying activity (69 mL/mL) than 'Triumph' flour (58 mL/mL) (Lucas-González *et al.,* 2017). Furthermore, the higher soluble dietary fiber content reported for 'Rojo Brillante' flour compared with 'Triumph' flour (Lucas-González *et al.,* 2017) could increase the viscosity, which some authors highlight as an important factor for maintaining meat emulsion stability (Chasoy & Cock, 2017), by contributing to gel formation. Whatever the case, none of the pâté samples showed any visual evidence of emulsion problems at the moment they were elaborated.

Texture

Texture profile analysis (TPA) simulates the mastication process by submitting products to two compressions. The results of TPA are shown in Table 4. The control pâté and PRB-3 showed the highest hardness and springiness values ($p<0.05$), whereas the pâtés containing 'Triumph' flour showed the lowest values ($p<0.05$). Springiness is a measurement of elastic recovery after deforming force is removing. In this case, this property showed decrease trends when persimmon flours were added to pâté, with the exception of PRB-3 sample which value was similar to control ($p>0.05$). The negative value observed for adhesiveness indicated that liver pâtés were sticky, a property that the persimmon flours increased ($p<0.05$), probably due to the presence of monosaccharides (mainly glucose and fructose) (Lucas-González *et al.,* 2018) in the flours and their consequent caramelization during heat treatment. The addition of 'Rojo Brillante' flour increased the cohesiveness of pâté, whereas 'Triumph' flour diminished the same ($p<0.05$). Gumminess, defined as the energy needed to disintegrate a semi-solid food until it is smooth to swallow, decreased in the pâtés containing persimmon flours. This effect was concentration-dependent and also affected by the type of persimmon flour, the addition of 'Triumph' flour producing a higher reduction ($p<0.05$) than 'Rojo Brillante' flour. Pâté is a finely comminuted meat product composed of a mixture of proteins (soluble and insoluble proteins with particles of muscle fibers and connective tissue), fat globules, water, salt and spices. The addition of other ingredients disrupts this matrix and modifies all textural properties, as reported by other authors (Georgantelis *et al.,* 2007; Savadkoohi *et al.,* 2014). In this case, the texture changes observed in pâtés due to the addition of persimmon flours could be due to the differences in their fiber type and total fiber content, monosaccharides content, emulsion properties and gelling properties, among others (Lucas-González *et al.,* 2017).

Sensory evaluation

The sensory evaluation results are presented in Fig. 1. Among all the sensory attribute studied in pâtés (color intensity, brightness, rancidity, hardness, juiciness, particle detection, cohesiveness, greasiness, saltiness and sweetness) the panelists only found differences in color intensity between the control pâté and the others ($p<0.05$), a difference that was also detected in the objective color measurement (Table 3). Furthermore, all pâtés with persimmon flour obtained

good general acceptance scores of 6.72-7.16 on a scale of 10 (without differences between them, $p<0.05$). Surprisingly, the control pâté had the lowest scores for general acceptance (4.53). It is true that the only attribute detected by the panelists as different due to the addition of persimmon flour was the color intensity, but small changes in other attributes (while not significant) might also affect general acceptance.

CONCLUSIONS

All of these finding suggested than persimmon flours (obtained from persimmon juice coproducts from the cultivars 'Rojo Brillante' and 'Triumph') at two concentrations (3 and 6%) could be successfully used as natural ingredients in pork liver pâté, where they were found to improve general acceptation, and reduce both lipid oxidation and residual nitrite levels. The flour from 'Rojo Brillante' stood out for reducing pH and lipid oxidation, and its general control of emulsion stability in pâtés, whereas the flour from 'Triumph' persimmon was better at decreasing water activity and residual nitrite levels, and improving redness. For these reasons, although both persimmon flours could be used in this kind of meat product, the selection of either would depend on the main purpose of their application. The major applications of this ingredient in meat products are confirmed as being to act as natural antioxidant, colorant and nitrite reducing agent.

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Table 1.- Ingredients used for manufacturing the different pork liver pâté formulations.

Ingredients	CP	PRB-3	PRB-6	PT-3	PT-6
Dewlap (%)	65	65	65	65	65
Liver (%)	25	25	25	25	25
Backfat (%)	10	10	10	10	10
Water (%)	15	15	15	15	15
Salt (%)	2	2	2	2	2
Polyphosphates (mg/kg)	300	300	300	300	300
Nitrite (mg/kg)	125	125	125	125	125
Ascorbate (mg/kg)	500	500	500	500	500
Caseinate (%)	1	1	1	1	1
White pepper (%)	0.05	0.05	0.05	0.05	0.05
Nutmeg (%)	0.03	0.03	0.03	0.03	0.03
Thyme (%)	0.03	0.03	0.03	0.03	0.03
'Rojo Brillante' flour (%)	-	3	6	-	-
'Triumph' flour (%)	-	-	-	3	6

Percentages of non-meat ingredients are related to 100% meat block (dewlap, liver and backfat)

Control pâté (CP), pâté whit 3% of 'Rojo Brillante' flour (PRB-3), pâté whit 3% of 'Triumph' flour (PT-3), pâté whit 6% of 'Rojo Brillante' flour (PRB-6) and pâté whit 6% of 'Triumph' flour (PT-6).

Table 2.- Chemical composition of pork liver pâtés

	CP	PRB-3	PT-3	PRB-6	PT-6
Moisture (%)	56.96±0.26[a]	53.97±0.26[b]	54.77±0.51[b]	56.15±2.09[a]	57.40±0.40[a]
Fat (%)	27.49±0.34[b]	30.69±0.60[a]	28.55±0.70[b]	27.19±0.79[b]	24.67±0.02[c]
Protein (%)	13.45±0.36[a]	12.42±0.13[ab]	13.00±0.21[ab]	12.28±0.32[b]	12.46±0.14[ab]
Ash (%)	1.55±0.03[c]	1.70±0.02[c]	2.17±0.02[b]	2.19±0.03[b]	2.45±0.08[a]
Nitrite ($NaNO_2$ ppm)	28.96±0.30[a]	22.49±0.21[b]	14.20±0.19[c]	11.61±0.14[d]	10.58±0.14[e]

[a-d]Different letters within the same row indicate significant differences (P<0.05)

Control pâté (CP), pâté whit 3% of 'Rojo Brillante' flour (PRB-3), pâté whit 3% of 'Triumph' flour (PT-3), pâté whit 6% of 'Rojo Brillante' flour (PRB-6) and pâté whit 6% of 'Triumph' flour (PT-6)

Table 3.- Physicochemical properties of pork liver pâté formulations.

	Aw	pH	L*	a*	b*

PC	0.933±0.004^{a}	6.43±0.02^{a}	64.15±0.42^{a}	4.25±0.31^{c}	14.05±0.32^{b}
PRB-3	0.926±0.001^{ab}	6.37±0.02^{ab}	59.59±0.45^{b}	5.20±0.42^{b}	13.31±0.71^{c}
PT-3	0.925±0.002^{b}	6.38±0.04^{ab}	54.87±0.99^{d}	6.88±0.72^{a}	15.43±0.42^{a}
PRB-6	0.929±0.001^{a}	6.24±0.01^{c}	56.38±0.33^{c}	6.79±0.15^{a}	13.45±0.24^{bc}
PT-6	0.921±0.002^{b}	6.34±0.05^{bc}	54.46±0.21^{d}	7.41±0.53^{a}	15.59±0.42^{a}

$^{a-d}$Different letters within the same column indicate significant differences (P<0.05)

Control pâté (CP), pâté whit 3% of 'Rojo Brillante' flour (PRB-3), pâté whit 3% of 'Triumph' flour (PT-3), pâté whit 6% of 'Rojo Brillante' flour (PRB-6) and pâté whit 6% of 'Triumph' flour (PT-6).

Table 4.- Texture values of TPA analysis of pork liver pâtés.

	CP	PRB-3	PT-3	PRB-6	PT-6
Hardness (Kg)	2.12±0.00^{b}	2.96±0.56^{a}	1.70±0.03^{bc}	1.86±0.00^{bc}	1.24±0.08^{c}
Adhesiveness (g/s)	-64.21±20.80^{b}	-111.12±30.94^{ab}	-171.52±1.98^{a}	-161.79±0.00^{a}	-91.75±7.13^{b}
Springiness (mm)	0.21±0.02^{a}	0.26±0.01^{a}	0.15±0.01^{b}	0.14±0.01^{b}	0.15±0.01^{b}
Cohesiveness	0.48±0.07^{b}	0.76±0.05^{a}	0.34±0.02^{cd}	0.44±0.02^{bc}	0.27±0.01^{d}
Gumminess (g)	934.67±47.41^{a}	809.35±70.03^{b}	604.14±17.60^{c}	769.41±20.30^{b}	337.67±31.45^{d}

$^{a-d}$Different letters within the same row indicate significant differences (P<0.05)

Control pâté (CP), pâté whit 3% of 'Rojo Brillante' flour (PRB-3), pâté whit 3% of 'Triumph' flour (PT-3), pâté whit 6% of 'Rojo Brillante' flour (PRB-6) and pâté whit 6% of 'Triumph' flour (PT-6)

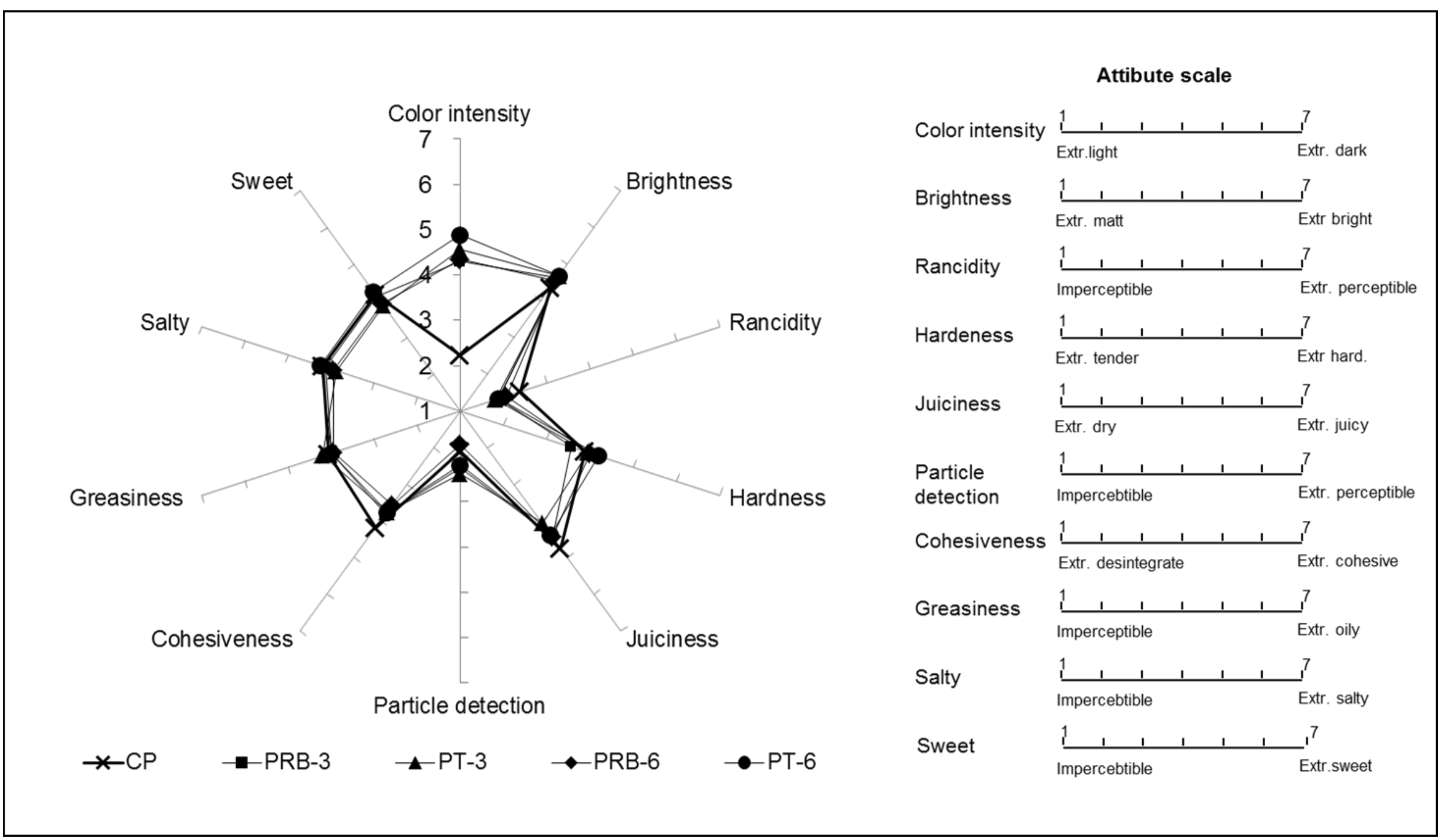

Figure 1.- Sensory evaluation: results of quantitative descriptive analysis carried out in control patê (CP), and patês with 3% 'Rojo Brillante' flour (PRB-3), 3% 'Triumph' flour (PT-3), 6% 'Rojo Brillante' flour (PRB-6) and 6% 'Triumph' flour (PT-6). The attribute scale used by panelists to perform the sensory analysis is also shown.

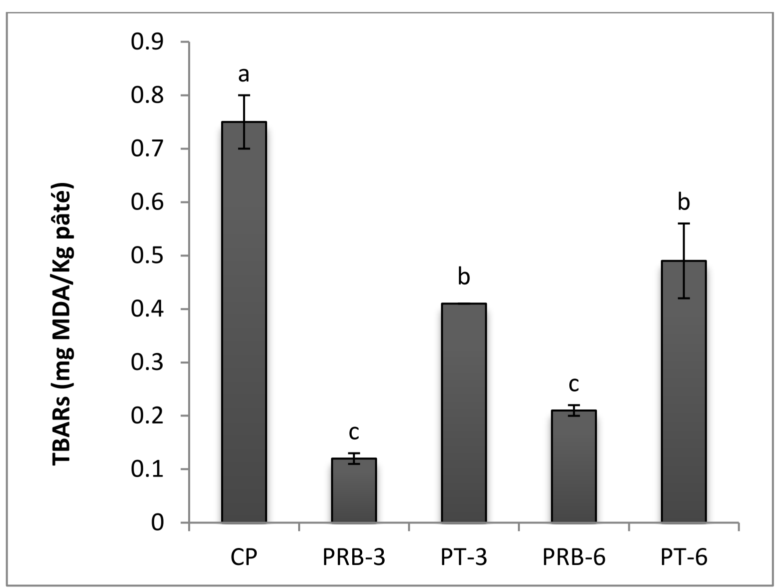

Figure 2.- Lipid oxidation (TBARS; mg MDA/kg pâté) of different pork liver pâté formulations.

The bars correspond to the standard deviations of mean values. [a-c]Different letters indicate significant differences ($p<0.05$).

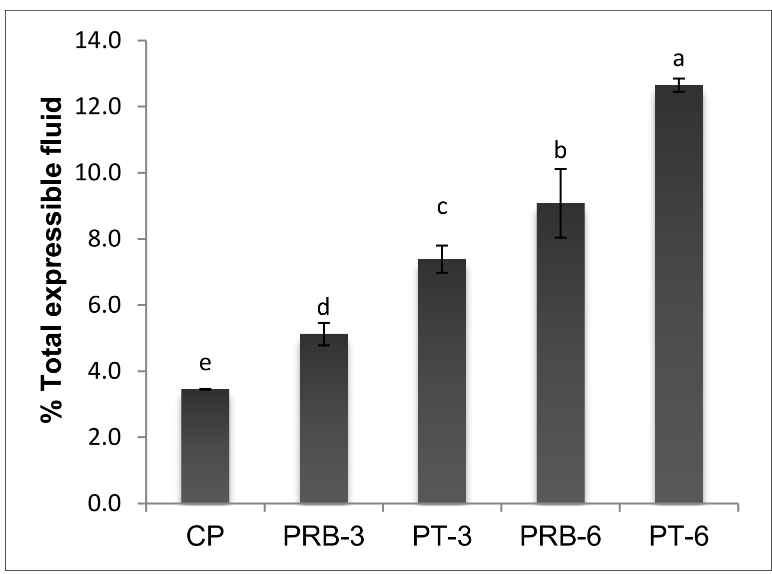

Figure 3.- Emulsion stability of the pork liver pâté formulations.

The bars correspond to the standard deviations of mean values. [a-e]Different letters indicate significant differences ($p<0.05$).

7.6.- PUBLICACIÓN 6

Título: Persimmon flours as functional ingredients in spaghetti: chemical, physico-chemical and cooking quality

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Persimmon flours as functional ingredients in spaghetti: Chemical, physico-chemical and cooking quality

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Abstract

The aims of the current work were to enrich durum wheat semolina spaghetti with two types of persimmon flours (from cv. "Rojo Brillante" and "Triumph") obtained from persimmon juice coproducts, at two concentrations (3% and 6%), to evaluate their chemical composition, physicochemical properties and cooking quality and to asses if they can be detected as different from control wheat semolina spaghetti (without any persimmon flours added) by sensory analysis.

Persimmon flour enriched spaghetti had higher total dietary fiber than control spaghetti, which allows applying the nutritional claim 'source of fiber". The addition of persimmon flours also increased their total yellow content (related to carotenoid content) in a dose-dependent way, which produced a higher yellow colour, typical and well appreciated by consumers in this type of pasta. Another positive characteristic of these spaghetti enriched with persimmon flours is that they need a short optimum cooking time in comparison with control spaghetti without it imply any significant change in their cooking quality. The type of persimmon flour and its concentration caused differences in colour of uncooked and cooked spaghetti, optimum cooking time, total organic matter, weight increase, fracturability and stickiness. Furthermore, 3% spaghetti formulations were not different from the control by sensory evaluation. In conclusion, the enrichment of durum wheat semolina spaghetti with persimmon flours allowed the valorization of persimmon coproducts and the production of spaghetti with similar cooking quality to traditional durum wheat semolina spaghetti, fuerthermore, the best results were obtained when persimmon flour from 'Rojo Brillante' was added at 3%.

Key words: kaki, co-products, spaghetti, cooking quality, fiber, yellow pigments

Introduction

Agri-food industry coproducts are an environmental problem and have repercussion in economy and society. Coproducts valorization to obtain new ingredients with high nutritional value and healthy properties (high content of dietary fibre and other bioactive compounds) can be a potent strategy to reduce waste and respond to the demand of consumers for healthier processed foods [1].

Persimmon fruit (*Diospyros kaki* L.f.) is considered an excellent source of insoluble fiber, provitamin A carotenoids, calcium, gallic acid, sugar and ascorbic acid [2, 3]. Furthermore, several authors have reported hypocholesterolemic, hypolipidemic, anti-atherogenic, antiobesity, antidiabetic and antioxidant effects in rats fed with persimmon [4-7]. All these evidences suggest that regular persimmon consumption can prevent and improve the treatment of several non-communicable diseases that currently have an important impact in public health [8].

In recent years, the increase of persimmon cultivation in Spain [9], has led not only to its fresh fruit consumption but also to their industrialization towards juices, jams or dehydrated fruit. Persimmon fruit industrialization has generated high amount of coproducts whose composition and properties could make them functional ingredients [10, 11].

Pasta products are widely consumed all around the world and have been considered as good carriers of bioactive compounds. This makes them good candidates for the addition of agri-food industry coproducts. Different coproducts have been previously used in the formulation of wheat spaghetti such as, carrot pomace [12] potato pulp [13], tomato by-products [14], carob fiber [15], partially-defatted chia [16], or unripe banana and plantain flour [1, 17].

Pasta is usually made with durum wheat semolina and water; the dough obtained by mixing these ingredients is submitted to extrusion and eventually to a dehydration process. The quality of pasta primarily depends on the compounds present in durum wheat, especially proteins [18], which are responsible for the gluten network formation. Others ingredients intentionally added to the main one, wheat semolina, can compete with the gluten-starch matrix for water sorption [19], hence affecting the correct hydration and the development of the pasta matrix, with possible negative effects on its cooking quality.

The aims of the current work were: i) to enrich durum wheat semolina spaghetti with two types of persimmon flours (“Rojo Brillante” and “Triumph”), obtained from persimmon juice co-products, in order to improve their functionality, ii) to process spaghetti by avoiding defective hydration during processing, and iii) to evaluate the effects of persimmon flour addition on the chemical composition, cooking quality, texture and sensory properties of spaghetti.

Materials and methods

Raw materials

Durum wheat semolina was obtained by grinding wheat (cv. Marco Aurelio) grains. The semolina obtained had the following characteristics: moisture 13.7 g/100 g, protein content 12.0 g/100 $g_{d.w.}$, gluten content 10.0 g/100$g_{d.w.}$, and ashes 0.67 g/100 $g_{d.w.}$. The average particle size of the flour was 280 m. Persimmon flours (particle size < 210 μm) were obtained from juice co-products of cultivars 'Rojo Brillante' and 'Triumph' as reported by Lucas-González et al. [10].

Pasta formulation and processing

Five formulations of spaghetti were obtained by adding different amounts (3% and 6%) of both persimmon flours, 'Rojo Brillante' and 'Triumph' to the basic formulation of spaghetti (Table 1). Control spaghetti (CS) was made with wheat semolina and water without persimmon flours addition. SR-3 spaghetti with 3% of 'Rojo Brillante' flour, ST-3 spaghetti with 3% of 'Triumph' flour, SR-6 spaghetti with 6% of 'Rojo Brillante' flour and ST-6 spaghetti with 6% of 'Triumph' flour.

Batches of 2 kg for each sample were processed using a Namad (Rome, Italy) pilot extruder with two mixing vessels and one extruder. The dry ingredients (wheat semolina and persimmon flour) were mixed in the first vessel by a shaft mixer for 15 min. Water (18 °C) was added to the dry mix in different amounts that were calculated by taking into account the water absorption index of the semolina and of those of the different flours, then the hydrated semolina was mixed for 12 min to obtain a dough. The final water content of the five dough is reported in Table 1. The dough was eventually mixed for 10 min in the second vessel and extruded in a single screw extruder with a barrel length of 350 mm and a diameter of 40 mm, provided with a water cooling system. The extruder was equipped with bronze round drawplate of 100 mm diameter provided with 18 bronze inserts of 12 mm having 7 dies of 1.2 mm diameter each. The input feed rate was of 15 kg h^{-1}, the screw speed was set to 42 rpm and temperature of cooling water was of 5 °C. In these experimental conditions the temperature reached a maximum of 30 °C and pressure behind the die reached 10 MPa. The output rate was of 1.0 m min^{-1} and spaghetti strands were cut in 300 mm long spaghetti using a cutting bar. Spaghetti were dried in an AFREM (Lyon, France) fan-assisted experimental dryer for 24 h, applying a low temperature (50 °C) drying process. At the end of the drying process, the spaghetti were packed in sealed plastic pouches and stored at room temperature for 10 days in order to stabilize the pasta and prevent it from absorbing water. Spaghetti were further transported and were stored at room temperature prior to analysis. Fig. 1 shows the 5 types of processed spaghetti.

Proximate composition

Moisture, ash, protein, total dietary fiber (TDF) and starch were determined following their corresponding AOAC method [20].

Sugars analysis

Sample preparation and sugars analysis was performed as described by Cavazza et al. [21]. Two g of pasta were weighed, added to 50 mL of HPLC-grade water, and homogenized with an Ultra-Turrax T 18 basic (IKA® Werke GmbH & Co. KG, Staufen, Germany) homogenizer for 2 min. The dispersion was then heated at 80 °C for 60 min under continuous stirring, centrifuged for 30 min at 3606 ×g using a ALC4218 (ALC Intl. srl, Cologno Monzese, Italy) centrifuge, and filtered through a 0.20 µm Millex nylon membrane (Merck Millipore Ltd, Cork, Ireland).

High-Performance Anion Exchange Chromatography with Pulsed Electrochemical Detection (HPAEC-PED) was used for separation and quantification using a Dionex (San Donato Milanese, Italy) ICS 3000 Ionic Chromatography System. Samples were injected using a 10-µL loop and carbohydrates were separated on a Dionex Carbopac PA20 column (3 × 150 mm), preceded by a Dionex Carbopac PA20 guard column (3 × 30 mm) at a flow rate of 0.5 ml min^{-1} and 30 °C. NaOH 150 mM was used as mobile phase. Sugars separation was carried out using the time/potential waveform A as indicated by Dionex (Technical note 21). All mobile phases were sparged and pressurized with helium to prevent adsorption of atmospheric carbon dioxide and eventual production of carbonate. Sugar detection was carried out by a Dionex ICS3000 ED detector working in pulsed amperometric mode. For sugar identification, glucose, fructose, sucrose and maltose standards (Sigma, Milan, Italy) were used and quantification was carried out by means of external standard curves.

Total yellow pigments

Total yellow pigment of uncooked spaghetti samples was measured by spectrophotometric analysis following AACC International Approved Method 14-50.01 [22]. The absorbance was measured a 440 mm. A standard curve was carried out with α-carotene, and the results were expressed as mg α-carotene equivalent/100 g spaghetti.

Microbiological analysis

Following the techniques described by the American Public Health Association [23], mesophilic aerobic bacteria, enterobacteriaceae, total coliforms, yeast, moulds and sulfite-reducing clostridia were quantified.

Colour

Colour of spaghetti was measured with a Minolta (Tokyo, Japan) CR300 colourimeter with CIE D_{65} illuminant and 10° standard observer conditions. Colour was measured using the CIELab colour space where L* corresponds to lightness, a* to red/green chromaticity and b* to yellow/blue chromaticity). The instrument was calibrated with a white tile (L* = 98.03, a* = -0.23, b* = 2.05) before measurements. Total colour differences (ΔE) of each sample (S) respect to control spaghetti (CS) were also calculated as:

$$\Delta E = \sqrt{(L_S^* - L_{CS}^*)^2 + (a_S^* - a_{CS}^*)^2 + (b_S^* - b_{CS}^*)^2}.$$

Diameter

The spaghetti diameter was calculated using an Instron U.T.M. (mod. 5542) electronic dynamometer equipped with 100 N load cell and a flat blade as testing device. Diameter was calculated from the force-displacement curve as the difference between the total displacement of the cutting edge and the displacement at the contact point with the pasta samples. The latter, in turn, was measured using a trigger force of 0.004 N [24].

Fracturability

Fracturability of spaghetti were measured with a texturometer TA.XT plus equipped with 30 kg load cell and Stable Micro Systems software (Stable Micro Systems Ltd, Surrey, UK). For carried out fracturability assay a compression force was applied on 4 adjacent strands of uncooked spaghetti using a blade. Condition parameters: speed 1 mm/s and 3 mm of distance. Fracturability was expressed as the maximum force (N), required to break 4 strands of uncooked spaghetti.

Cooking properties

Spaghetti cooking and optimum cooking time evaluation

Ten grams of spaghetti were cooked in a durex beaker using 100 ml of boiling tap water. The optimum 1:10 pasta to water ratio was chosen according to Menger [25]. The spaghetti were cooked for different times, and immediately drained for 30 s just after cooking. Optimum cooking time (OCT) was evaluated by the disappearance of the white uncooked core in small pasta [24].

Moisture after cooking

Moisture of cooked spaghetti at their optimum cooking time was gravimetrically measured after dehydration of 5 g of minced cooked spaghetti in an oven set at 105 °C until constant weight.

Water absorption, cooking loss and total organic matter released after rinsing

Water absorption, cooking loss (CL) and total organic matter released after rinsing (TOM) of spaghetti were determined. Three independent samples of each batch were cooked until their optimum cooking time. For water absorption determination, cooked spaghetti were drained for 30 s and weighed; their weight increase upon cooking (WI) was calculated and expressed as a percentage of the sample weight before cooking. Cooking loss (CL) was determined gravimetrically: 40 mL of cooking water was precisely weighed, then was evaporated and dried in an oven at 105 °C until constant weight. Cooking loss was expressed as the percentage of solids solubilized in cooking water [26]. Total organic matter released after rinsing (TOM) was determined as described by D'Egidio et al. [27].

Texture of cooked spaghetti

To determine firmness, 8 adjacent strands of spaghetti were cut using an Instron U.T.M. (mod. 5542) electronic dynamometer equipped with 100 N load cell and a flat blade as testing device. The test velocity was of 50 mm/s and the displacement was set to 10 mm. Firmness was expressed as maximum cutting force (N) required to shear one strand of spaghetti.

To determine stickiness, 4 adjacent strands of spaghetti underwent to compression (10 N) with a texturometer TA.XT plus equipped with 30 kg load cell, P/50 cylinder probe (50 mm diameter) and Stable Micro Systems software (Stable Micro Systems Ltd, Surrey, UK) for 2 seconds. Stickiness was expressed as the maximum peak force (N) to separate the probe from the samples surface on probe retraction.

Sensory analysis

Triangle tests were performed to compare spaghetti without persimmon flour addition (CS) to those added with 3%, and 6% persimmon flour.

Sensory analysis was carried out by a panel, constituted of a total of 8 initiated assessors aged from 25 to 44 years old, in a laboratory designed according to ISO 8589 [28] and equipped with individual booths and dark red light to prevent the samples colour from being seen. For panellist initiation, a short practical seminary about triangular test definition, instructions, and finally an example of a triangular test with commercial spaghetti, was carried out following the ISO 4120 guidelines [29].

For the test purpose, 500 g of spaghetti both without and with persimmon flour (either 3 or 6%) were cooked in 5 L of water (without salt addition) to their optimal cooked time and allowed to cool for 10 minutes in order to equilibrate samples at room temperature (22 °C). Two identical and one different sample of cooked spaghetti (≈ 10g) were taken, identified by random three-digit codes and contemporarily presented to judges in a random order. Each test was repeated in two sessions, in the morning and in the afternoon. The order of sample presentation was changed in the two sessions in order to balance the test and to avoid the alone effect.

Panellists were invited to taste three samples in each session and to identify the odd sample even if a difference was not perceived (forced-choice method). The triangle test was extended by applying a directional variation that requires the assessor to identify the sample that is more intense in sweet taste, and assessors were asked which sample presented the highest sweet taste intensity. Mineral water was used as palate cleaner between samples.

Statistical analysis

For each experiment, three independent samples were examined with three replications per sample. The data obtained were analyzed by means of one-way ANOVA test. Tukey's HSD test was applied for comparisons of means. Differences were considered significant at $p<0.05$ probability level. Correlations between the variables were carried out and the Pearson's correlation coefficient and the associated probability (p) value were calculated. Statistical analyses were carried out using the statistical package SPSS 19.0 (SPSS Inc., Chicago, IL).

The statistical significance of triangle tests (discrimination among samples) was verified by comparing the total number of responses and the number of correct responses with the values reported in unilateral $p = 1/3$ probability tables. The statistical significance of the triangle directional tests was verified by comparing the number of correct responses in the first triangle test and the number of responses referred to the most frequently selected odd sample in the directional test with the values reported in bilateral $p = 1/2$ probability tables.

Results & discussion

Microbiological analyses of persimmon flours and spaghetti

Persimmon flours, obtained by grinding dried by-products of persimmon juice processing, were analysed for their microbiological profile in order to test their suitability to be used in pasta formulation.

Even though these flours are obtained from raw industrial residues, microbial counts in persimmon flours are within the range observed for dried fruits and wheat flours [30] and the further mixing and drying process contributes to decrease their initial microbial load (Table 2). The total mesophilic bacteria count, mould count and *Salmonella* absence in spaghetti are conform to the Italian legislation for pasta products [31], thus persimmon flour could be intended as a suitable ingredient for pasta processing.

Spaghetti processing and quality

Semolina was mixed with water to obtain a water content of 32 g/100 g in the dough. The water added to semolina in the first step of spaghetti production plasticizes the wheat proteins and allows the shaping of pasta by extrusion or sheeting [26]. However, when a fiber riche source is added to semolina, a relevant interaction with the water could be attributed to fiber, which could cause modifications on the supramolecular assessment of gluten network and would affect to the final structure of spaghetti [32]. In order to avoid protein-fibre competition for water availability, in semolina/persimmon flour mixes the hydration of semolina was kept constant by using an additional amount of water to the mixtures (Table 1).

The amount of water addition was calculated by taking into account the water holding capacity of persimmon flour: 7.37 ± 0.73 g/g ('Rojo Brillante flour) and 11.23 ± 0.53 g/g ('Triumph' flour) [10]~~,~~ which is much higher than that of semolina.

The water addition calculated on the basis of water holding capacity data permitted to obtain spaghetti without white spots due to lack of hydration (Fig. 1).

The diameter of spaghetti enriched with persimmon flour was not statistically different from that of control spaghetti except for spaghetti enriched with the highest percentage of Triumph persimmon flour (ST-6), which showed a lower diameter (Table 3), due to shrinkage phenomena that take place during drying. Shrinkage occurs in the amorphous matrix of spaghetti when water is removed from the material due to a pressure unbalance between the inner of the material and the external pressure and depends on the amount of water removed [33]; thus the spaghetti enriched with 6% Triumph persimmon flour, which needed the highest amount of hydration water due to the flour water holding capacity, showed also the highest shrinkage upon drying.

Fracturability of spaghetti, defined as the force needs to break an uncooked strand of spaghetti when a mechanical strength is applied, was also measured. This parameter is important to determine the commercial quality of final product and their resistance to the handling and shipping [18]. Persimmon flour is a natural source of sugars and fibre [11]. Several studies in literature reported that the addition of sources of insoluble fibre to wheat spaghetti generates cracks and holes in their continuous structure, making the uncooked pasta more fragile [19, 32, 34]. Other authors reported that, besides fibre, also sugars could disturb pasta structure [16] by inducing the formation of clumps of material between the starch granules. As can be seen in Table 3, the addition of 3% persimmon flours (Rojo Brillante and Triumph) to durum wheat spaghetti did not influence their fracturability (similar values to control), however, when they were added at higher concentrations (6%), their fracturability was increased ($p<0.05$), showing ST-6 sample the highest values. These results seem to be in accordance with the previously cited references, since spaghetti with 6% persimmon flour (ST-6 and SR-6) had the highest sugar content (Table

4). In addition, the highest fracturability showed in ST-6 sample could be related with their higher fiber content (Table 4). It seems clear that the negative effect of fiber and sugar content on the fracturability of spaghetti could depend on their amount.

Also, this indicates that the proper hydration of flour could counterbalance the negative effects induced by fibre and sugars presence, and only the addition of a high amount of water, whose removal promotes shrinkage, decreases the mechanical resistance of spaghetti.

Chemical composition

The enrichment of durum wheat semolina spaghetti with both persimmon flours, 'Rojo Brillante' and 'Triumph' at different concentrations (3% and 6%), caused changes in their chemical composition, especially in their content of protein, fibre, sugars and total yellow pigments, as it can be seen in Table 4.

As regards moisture content, it decreased as the amount of persimmon flour increased ($p<0.05$), indicating that both the persimmon flours under study have lower ability of to retain water after drying than semolina, even though their water binding capacity is higher than that of semolina. Aranibar et al. [16] reported similar moisture values in wheat flour pasta enriched with partially-deoiled chia flour.

The enrichment with persimmon flour affected the sugar content of spaghetti by decreasing their maltose content and increasing their content in fructose and glucose, whose concentration was influenced by the type of persimmon flour added (with 'Triumph flour' causing a greater increase than 'Rojo Brillante' flour) and also by its percentage. The increase of total sugar content, which is due to the high abundance of glucose and fructose in persimmon flours [11], is of about 1.5 g/100g and 3 g/100g for 3 and 6% persimmon flour addition respectively.

The starch content of spaghetti decreased with persimmon flour enrichment of 6% (SR-6 and ST-6) ($p<0.05$) whilst the persimmon flour enrichment of 3% did not evidence a significant decrease of starch content in comparison to CS ($p>0.05$). These results are in agreement with those reported by Padalino et al. [35] who observed that the starch content in spaghetti decreased as inulin content increased, and with Lu et al. [36], who observed a decrease in the starch amount in durum wheat semolina pasta as mushroom powder increased.

The protein content of spaghetti enriched with increasing amounts of persimmon flours decreased in a dose-dependent behaviour and the enrichment with 6% of persimmon flour resulted in a decrease of about 1 g/100 $g_{d.w.}$ of protein. In any case, the wheat protein content, calculated by taking into account the percentage of persimmon flour addition, was above 10.5 g/100 $g_{d.w.}$, which is the minimum amount of protein which is required in a semolina for pasta making process by the Italian law.

As far as TDF is concerned, spaghetti samples enriched with 'Triumph' flour showed the highest content, following by the spaghetti added with 'Rojo Brillante' flour. These results were

expected since ‘Triumph’ flour has more TDF than ‘Rojo Brillante’ flour (40 % and 32 %, respectively) on the basis of the data reported by Lucas-González et al. [10]. In any case, all spaghetti enriched with persimmon flours (for both flour types and concentrations) could be labelled as “source of fibre” in accordance to the Regulation (EC) No 1924/2006 of the European Commission on nutrition and health claims made on foods [37] because the fiber content is higher than 3 g/100 g of food but lower than 6 g/100 g of food. The increase in the TDF content of spaghetti due to its enrichment with vegetable sources has been previously reported by Padalino et al. [38] and Patiño-Rodríguez et al. [1], who highlighted the potential of vegetable flours to improve the fiber content of pasta.

Spaghetti enriched with persimmon flours (ST-3, SR-3, ST-6 and ST-6) showed TYP amounts higher than CS ($p<0.05$). This increase in TYP depends on both factors: type of persimmon flour (Triumph and Rojo Brillante) and concentration (0, 3 and 6%). Spaghetti added with ‘Triumph flour’ (ST-3 and ST-6) showed higher amount of TYP than spaghetti added with ‘Rojo Brillante’ flour (SR-3 and SR-6) at the same concentrations, and this reflect the carotenoids differences between the two flours [11]. The higher the amount of persimmon flour added, the higher the TYP amount. The chemical profile of yellow pigments present in wheat flour consists mainly of carotenoids, mostly lutein and its esters and in less amount zeaxanthin [39]. The increase in TYP could be related with the content in carotenoids, especially provitamine A carotenoids (β-cryptoxanthin and β-carotene) which have been reported in persimmon flours [2]. Carotenoids enrichment in wheat semolina spaghetti has been recently performed also by other authors [14] through the addition of tomato co-products.

Colour

Pasta (spaghetti) colour is an important quality parameter with a high influence in its acceptance by consumers, who generally prefer pasta with a bright yellow colour rather than a brown or cream one. Nevertheless, the final colour of pasta is the result of changes of the occurrence of the natural carotenoid yellow pigments in durum wheat grain [21]. In the case of using new ingredients in pasta processing also the presence of compounds with colourant properties and their chemical and enzymatic transformations must be taken into account.

Table 5 reportes spaghetti (uncooked and cooked) colourimetric parameters. As can be seen in this table, both in the uncooked and cooked spaghetti the colour changes upon of persimmon flour were the same: lightness (L^*) decreased ($p<0.05$), whereas redness (a^*) and yellowness (b^*) increased ($p<0.05$). These changes observed in the colour coordinates were concentration-dependent ($p<0.05$), and only b^* values were affected by persimmon flour type (higher values in spaghetti added with ‘Triumph’ persimmon flour). The decrease in L^* values in foods due to the addition of some colourants (natural or synthetic) or coloured ingredients has been previously reported [40]. The increase in a^* and b^* values due to the persimmon flour

addition are due to their carotenoids content [11], this is supported by the strong positive correlation observed between a* values and yellow pigment content ($r= 0.92$; $p<0.05$). Carini et al. [34] and Gull et al. [12] also reported that a* and b* values increased in pasta samples enriched with carrot flour.

The cooked process caused an increase in L* values and a decrease in a* and b* values, in all samples. The increase in the free water on the spaghetti surface and the starch gelatinization could be related with the increase in lightness. Similar behaviour for a* and b* values in tagliatelle added with tiger nut due to cooking process has been reported [41].

The total colour differences between the spaghetti added with persimmon flours and control sample, before or after cooking, are high enough to be visible to the human eye, as it was confirmed in Fig. 1 and 2. In both cases (cooked and uncooked samples) total colour differences were higher in the spaghetti with higher amount of persimmon flour added (ST-6 and SR-6). It is important to denote that cooking process decreased total colour differences in all samples.

Cooked spaghetti diameter

As it was expected, the diameter of the spaghetti after cooking increased respect to the uncooked spaghetti (Table 6), reaching all the samples similar values (2 mm approximately). In this case, differences between samples were not observed ($p>0.05$), indicating that the persimmon flour addition did not affected the product swelling during cooking.

Spaghetti cooking quality

Spaghetti cooking quality is closely related to the network gluten and starch gelatinization and their capacity to trap water. During cooking the hot-water induces starch gelatinization and protein polymerization; furthermore both compounds interact between them forming a continuous gluten-starch matrix, where starch is trapped in gluten networks [42]. The addition of others ingredients to wheat semolina can interact with the gluten-starch matrix and compete with water [19, 32] modifying the correct development of common pasta matrix, which could affect the cooking quality of spaghetti. In persimmon flours the compounds with the highest affinity to water are fiber and sugar, which also are its main components.

Persimmon flours addition showed a significant effect on the OCT of spaghetti. The longest OCT was required for the CS (8.5 min). The addition of persimmon flours (‘Triumph’ and ‘Rojo Brillante’) resulted in a shortening of the OCT. The spaghetti with the highest amount of persimmon flours (SR-6 and ST-6) showed the lowest OCT. Previously, others authors have reported some reduction in OCT in pastas enriched with different vegetable ingredients [15, 38]. This fact could be due to the weakening of the gluten-starch network that allows a rapid entry of water into the food matrix [16]. The lower starch and protein content showed in spaghetti enriched with the highest persimmon flours concentrations also can contribute to reduce the OCT.

Reducing the OCT of spaghetti could be interesting to provide to the market products that require less processing time, which will be well accepted by those consumers who due to their pace of life have less time to prepare food, or simply do not want to dedicate a lot of time to the preparation of their dishes.

Total organic matter (TOM) is defined as the surface material released from cooked pasta during exhaustive rinsing. Small TOM differences were found between samples; ST-3 showed the lowest value, significantly different to the CS ($p<0.05$). According to the classification proposed by D'Egidio et al. [27] about TOM values and pasta quality (<1.4%: very good quality; 1.4% and 2.1%: good quality; >2.1 poor quality), all samples had good quality.

As regards cooking loss (CL), the addition of persimmon flours did not modify this parameter, with all the samples showing similar values. The CL values observed in the current work are in accordance with those reported by Chillo et al. [43]. Although the addition of vegetable ingredients to wheat semolina spaghetti have been related to an increase in cooking loss [34, 44] mainly due to fiber interference with the gluten-starch network, it is possible that in this case, the already reported gelation ability of persimmon flours [9] would have favoured the retention of solids in the spaghetti matrix. This hypothesis is supported by the results obtained by Padalino et al. [38], who reported that compounds with gelling ability, such as gums, forms a network around starch granules by encapsulating them during cooking.

CS and ST-3 samples presented similar water absorption values, which decreased ($p<0.05$) when persimmon flours were added at 6% (SR-6 and ST-6). These results pointed out that persimmon flours decrease water absorption in spaghetti samples, probably due to their sugar content, which interact with water and decrease the swelling of starch [19]. Other authors have reported a decrease in water absorption of spaghetti enriched with different vegetable ingredients such as pea flour, popped amaranth flour and pollard [19, 44]. It is important to denote that the shorter OCT and the lower starch content found in spaghetti added with persimmon flours could have also contributed to their lower water absorption capacity.

As far as moisture of cooked spaghetti is concerned, samples enriched with persimmon flours showed lower ($p<0.05$) values than CS showing a behaviour very similar to that reported in the case of water absorption ($r=0.98$; $p<0.05$).

Texture

Texture of cooked spaghetti samples is also showed in Table 6. Firmness of spaghetti was not affected by the addition of persimmon flours ($p>0.05$), with all the enriched samples showing values similar to CS.

Persimmon flours were shown to have gelation ability and, upon cooking, this could have contributed to eliminate the small texture differences found in uncooked samples. These results

are positive since the texture of spaghetti has a great effect on the general acceptability of the final product by consumers.

Stickiness, defined as the work/force necessary to overcome the attractive forces between the surface of the product and the surface of the material with which the product comes in contact, was determined on spaghetti. Stickiness is determined by the accumulation of starch on pasta surface due to the weakening or partial breakdown of the surface protein network, resulting in the formation of a paste on the surface of the pasta that make it sticky [45] and is related to the stickiness. Only spaghetti enriched with 6% of Triumph flour (ST-6) showed higher ($p<0.05$) stickiness values than CS. Although stickiness values are generally correlated to TOM, in the present work this relation has not been observed, this is likely due to the fact that in this study small or no differences among sample were observed for both these quality indices.

Sensory evaluation

Spaghetti enriched with 'Rojo Brillante' and 'Triumph flour' at 3 and 6% were compared with control sample by a triangular test. The results (Table 7) showed that panelists did not find significant differences between spaghetti added with 3% of persimmon flours (ST-3 or SR-3) and CS ($p<0.01$). However, as regards spaghetti added with 6% persimmon flour (both types, ST-6 and SR-6), the panelists identified that these sample were different ($p<0.01$) from the control and that they showed a significantly ($p<0.01$) sweeter taste. As previously discussed the increase of sugar content in spaghetti added with 3% persimmon flour is about 1.7% and the increase of sugar content in spaghetti added with 6% persimmon flour is above 3% and the latter increase of sugar content.

Conclusions

The use of persimmon flours, obtained from juice coproducts (cv 'Rojo Brillante' and 'Triumph') could be a technologically viable option for the production of enriched durum wheat semolina spaghetti and also would allow the valorization of these coproducts. As regard to nutritional value, these spaghettis have higher sugar and total dietary fiber which could allow to label the products as a 'source of fiber'. The addition of persimmon flours also increases the total yellow pigment content, which enhanced the typical yellow colour of pasta.

Other positive characteristic of the persimmon flour enriched spaghetti is that they need a short optimum cooking time without implying any significant change in their cooking quality and final texture. However, it should be stated that the addition of Triumph persimmon flour, which showed the highest fiber and sugar content, in the highest percentage 6% determined adverse effect on raw spaghetti fracturability.

Although in the current work some positive results due to persimmon flour enrichment of spaghetti have been showed, more researches are needed to study the bioaccessibility of sugars

during spaghetti digestion since the high sugar content of persimmon flour and the enhancement of fiber content could have adverse effect on the glycemic index of spaghetti enriched with persimmon flour. Moreover, the antioxidant activity of enriched spaghetti, as well as the stability of both their bioactive compounds and antioxidant activity during digestion should be investigated.

Compliance with ethical standards

Conflict of interest: There is no conflict of interest.

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Fig. 1.- Visual appearance of uncooked spaghetti after their processing.

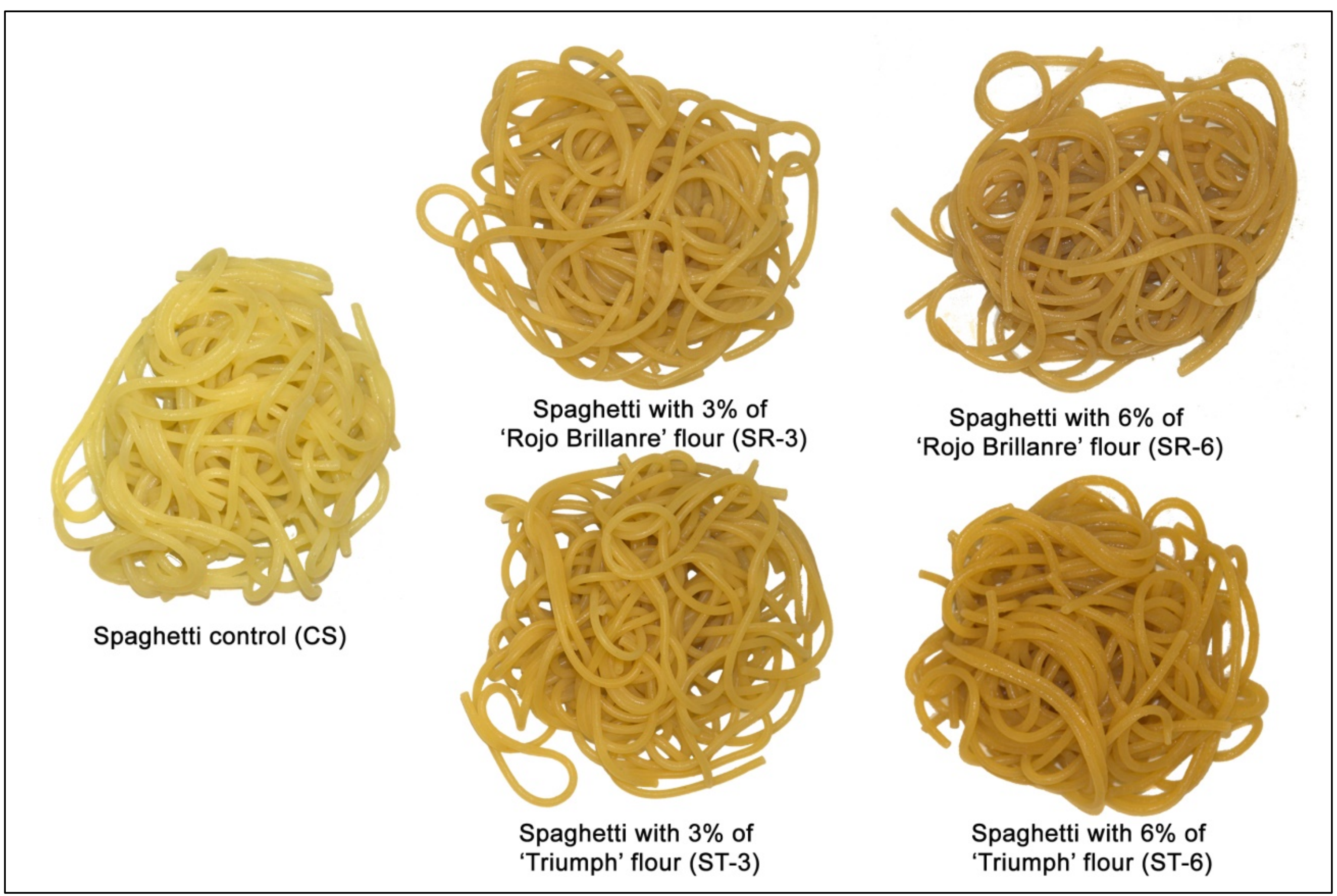

Fig. 2.- Cooked spaghetti samples at their optimum cooking time.

Table 1.- Formulation of spaghetti: control spaghetti (CS), spaghetti whit 3% of 'Rojo Brillante' flour (SR-3), spaghetti whit 3% of 'Triumph' flour (ST-3), spaghetti whit 6% of 'Rojo Brillante' flour (SR-6) and spaghetti whit 6% of 'Triumph' flour (ST-6).

Ingredients	CS	SR-3	SR-6	ST-3	ST-6
Wheat flour (g)	2000	2000	2000	2000	2000
Water (mL)	550	600	700	750	850
'Rojo Brillante' flour (g)	-	60	120	-	-
'Triumph' flour (g)	-	-	-	60	120

Table 2.- Microbiological profile of persimmon flours (cv. 'Rojo Brillante' and 'Triumph') and spaghetti with 6% of persimmon flours added.

Microbiological group	'Rojo Brillante' flour (Log CFU/g)	SR-6 spaghetti (Log CFU /g)	'Triumph' flour (Log CFU /g)	ST-6 spaghetti (Log CFU /g)
Mesophilic aerobic bacteria	3.35±0.71	< 2	< 2	< 2
Enterobacteriaceae	2.97±0.52	< 2	2.58±0.56	< 2
Total coliforms	3.03±0.69	< 2	2.68±0.32	< 2
Micrococcus	< 2	< 2	< 2	< 2
Lactobacillus	3.49±0.78	< 2	2.79±0.69	< 2
Lactococcus	3.85±1.02	< 2	< 2	< 2
Spore-forming bacteria	1.78±0.26	< 2	2.48±0.96	< 2
Yeasts	1.78±0.36	< 2	2.08±0.32	< 2
Moulds	1.48±0.45	< 2	2.00±0.52	< 2
Salmonella	n.d	n.d	n.d	n.d

For sample denomination see Table 1

n.d. not detected in 25 g.

Table 3.- Diameter and fracturability of uncooked spaghetti

Sample	Diameter (mm)	Yield force (N)
CS	1.70±0.00[a]	68.18±6.18[a]
SR-3	1.65±0.04[ab]	66.97±5.68[a]
ST-3	1.61±0.01[b]	65.88±8.53[a]
SR-6	1.66±0.02[ab]	55.69±7.19[ab]
ST-6	1.50±0.02[c]	48.09±8.31[b]

For sample denomination see Table 1

Values in the same column with different letter are significantly different, according with Tukey test ($p<0.05$).

Table 4.- Proximate composition (g/100g), sugar content (g/100g) and total yellow pigment (mg α-carotene equivalent/100 g) of spaghetti.

	CS	SR-3	ST-3	SR-6	ST-6
Moisture	9.99±0.08[a]	9.42±0.06[b]	9.48±0.06[b]	9.15±0.05[c]	8.96±0.07[d]
Starch	62.98±2.91[a]	63.03±3.54[a]	62.84±1.62[a]	56.03±2.79[b]	56.72±1.37[b]
Protein	10.85 ±0.09[a]	10.35±0.09[b]	10.11±0.13[bc]	10.04±0.02[c]	9.97±0.13[bc]
TDF	2.96±0.12[e]	3.57±0.14[d]	3.94±0.01[c]	4.39±0.30[b]	4.87±0.26[a]
Ash	0.60±0.01[a]	0.62±0.02[a]	0.64±0.03[a]	0.67±0.01[b]	0.67±0.00[b]
Maltose	6.24±0.22[a]	6.03±0.07[b]	5.77±0.11[c]	5.33±0.06[d]	5.33±0.03[d]
Fructose	0.39±0.01[c]	1.72±0.03[b]	1.88±0.00[b]	2.37±0.03[a]	3.02±0.02[a]
Glucose	0.39±0.01[c]	1.55±0.02[b]	1.64±0.00[b]	2.23±0.02[a]	2.92±0.04[a]
Sucrose	0.06±0.00[a]	tr[b]	tr[b]	tr[b]	tr[b]
TYP	1.37±0.10[e]	2.27±0.16[d]	2.97±0.04[c]	3.72±0.25[b]	4.49±0.31[a]

For sample denomination see Table 1

TDF: Total dietary fiber; TYP: Total yellow pigment

[a-e]Values in the same row with different letter are significantly different, according with Tukey test ($p<0.05$).

tr: traces

Table 5.- Colour coordinates of both uncooked and cooked spaghetti and total colour differences (ΔE) from control sample (CS).

	Uncooked spaghetti samples				Cooked spaghetti samples			
	L*	**a***	**b***	**ΔE**	**L***	**a***	**b***	**ΔE**
CS	63.54±1.74^{a}	-7.85±0.35^{c}	44.48±2.89^{c}		66.25±0.84^{a}	-9.03±0.38^{d}	23.22±1.38^{d}	
SR-3	59.83±1.56^{b}	-0.34±0.60^{b}	47.23±1.37^{b}	8.81	60.17±1.16^{b}	-4.21±0.34^{c}	26.55±1.22^{c}	8.45
ST-3	58.73±2.39^{b}	0.27±0.29^{b}	49.75±1.96^{a}	10.81	61.08±0.91^{b}	-4.57±0.31^{c}	27.70±0.98^{bc}	8.17
SR-6	55.13±1.85^{c}	4.62±0.37^{a}	45.26±1.58^{c}	15.09	57.64±1.16^{c}	-1.72±0.55^{a}	28.44±1.18^{b}	12.45
ST-6	54.57±1.92^{c}	4.04±0.43^{a}	48.64±1.33^{ab}	15.46	58.85±1.09^{c}	-2.57±0.53^{b}	31.80±1.48^{a}	13.05

For sample denomination see Table 1

$^{a-d}$Values in the same column with different letter are significantly different, according with Tukey test ($p<0.05$).

Table 6.- Cooking quality of cooked spaghetti

	CS	SR-3	ST-3	SR-6	ST-6
OCT (min)	8.5	6	7.5	5.5	5.5
Diameter (mm)	2.15±0.15^{a}	2.11±0.15^{a}	2.15±0.09^{a}	2.09±0.16^{a}	2.10±0.13^{a}
Firmness (N)	3.51 ±0.18^{a}	3.40 ±0.35^{a}	3.45 ±0.30^{a}	3.56 ±0.24^{a}	3.41 ±0.25^{a}
Stickiness (N)	1.95±0.08^{b}	2.38±0.31^{b}	2.13±0.05^{b}	2.03±0.15^{b}	2.65±0.13^{a}
WA (%)	132.95±0.78^{a}	115.46±3.03^{b}	130.03±1.81^{a}	103.46±3.28^{c}	105.99±4.11^{c}
CL (g/100g)	8.93±0.24^{a}	8.15±0.05^{a}	8.69±0.42^{a}	8.06±0.44^{a}	8.93±0.41^{a}
TOM (g/100g)	2.05±0.20^{b}	1.85±0.06^{ab}	1.60±0.14^{a}	1.99±0.02^{b}	1.93±0.01^{ab}
Moisture (g/100g)	62.57±0.37^{a}	59.19±1.49^{b}	61.98±0.92^{ab}	58.36±1.51^{b}	58.78±0.30^{b}

For sample denomination see Table 1

$^{a-c}$Values in the same row with different letter are significantly different, according with Tukey test ($p<0.05$).

OCT: optimum cooking time; % WA: Water absorption; CL: Cooking loss; TOM: Total organic matter.

Table 7.- Results of the Triangle Test performed on the spaghetti with persimmon flours added (SR-3, SR-6, ST-3 and ST-6) compared to control spaghetti (CS).

Test	Responses Total number	Difference Number of correct responses	Sweet taste Frequency of the most frequent sample
SR-3 vs. CS	8	1	0/1
SR-6 vs. CS	8	6*	6/6*
ST-3 vs. CS	8	0	0/0
ST-6 vs. CS	8	6*	6/6*

For sample denomination see Table 1

* $p < 0.01$ significance level

7.7.- PUBLICACIÓN 7

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Evaluation of polyphenol bioaccessibility and kinetic of starch digestion of spaghetti with persimmon (*Dyospyros kaki*) flours coproducts during *in vitro* gastrointestinal digestion

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Abstract

The aim was to study the *in vitro* starch digestibility, the free and bound polyphenol profile and their bioaccessibility and antioxidant activity during *in vitro* gastrointestinal digestion of durum wheat semolina spaghetti added with two types of persimmon flour concentrates ("Rojo Brillante" flour and "Triumph" flour) at two concentrations (3 and 6%). Results obtained showed that persimmon flour improves the polyphenol profile of spaghetti by addition gallic acid and coumaric acid-o-hexoside, and increasing 2-fold and around 3-fold its content in spaghetti with 3% and 6% persimmon flours, respectively. Cooked process and digestion affected more to free polyphenol content than bound. Furthermore, 3% persimmon flour enriched spaghetti reduce kinetic of starch digestion, while 6% enriched spaghetti increased it. In conclusion, persimmon flours (Rojo Brillante and Triumph) at low concentrations could be used to develop spaghetti with more polyphenol content and less starch digestibility than traditional spaghetti.

Keywords: kaki, phenolic acids, *in vitro* digestion, glycaemic index, gallic acid.

Chemical compounds studied in this article: gallic acid (PubChem CID: 370), protocatechuic acid (PubChem CID: 72), catechin (PubChem CID: 9064), epicatechin (PubChem CID: 72276) 4-Hydroxybenzoic acid (PubChem CID: 135), p-Coumaric acid (PubChem CID: 637542), ferulic acid (PubChem CID: 445858), vanillic acid (PubChem CID: 8468), vanillin (PubChem CID: 1183), sinapic acid (PubChem CID: 637775), L-Tryptophan (PubChem CID: 6305), D-glucose (PubChem CID: 107526).

1. Introduction

Nowadays, the population and scientific community are more aware of the relationship between diet and health, as well as the environmental impact of food production. Since diets rich in easy digestive carbohydrates, saturated fats, ultraprocessed foods and poor in fiber and bioactive compounds are related with development many non-communicable diseases such as cardiovascular diseases, diabetes or cancer, which are currently a public health issue, (WHO, 2013). For that reason, consumers and World Health Organization (WHO) claim to agro-food industry healthier and sustainable foods, but also, cheap, tasty and useful.

In the search for novel and sustainable ingredients with healthy and technological properties to formulate potential functional foods, many scientists have focused their research in agro-food industry coproducts, since these are rich in bioactive compounds: vitamins, polyphenols, carotenoids, etc. and macronutrients, especially fiber, but also protein and oil; furthermore, it have technological potential as water and oil holding agents, emulsifiers, colorants and antioxidants (Gullon, Pintado, Fernández-López, Pérez-Álvarez & Viuda-Martos, 2015; Lucas-González, Viuda-Martos, Pérez-Álvarez, & Fernández-López, 2017; Simonato, Trevisan, Tolve, Favati & Pasini, 2019), and also, use them in the food formulation is a strategy to reduce waste and value them.

Persimmon (*Dyospyros Kaki*) is an orange-red fruit of Asian origin, whose composition has been related with the prevention of heart diseases and diabetes for different *in vitro* and *in vivo* studies (Gorinstein et al., 1998; Sindu et al., 2019). Being their mechanisms of action related with: i) their ability to bile retention, which activates bile acid pathway, (and that are associated with modulation of metabolism of carbohydrate, protein, and lipids), ii) antioxidant activity and iii) inhibition of alpha-amylase and amiloglucosidase by their polyphenols compounds (Gorinstein et al., 1998; Kawakami, Aketa, Nakanami, Izuka & Hirayama, 2010; Matsumoto, Yokoyama & Gato, 2010; Sindu et al., 2019). Persimmon is found widely distributed in Mediterranean area, especially Spain, which experimented in the last decade and important increase in its production, specifically of "Rojo Brillante" and "Triumph" cultivars. Furthermore, a recent industry has been created around persimmon, being juice manufactured one of them. The coproduct generated (peel and pulp) by persimmon manufactured present potential intermediate ingredients, due to their high gallic acid, carotenoids and dietary fiber content (Lucas-González et al., 2017; Lucas-González, Fernández-López, Pérez-Álvarez & Viuda-Martos, 2018a).

Pasta is widely consumed in Europe and greatly appreciated worldwide since their easy elaboration, versatility, carbohydrate value and protein content. Furthermore, in the market, a high diversity of pasta formulations are available, like pasta enriched with carrot, spinach, tomato, squid ink, spirulina, even chocolate. Among cereal-based foods, like bread, breakfast cereals, pizza, and others, pasta has the lowest glycemic index (GI) due to its complex matrix. However, based in

the carbohydrate food classification (FAO/WHO, 1998) [GI: low (<55), medium (>55 to <70) and high (GI>70)], pasta made with durum wheat semolina has a GI between medium and high (Foster-Powell, Holt, & Brand-Miller, 2002). Furthermore, it can be accompanied by sauces and ingredients with high-fat, energy meals, since generating high postprandial oxidative stress (Miglio et al., 2014). So, many scientists have enriched pasta with other ingredients, such buckwheat, oats, barley, sorghum, fibers, mushroom or also agro-food co-products, with the purpose to modify their carbohydrate digestibility and or improve their polyphenolic content, showing in many case positive results in the modification of traditional spaghetti. (Chillo, Ranawana & Henry, 2011; Khan, Yousif, Johnson & Gamlath, 2013; Biney & Beta, 2014; Lu et al., 2019; Padalino, et al 2017; Tackas et al. 2018; Simonato et al, 2019); However, pasta matrix stability can be modified with the inclusion of ingredients other than wheat semolina, which can cause porous in their structure (Padalino et al., 2017). So, although healthy ingredients are added to pasta, a functional study must be performed. In this sense, *in vitro* digestion models, are a useful screening tool to study the functional potential of various formulations, being able to compare between them their digestibility, bioaccessibility, interaction between nutrients, etc.

Taking into account the persimmon consumption benefits mentioned before, the hypothesis of the current study was that the use of persimmon juice coproducts to enrich durum wheat spaghetti could contribute to reduce postprandial insulin respond, to increase diversity of polyphenols and to reduce postprandial oxidation. However, for carrying out these benefits, polyphenol compounds must be released from the matrix.

For all exposed, the aim of this work was to study the *in vitro* starch digestibility and the free and bound polyphenol profile and their bioaccessibility and antioxidant activity after the simulated *in vitro* gastrointestinal digestion of durum wheat semolina spaghetti added with two types of persimmon flour concentrates (“Rojo Brillante” flour and “Triumph” flour) at two concentrations (3 and 6%). The effect of cooking treatment in the free and bound polyphenol profiles in spaghetti samples were also studied.

2. Materials and methods

2.1 Raw material

Durum wheat (cv. Marco Aurelio) was used to obtain durum wheat semolina with a particle size of 280 μm and with the following chemical composition: moisture 13.7 g/100 g, protein content 12.0 g/100 g d.w., gluten content 10.0 g/100 g d.w., and ashes 0.67 g/100 g d.w. Juice persimmon co-products from cultivars ‘Rojo Brillante’ and ‘Triumph’ were processed as described by Lucas-González et al. (2017) to obtain both persimmon flours with a particle size <210 μm. White bread was purchased in a local bakery of Orihuela (Alicante, Spain).

2.2 Pasta formulation and processing

Five batches (2 Kg) corresponding with different spaghetti formulations [spaghetti with 100 % durum wheat semolina (CS), spaghetti with 3% of Rojo Brillante flour (SR-3), spaghetti with 3% of Triumph flour (ST-3), spaghetti with 6% of Rojo Brillante flour (SR-6) and spaghetti with 6% of Triumph flour (ST-6)], were processed using a Namad (Rome, Italy) pilot extruder with two mixing vessels and one extruder.

In brief, durum wheat semolina and persimmon flours were mixed in a shaft mixer for 15 minutes, then different water (18 ºC) amounts: 500 g (CS), 600 g (SR-3), 750 g (ST-3), 700 g (SR-6) and 850 g (ST-6), which were calculated taking into account the water holding ability to dry ingredients, were added and mixed for 12 minutes. The formed dough was mixed again for 10 min in the second vessel and extruded in a single screw extruder with a barrel length of 350 mm and a diameter of 40 mm, provided with a water-cooling system. The extruder was equipped with bronze round drawplate of 100 mm diameter provided with 18 bronze inserts of 12 mm having 7 dies of 1.2 mm diameter each. The input feed rate was of 15 Kg/h, the screw speed was set to 42 rpm and temperature of cooling water was of 5 ºC. In these experimental conditions, the temperature reached a maximum of 30 ºC and pressure behind the die reached 10 MPa. The output rate was of 1.0 m/min and spaghetti strands were cut in 300 mm long spaghetti using a cutting bar. An AFREM (Lyon, France) fan-assisted experimental dryer was used to dry spaghetti formulations for 24 h at low temperature (50 ºC). Dry spaghetti formulations were then packed in sealed plastic pouches and stored at room temperature for 10 days in order to stabilize the pasta and prevent it from absorbing water. Formulations were kept at room temperature and dark conditions prior to analysis.

2.3 Extraction and identification of polyphenolic compounds (free and bound)

The extraction of phenolic compounds from crude and cooked spaghetti samples was divided in two fractions, free and bound. For the extraction of the polyphenolic compounds, 2 g of samples were mixed with 10 mL of acidified methanol (1% HCl), then the mix underwent sonication for 2 hours at room temperature and centrifuged 10 min at 4 ºC and 7100 *g*. The supernatant, containing free phenolic compounds, was collected and evaporated under vacuum, and then the solids were re-suspended with 5 mL of methanol:water (50:50 v/v). For extraction of bounded polyphenolic compounds, the method described by Mpofu, Sapirstein and Beta (2006) was followed, using the pellet remaining after the free polyphenolic compounds extraction. Ethyl acetate extracts were evaporated under vacuum, and then re-suspended in 5 mL of methanol: water in the case of non-digested samples (crude and cooked) and 2 mL in the case of digested samples.

Detection of free and bound polyphenols in the different samples (crude, cooked and digested) was carried out in a Hewlett-Packard HPLC series 1200 instrument equipped with a C_{18} column Mediterranean Sea 18, 25 × 0.4 cm, 5 μm particle size (Teknokroma, Barcelona, Spain).

In brief, 20 µL of the sample was eluted with a gradient of 1 mL/min. The mobile phases were composed of a mixture of two solvents. Solvent A contained formic acid in water (1:99, v/v) and solvent B was composed of methanol (100%). The solvent gradient was at 0 min- 5% solvent B, at 20 min- 25 % solvent B, at 40 min-50 % solvent B; and at 45 min-5 % solvent B. The detection of polyphenols was made by UV absorption at 270, 280, 325 and 360 nm. Identification was carried out comparing retention time and spectrum observed in the samples with the pure standards injected in the same conditions, while quantification was realized using calibrated curve of four point to following pure standards: gallic acid, protocatechuic acid, catechin, 4-hydroxybenzoic acid, epicatechin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, vanillin, p-coumaric acid, ferulic acid, sinapic acid and L-tryptophan (aromatic aminoacid).

2.4 Antioxidant activity

Antioxidant activity of crude, cooked and digested spaghetti samples of both extracted fractions (free and bound) was evaluated by two spectrophotometric assays, using a spectrophotometer HP 8451 (Hewlett Packard, Cambridge, UK), as described below,

2.4.1 Ferric ion Reducing Antioxidant Power (FRAP)

Reducing power of samples to reduce Fe^{3+} to Fe^{2+} was evaluated following the methodology describe by Oyaizu (1986). In brief, 1 ml aliquots of methanolic extracts (crude, cooked and digested) were mixed with phosphate buffer (0.2 M, pH 6.6) and Potassium ferricyanide (1%), then the samples were incubated a 50ºC during 20 minutes. Time elapsed; 2.5 mL of trichloroacetic acid (10 %) was added to the samples and vortex. Then 2.5 mL to the before sample were mixed with distillated water and iron trichloride (0.1%). After mixing the samples in a vortex, reaction was carried out 10 minutes, the absorbance was measured at 700 nm. The blank was made by replacing the sample with distilled water. A calibration curve was made with the Trolox reagent. Results were expressed as mg Trolox equivalent (TE)/g dry weight sample.

2.4.2 ABTS radical cation scavenging activity assay

ABTS assay was performed with the procedure carried out by Leite et al. (2011). In summary, ABTS radical active was prepared mixing the radical ABTS with Potassium persulfate ($K_2S_2O_8$) and distilled water. The ABTS radical active was diluted in distilled water until the absorbance (at a wavelength of 734 nm) was between 0.70-0.72. Then 10 µL of the methanolic extracts (crude, cooked and digested) was mixed with 990 µL of diluted ABTS radical. The reaction was led to 6 minutes. After that, absorbance was measured at 734 nm. The blank was made by replacing the sample with distilled water. A calibration curve was made with the Trolox reagent. Results were expressed as µg Trolox equivalent (TE) / g dry weight sample.

2.5 Simulated in vitro gastrointestinal digestion

Simulated *in vitro* gastrointestinal digestion was carried out following the consensus procedure describes by Minekus et al. (2014). In brief, three digestion phases were simulated: oral, gastric and intestinal. For each of them, simulated digestion fluids were prepared with its corresponding electrolyte stock solution, enzymes, $CaCl_2$ and water, in the quantities that are indicated in the protocol describe by Minekus et al. (2014). For adjust the pH, HCl 1N and NaOH 1N solutions were used. The digestion process was carried out in falcon 50 mL tubes, which were arranged horizontally in a reciprocal shaking bath (JP Selecta™ Unitronic Reciprocating Shaking Bat) and incubated with constant temperature (37 ^{o}C), agitation (30 rpm) and specific time of each digestion phase.

Before to start *in vitro* gastrointestinal digestion, crude spaghetti samples were cooked in boiling tap water (relation 1:10) during their optimum cooking time (disappearance of the white uncooked core in pasta) (CS: 8.5 min.; SR-3: 6.0 min.; SR-T: 7.5 min.; SR-6: 5.5 min.; ST-6: 5.5 min.), which was previously determined by Lucas-Gonzalez et al. (2020). Then, the cooked spaghetti samples were cooled for 10 minutes while these were manually cut with a knife into small pieces, around 2.0-5.0 mm, and weighed.

Simulated gastrointestinal digestion start with oral phase, in which 5.00 ± 0.05 g of each cooked spaghetti samples were mixed with 5 mL of simulated salivary fluid (SSF) and incubated for 2 minutes. At the end of oral phase 10 mL of simulated gastric fluid was added to oral bolus, and when the pH was adjusted to 2.00 ± 0.02, gastric phase was left running for 2 hours. For stop gastric phase 20 mL of simulated intestinal fluid was mixed with the gastric bolus and pH was adjusted to 7.00 ± 0.02. Another 2 hours was needed of incubation for complete intestinal phase. Blanks of reagents were carried out of each studied simulated phase.

2.6 Polyphenol stability and their bioaccesibility after in vitro gastrointestinal digestion

The released polyphenols and their stability in the spaghetti matrix were study following the procedure describe by Gullón, et al. (2015). After each digestion phase, samples were

centrifuged to 12 000 g 10 min 4ºC, with the purpose to separate pellet fraction (PF) and chyme soluble fraction (CSF). Then, separated samples were collected, weighted and lyophilized. Two different methodologies were used for the extraction of phenolic acids from both digestion fractions; pellet fraction was extracted following the procedure for bound phenolic acid extraction described in the section 2.4, while soluble chime fraction was extracted with 2.5, 5.0 or 10.0 mL of 50 % (v/v) aqueous methanol for 8 h at room temperature. After centrifugation at 127 *g* for 5.0 min, the supernatant was filtered through a 0.45 μm syringe filter prior to analysis by HPLC. Bioaccessibility index and colon available index (%) was calculated as following:

$$\%\ Bioaccessibility\ index = \frac{CSF_i}{TP_c} \times 100 \text{ (Equation 1)}$$

$$\%\ Colon\ available\ index = \frac{PF_i}{BP_c} \times 100 \text{ (Equation 2)}$$

Where,

CSF_i: polyphenols sum in chyme soluble fraction after intestinal phase (μg /g)

TP_C: Total polyphenols after cooking treatment (μg /g)

PF_i: polyphenols sum in pellet fraction after intestinal phase (μg /g)

BP_C: Total bound polyphenols after cooking treatment (μg /g)

2.7 Total starch content

Total starch content of crude spaghetti samples was determined following the AOAC Official Method 996.11 (AOAC, 2000) using K-TSTA Megazyme assay (Megazyme Ltd, Ireland) for total starch determination. Results were expressed as percentage of dry weight.

2.8 Kinetic of starch digestion and predicted glycemic index

The Protocol described by Goñi, Garcia-Alonso and Saura-Calixto (1997) was used for determining the *in vitro* kinetic of starch digestion and the predicted glycemic index. However, many modifications were carried out. So, in *vitro* digestion simulation was performed as describe above, but incubation time of gastric phase was of one hour. After 2, 30, 60, 75, 90, 120, 150 and 180 min, aliquots (100 µL) were collected and after enzyme inhibition by thermal shock (5 min to boiling water bath) were diluted. Then, 1 mL of the diluted aliquot was mixed with 3 mL of acetate buffer (pH 4.50) and 100 µL of amiloglucosidase (3,300 U/mL). The mixture was vortex mixed and incubated for 30 minutes at 50 °C. Then, the content was made up to 10 mL with distilled water and an aliquot (100 µL) was mixed with 3 mL of GOPOD reagent and incubated for 20 minutes at 50 °C. Absorbance of blank, sugar pattern and samples were measured a 510 nm. Results were expressed as percentage of starch hydrolyzed calculated following the equation 3

$$\% \, Starch = A \times F \times V_D \times \frac{D}{W_d} \times 0.9 \text{ (Equation 3)}$$

A= Absorbance sample

F= factor to convert absorbance values to µg of D-glucose (100 µg of D-glucose divided by the GOPOD absorbance value for 100 µg of D-glucose)

V_D= Digestion phase volume (mL)

D= Dilution factor

W_d= Sample dry weight (mg)

0.90= factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch.

Predicted glycemic index (pGI) was calculated as the area under the curve (AUC) of each studied spaghetti formulation, with the help of first order equation of hydrolytic process (Equation 4 and 5), using white bread as reference food. The equation 6 and 7 proposed by Goñi et al. (1997) was used for calculating the estimated glycemic index. The concentrations obtained at 120 minutes were used as final reaction time.

$$C = C_\infty \left(1 - e^{-kt}\right) \text{ (Equation 4)}$$

$$AUC = C_\infty (t_\infty - t_0) - (C_\infty / k) \left[1 - e^{[-k(t_\infty - t_0)]}\right] \text{ (Equation 5)}$$

$$HI = AUC_{Spaghetti} / AUC_{white\ bread} \times 100 \text{ (Equation 6)}$$

$$eGI = 39.71 + 0.549 \, HI \text{ (Equation 7)}$$

Where,

C= % hydrolyzed starch

C_{∞}= % hydrolyzed starch at final time

k= kinetic reaction constant

t_{∞} = final reaction time (120 minutes)

t_0 = start reaction time

2.9 Statistical

Values were expressed as mean ± standard deviation of three repetitions. To know the differences between, total starch content and pGI among the samples a simple ANOVA was carried out. The differences in kinetic of starch digestion and starch hydrolysis during *in vitro* gastrointestinal digestion were evaluated carried out a two-ANOVA assay. The differences among the free and bound polyphenols content of spaghetti formulations were studied through two-ANOVA assays: Firstly, the differences among crude and cooked samples for the different spaghetti formulations and then, the differences among cooked and digested phases (oral, gastric and intestinal) for the different formulations. Statistical significances were considered when p value was <0.05 after Tukey's post hoc test. Pearson correlation analysis was used to investigate the relations between polyphenols and ABTS and FRAP values. Statistical analyses were performed using the STAT graphic program.

3. Results and discussion

3.1 Phenolic profile of crude and cooked spaghetti

Among ten quantified bound polyphenols in crude CS, which can be shown in Figure 1, ferulic acid was found in the biggest amount, following by sinapic acid, ferulic acid derivatives (I and II), protocatechuic acid, catechin, 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid and vanillin. These results were in agreement with scientific literature, where bound ferulic acid is the major phenolic acid present in durum wheat spaghetti (Biney et al., 2014; Fares, Platani, Baiano & Menga, 2010, Ciccoritti et al., 2017).

The enrichment of durum wheat spaghetti with both studied persimmon flours, modified its polyphenolic profile, because, two new compounds, gallic acid and p-coumaric-o-hexoside, have been detected in the enriched samples (SR-3, ST-3, SR-6 and ST-6). Furthermore, while in CS the biggest polyphenol was ferulic acid, in the enriched samples was gallic acid. However, persimmon flour in spaghetti did not reduce or increase the content of phenolic compounds derived from wheat (p>0.05). In consequence, the content of total bound phenolic acid increased 2-fold and around 3-fold in spaghetti with 3% and 6% persimmon flours, respectively, in comparison with the content of CS (p<0.05). These results were expected, because gallic acid represented around 90% among of the total of polyphenols detected and quantified (gallic acid, 4-hydroxybenzoic acid, coumaric acid-o-hexoside, catechin, epicatechin, quercetin, kaempferol,

ellagic acid, and various glycosylated quercetin and kaempferol compounds) in both studied flours as can be observed in Lucas-González et al. (2018a,b). Thus, the presence of other polyphenols contributed by persimmon flours to spaghetti was limit by the low concentration in the persimmon flours and the possible loss during the making spaghetti process, although free phenol compounds are more susceptible to degradation during process than bound phenol compounds (Fares et al., 2010). However, as exception of this fact, it is the case of the coumaric acid o-hexoside, which although was found in fewer concentrations in Rojo Brillante and even in Triumph flour was not detected (Lucas-González et al. 2018b), it has been quantified in the enriched spaghetti. This fact could be due to the different extraction methods (in the referenced work about the polyphenol profile of persimmon flour, neither alkaline nor acid hydrolysis was carried out to determine bound phenol compounds), different harvest condition, postharvest treatment and fruit state maturation.

Significant differences between enriched crude spaghetti samples were observed, depending on persimmon flour type and dose; the amount of gallic acid and coumaric acid-o-hexoside was dose-dependent ($p<0.05$) and SR-6 showed the biggest amount of gallic acid ($p<0.05$), while ST-6 showed the highest amount ($p<0.05$) of coumaric acid-o-hexoside. Other authors (Khan, Yousif, Johnson & Gamlath ,2013) have previously reported dose-dependent rise in polyphenolic content of durum wheat spaghetti by addition of novel ingredients.

As regard to the content of free polyphenols in crude spaghetti (Figure 2), in the case of crude CS three phenolic acids (protocatechuic, vanillin and ferulic acid) were identified, and four (the same than for crude CS and the gallic acid) in the enriched spaghetti samples (SR-3, ST-3, SR-3 and ST-6). Other authors have detected another free phenolic acids, such as sinapic, caffeic, o-comuaric, hydroxybenzoic acid glucoside, 8-C-Glucosyl-6-C-arabinosyl-apigenin and vanillic acid, in whole wheat spaghetti (Takács, et al., 2018) or whole wheat noodles (Podio, Baroni, Pérez & Wunderlin, 2019). These differences could be due to the different extraction methods, the wheat varieties used treatment and environment (Mpofu et al., 2006). Furthermore, in all studied samples, L-tryptophan, an aromatic amino acid, was detected; this compound has been previously detected in free fractions of durum whole-wheat noodles (Podio et al., 2019).

Cooking treatment affected, indifferent ways, to bound and free phenolic acids (Figures 1 and 2); On the one hand, it caused insignificant changes in the content of bound phenolic acids present in all spaghetti samples, given that, only was observed a significant lost in the vanillic acid content ($p<0.05$). These results differed from those shown by other authors (Fares, Platani, Baiano, & Menga, 2010; Khan et al., 2013) who reported that cooked process increased the amount of bound phenolic acids in pasta, due to the links breakdown among phenolic acids and wall cells, and others that indicated losses after cooking process (Biney et al., 2014). These variations could be due to the different processing of spaghetti (Khan et al., 2013), the optimum

cooking time, or the wheat variety. For example, the optimum cooking time used to cook the studied spaghetti was low (around 5.5-8.5 min), if it is compared with other works (around 10-15 minutes) and so they reported a higher bound polyphenol content (Fares et al., 2010; Khan et al., 2013). On the other hand, the cooked process significantly decreased the content of free polyphenols present in studied spaghetti ($p<0.05$), probably due to the lixiviation of these compounds to cooking water. As in the case of bound polyphenols, conflicted results can be found in scientific literature, where cooking treatment can improve the free phenolic content in spaghetti (Ciccoritti, et al 2017) or reduce it (De Paula, Rabalski, Messia, Abdel-Aal, & Maeconi, 2017)

3.2 Polyphenol stability and their bioaccessibility after in vitro gastrointestinal digestion.

The evolution of polyphenols presents in spaghetti samples (CS, SR-3, ST-3, SR-6 and ST-6) during *in vitro* gastrointestinal digestion was studied in the three digestion steps (oral, gastric and intestinal), in both fractions, the chyme soluble fraction and in the pellet fraction (Figure 2.A), with the propose to have a complete vision of their matrix release and bioaccessibility during enzymatic and mechanic process. The variations showed in their polyphenol content after digestion, were dependent on the own compound, the fraction and the digestive phase.

After oral phase in the chyme soluble fractions only protocatechuic and ferulic acids (traces amounts) were observed in low concentrations in comparison with that showed in cooked samples. Furthermore, spaghetti samples presented the same concentrations ($p<0.05$). On the contrary, the highest content and diversity of free phenolic acids was found in gastric phase, since protocatechuic, vanillin, gallic acid (only in enriched spaghetti formulations), ferulic acid, catechin and epicatechin were detected. In the case of catechin and epicatechin, which have not been detected in the other studied extracts (crude, cooked and oral), their presence could be due to the acid degradation of proanthocyanidins (condensed tannins) (Zhu et al, 2002), a type of oligomeric flavonoids, whose common oligomers are (epi)gallocatechins, (epi)catechins, and (epi)afzelechins. However, after intestinal phase catechin, epicatechin, gallic acid and vanillin were not detected, which could be due to intestinal conditions, interaction with other dietary compounds or even biliary interactions (Kida, Suzuki, Matsumoto, Nanjo, & Hara, 2000; Zhu et al., 2002). On the other hand, a significant increase in the concentration of protocatechuic acid and ferulic acid, with respect to the other digestion phases (oral and gastric) were detected ($p<0.05$). These increases could be due to the release of both polyphenols from their spaghetti matrix or to the degradation of flavonoids, like catechin, in the case of protocatechuic (Sánchez-Patán, Monagas, Moreno-Arribas & Bartolomé, 2011). Podio et al. (2019) also showed that ferulic acid increased after intestinal phase with respect to oral and gastric phases. As regard to L-tryptophan (Figure 2.B), the content increased gradually after oral and gastric phases and strongly after intestinal phase, which was mediated by protease enzyme. Among studied spaghetti, CS showed

the highest polyphenols bioaccessibility (18.7%, Figure 1), whereas the bioaccessibility of polyphenols presents in enriched persimmon flours spaghetti (SR-3, ST-3, SR-6 and ST-6) was around 10 %, showing SR-6 samples the lowest values. These differences were due to the higher total polyphenol content in enriched persimmon flours spaghetti than in the CS, since after intestinal phase, the 3 % and 6% enriched spaghetti had 2 and 3 fold polyphenol content than CS, respectively (p<0.05). The low bioaccessibility of wheat pasta determined *in vitro* have been previously reported by Takács et al. (2018) and Podio et al. (2019).

As regard to bound fraction, in general, intestinal phase promoted a decrease in their concentration. However, if the behavior of each compound is detailed, substantial differences can be observed among individual compounds (Table 1). So, the amount of ferulic acid in studied spaghetti (CS, SR-3, ST-3, SR-6 and ST-6) increased discreetly in oral and gastric phases, while significantly decreased after intestinal phase (p<005). In the case of *p*-coumaric acid, vanillic acid, vanillin, 4-hydroxybenzoic acid and both derivative ferulic acids (I and II), the oral digestion conditions caused significant loses in their concentration, in comparison with the amounts showed in cooked spaghetti (p<0.05), which were remained along digestive process (p>0.05). As regard to the content of sinapic acid and catechin it was also downed, but in a gradual way through gastrointestinal digestion. The protocatechuic acid content in the three digested samples (oral, gastric and intestinal) of the studied spaghetti was always lower than in cooked samples, however after gastric phase a light raise was showed (p<0.05). The behavior of gallic acid was similar than reported for the ferulic acid, briefly, a significant increase was observed in oral phase, then its concentration decreased in gastric and intestinal phases, although these differences were only significant respect to the cooked samples at intestinal phase. Furthermore, 6% persimmon flour enriched spaghetti showed higher amounts of gallic acid than those showed in the 3% formulations (p<0.05). In addition, a dose-dependent increase was showed in the content of coumaric acid-*O*-hexoxide, but only after intestinal phase. After digestion process, the most remarkable differences in the polyphenols content between CS and enriched spaghetti were for the ferulic acid content, showing higher concentration in the persimmon flours enriched spaghetti (SR-3, ST-3 SR-6 and ST-6) than in the CS at the end of intestinal phase, and also the presence of gallic acid and coumaric acid-*O*-hexoside. Therefore, the bound polyphenols release from spaghetti matrix after *in vitro* gastrointestinal digestion were poor. Chait, Gunenc, Bendali & Hosseinian (2020) reported low matrix release of bound polyphenols, like gallic acid, vanillic acid and protocatechuic acid content in carob after *in vitro* digestion process. Therefore, after digestion process a high number of polyphenols continue linked to cell walls and to indigestible polysaccharides. Spaghetti samples showed different colon available polyphenols index (Figure 1), following this serie ST-6>SR-6>ST-3>SR-3>CS. So, a high amount of polyphenols could arrive to colon and could be used by gut microbiota. Polyphenols present in wheat can the ability to

modulate gut microbiota in a positive way, since promote the grow of *Bifidobacterium* and *Lactobacillus*, and reduce the proliferation of *Escherichia coli* and *Clostridium spp.* (Costabile et al., 2008). Gallic acid has also b linked to promoting the grow of beneficial bacteria and the inhibition of harmful bacteria (Li et al., 2019). Actually, many scientists start to point that cancer colonic prevention derive to cereals consumption could be associated with their polyphenols content although these polyphenols have not a high bioaccessibility (Mileo, Nisticò & Miccadei, 2019). Regarding these investigations, it could be said that although the majority of bound polyphenols have not been released from their food matrix during gastrointestinal digestion, these can still have a health-promote action, which in the case of studied spaghetti samples could be enhanced by the presence of gallic acid.

3.3 Antioxidant activity of crude and cooked spaghetti

The antioxidant potential of crude spaghetti samples and their changes after cooked and digested process were studied using two different *in vitro* antioxidant essays, ABTS and FRAP.

As regard to crude samples, persimmon flour enriched spaghetti showed higher ABTS and FRAP values than CS samples in both polyphenol fractions ($p<0.05$), as can be observed in Table 1. That increase was dose dependent ($p<0.05$), except for the activity of the free fraction in the ABTS assay, where not differences were showed between persimmon flour enriched spaghetti ($p>0.05$). These results agreed with those reported by Khan et al. (2013) and De Paula et al. (2017), who reported a dose-dependent increase in antioxidant activity in spaghetti enriched with other vegetable ingredients at increasing concentrations.

Cooking treatment caused a significant loss in the ABTS radical scavenging ability and in the ability to reduce iron in the free fraction, respect to the crude CS sample, while these antioxidant activities were not modified in the bound fractions. Enriched spaghetti showed the same behavior than control during the cooking ($p<0.05$), however the FRAP and ABTS values in both fractions were higher and dose-dependent than in control samples ($p<0.05$). These results can be correlated with the results found in polyphenol profile (Figure 2.A). Podio et al. (2019) reported no changes in the antioxidant activity of whole noodle samples make with whole wheat flour in free fractions and an increase in bound fraction while other authors (Takács et al.,2018; Khan et al., 2013) have observed loses in wheat spaghetti and durum wheat spaghetti enriched with sorghum, respectively, after cooking process.

3.4 Antioxidant activity during in vitro gastrointestinal digestion

As regard to the antioxidant activity in the chyme soluble fractions of studied spaghetti, their ABTS and FRAP values showed a slight increase after oral and gastric digestion that was higher after intestinal phase, in comparison with values reported for liquid fractions in cooked samples ($p<0.05$). Furthermore, no differences in ABTS radical scavenging activity and ferrous-

ion chelating capacity between the CS and persimmon flour enriched spaghetti (p>0.05) were found for any simulated digestion step, with the exception of the Triumph flour enriched spaghetti, whose FRAP values after intestinal digestion were the highest (p<0.05). Podio et al. (2019) also reported a progressive increase in the FRAP values of free fractions in durum wheat noodles after simulated gastrointestinal digestion with the highest values observed in the intestinal step. It is important to note that the antioxidant activity in chyme soluble fractions measured by ABTS and FRAP assays was strongly correlated with the L-tryptophan content in the spaghetti samples (R^2= 0.984 p<0.001; R^2=0.917, p<0.001, respectively). ABTS assay seem to be more sensitive to the antioxidant activity of L-tryptophan than FRAP assay, since a strong positive correlation was also found between protocatechuic acid content and FRAP values in free fraction (R^2=0.999 p<0.001). These results were in agreement whit those reported by Pešić et al. (2019), who attributed the increase in ABTS radical scavenging activity and ferrous-ion chelating capacity observed in digested meat food matrix enriched with grape extracts, to the presence of meat protein hydrolysates and carnosine, being ABTS the most affected.

In the case of the ferrous-ion chelating capacity showed by CS in the pellet fraction, the oral digestion improved it (p<0.05), when compared with the antioxidant activity showed after cooking, but then, the subsequent digestions caused a gradual decrease in their antioxidant activity (p<0.05). Whereas, in the case of persimmon enriched spaghetti samples (SR-3, ST-3, SR-6 and ST-6), the losses in their ferrous-ion chelating ability caused by digestion process (p<0.05) were similar between the three simulated digestion phases (p>0.05). However, in spite of the mentioned reduction in their antioxidant activity, their FRAP values were always higher than those of CS (p<0.05). These results were correlated with the polyphenol content in studied spaghetti.

As regard to the ABTS+ radical scavenging activity in the pellet fraction of persimmon enriched spaghetti, their ABTS values increased significantly after digestion process, with respect to the values showed in the bound fractions of the respective cooked spaghetti (p<0.05). However, their antioxidant activity was gradually reduced through the gastrointestinal digestion, so the lowest values were showed after intestinal phase (p<0.05). Furthermore, the enrichment with persimmon flours (Rojo Brillante and Triumph) improved their ABTS+ radical scavenging ability after digestion process, in a concentration-dependent manner (p<0.05). These increases could be due to the presence of other bioactive compounds, like carotenes or terpenes in the digested bound extract. Other authors (Chait, Gunenc, Bendali & Hosseinian, 2020) in studies about the polyphenol bioaccessibility in several foods reported increases in the antioxidant activity measured in the free fractions and reductions in the bound fractions, as occurred in the current work.

3.5 Kinetic of starch digestion

As can be seen in Figure 4, the starch hydrolysis of the five studied spaghetti started in the oral phase by the action of alpha-amylase, where around 10 % of starch was released from the spaghetti matrix ($p<0.05$). The starch hydrolysis continued in gastric phase, where, after 30 minutes, around 13-19 % of starch was transformed into glucose, then, the hydrolysis process remained without significant changes ($p>0.05$) until the end of gastric phase. Although the action of salivary alpha-amylase has been usually ignored in the past, due to the short contact with the food and the optimal pH action (6.9), it seems that during gastric phase it have residual activity (Bustos, Vignola, Pérez & León, 2017; Freitas & Le Feunten, 2019). The highest ratios of starch hydrolysis (around 50-60%) were observed during the intestinal phase. The released glucose continuously increased until 120 minutes and then a plateau was observed until the end of digestion process, since not differences were observed between the starch digestibility at 120, 150 and 180 minutes of *in vitro* digestion models ($p<0.05$). As regard to kinetic of starch digestion of white bread, the highest starch hydrolysis rate was observed in gastric phase, where of 66 % of starch was releases from white bread matrix after 30 minutes. These results were similar to that reported by Bustos et al. (2017) for both, white bread and whole-grain pasta. The kinetic of starch digestion was similar for the 3% enriched persimmon flour spaghetti ($p>0.05$) and was different from the rest of studied spaghetti ($p<0.05$). Therefore, persimmon flours (Rojo Brillante and Triumph) enrichment to durum wheat spaghetti modified their kinetic of starch hydrolysis, being these modifications concentration-dependents and in the highest studied concentrations (6%), also to persimmon flour type. Lu et al. (2018) reported changes in the kinetic of starch digestion in an *in vitro* digestion model of fresh pasta (durum wheat semolina) with different amounts (5, 10 and 15%) of mushroom powders (white button, shiitake and porcini mushrooms) depending on the concentration and type of mushroom, however these results showed an inverse proportionality between concentration and starch digestibility. Chillo et al. (2011) studied the effect of two barley β-glucan concentrates (Glucagel™ and Barley Balance™) on *in vitro* glycaemic impact of durum wheat spaghetti, reporting that the starch hydrolysis of the spaghetti was reduced by Barley Balance™ (reduction dose-dependent) but not by Glucagel™.

The differences found in the starch hydrolysis between the 6% persimmon flour enriched spaghetti and the rest of studied samples (SR-3, ST-3 and CS) could be related with insufficient semolina hydration during spaghetti processing, derived from the competition for water between semolina and the fiber and sugars present in persimmon flours. The consequence of a low semolina hydration is the supramolecular modification in the gluten network (Lucas-González et al 2020). In this sense many researchers have pointed that the defects in the gluten network induce by fiber, especially insoluble fiber, causes open pores in their structure which could promote the greater accessibility of enzyme attack to starch granules (Bustos et al., 2017; Padalino, et al. 2017). Although the water-holding ability of both studied persimmon flours was

taking into account when pasta where produced, Lucas González et al. (2020) reported a weaker structure in SR-6 and ST-6 samples. Since these samples were easily fracturable in comparison with the other studied spaghetti, being ST-6 which showed lower fracturabilty value (defined as the force needs to break an uncooked strand of spaghetti when mechanical strength is applied). Furthermore, this sample also showed the lower value of uncooked diameter, which are related to shrinkage phenomena during drying of spaghetti. As regards to the total fiber content in spaghetti samples, this content increased depending on the persimmon flour concentration and type, so the ST-6 showed the highest value, followed by SR-6>ST-3>SR-3>SC (Lucas-González et al. 2020). Furthermore, insoluble fiber content is higher in Triumph flour than Rojo Brillante flour (Lucas-González et al., 2017). These facts point out the negative effect of the high fiber content contributed by persimmon flours to the durum wheat semolina. However, to improve the final structure of spaghetti with a higher concentration of persimmon flours and to reduce the starch hydrolysis index, the effect of proper hydration could be studied, perhaps increasing the water in the spaghetti formulation or even mixing the flours (semolina and persimmon flours) with the water separately.

3.6 Predicted Glycemic index

The glycemic index is defined by FAO/WHO experts as "the incremental area under the blood glucose response curve of a 50g carbohydrate portion of a test food, expressed as a percent of the response to the same amount of carbohydrate from a standard food taken by the same subject" (FAO/WHO, 1998). It is considered a useful nutritional concept to classify foods in function of its ability to increase postprandial insulin respond. Nowadays, it is known that the post-prandial insulin respond is a good indicator of lipid anabolism and consequently a weight increase indicator in form to visceral fat. The predicted glycemic index (pGI) of studied spaghetti ranged from 79.3 to 91.8 (Table 2), being the 3% persimmon enriched formulations which showed the lower value ($p<0.05$), and the 6% persimmon enriched formulations the highest, although these differences were not significant respect to the control samples. The differences between pGI among enriched spaghetti could be due to gluten network modification and the greater amylase attack to starch as previously have been discussed. All values were into the range of *in vivo* spaghetti GI (Foster-Powell et al., 2002) and following the carbohydrate food classification in base of their GI, mentioned before, all formulations showed a high glycemic index. These results were higher than that reported by other authors (Goñi et al., 1997; Simonota et al., 2019). These difference could be due to *i*) differences in the *in vitro* methods used, since these authors did not carry out oral phase; *ii*) different size of spaghetti strand, since a less size is more easy to be attacked by digestive enzymes, so in the present work small spaghetti strand was used (2-5 mm) following the recommendations of Minekus et al. (2014), however other authors used higher sizes, which are more close to real mastication of spaghetti, and iii) spaghetti processing (extrude, dry temperature, etc.).

4. Conclusions

Polyphenol profile of spaghetti (control and persimmon enriched) was affected by cooking treatment, being these changes stronger when persimmon flours were added at higher concentrations and depending on the polyphenol form (free or bound) and on the individual compound.

The enrichment of durum wheat semolina with both persimmon flours (3, 6%) increases the total polyphenol content in a dose-dependent way and apports gallic acid and coumaric acid-o-hexoside, which are not present in the control spaghetti. The polyphenols bioaccessibility in the spaghetti was poor and it was not improved by the addition of persimmon flours. After *in vitro* digestion a high number of polyphenols continued link to cell wall or indigestible polysaccharides and so they could be used by gut microbiota. The enrichment of durum wheat spaghetti with both persimmon flours (Rojo Brillante and Triumph) modified the kinetic of starch digestion, decreasing it, only in the case of the 3% enriched persimmon spaghetti. However, the predicted

glycemic index was similar between control and enriched spaghetti. Spaghetti with 3% persimmon flours showed glycemic index lower than spaghetti with 6% persimmon flours. In conclusion, both persimmon flours (Rojo Brillante and Triumph) added at 3% could be used to develop spaghetti, with more polyphenol content and less starch digestibility than traditional spaghetti. Nevertheless, further *in vivo* assays are needed to confirm these *in vitro* results.

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Table 1.- Values of antioxidant activity determined by ABTS and FRAP assays in both fractions (free and bound) of studied spaghetti samples (crude, cooked and digested).

			Crude	Cooked	Oral	Gastric	Intestinal
ABTS (µg TE/ g d.w)	Free fraction	CS	24.2 ± 1.4^{Yb}	12.4 ± 0.5^{ZBa}	11.4 ± 1.7^{Ba}	15.2 ± 3.6^{Ba}	432.0 ± 138.9^{Aa}
		SR-3	31.1 ± 2.6^{Ya}	16.5 ± 1.8^{ZBa}	8.5 ± 1.4^{Ba}	15.3 ± 3.7^{Ba}	457.1 ± 29.0^{Aa}
		ST-3	26.4 ± 0.8^{Ya}	18.7 ± 1.9^{ZBa}	8.9 ± 0.7^{Ba}	15.4 ± 2.2^{Ba}	492.0 ± 6.5^{Aa}
		SR-6	30.7 ± 3.0^{Ya}	16.2 ± 1.1^{ZBa}	8.2 ± 0.8^{Ba}	15.6 ± 2.3^{Ba}	427.0 ± 9.0^{Aa}
		ST-6	33.6 ± 0.8^{Ya}	16.5 ± 0.6^{ZBa}	7.5 ± 1.5^{Ba}	16.3 ± 4.7^{Ba}	447.2 ± 32.1^{Aa}
	Bound fraction	CS	13.7 ± 1.5^{Yc}	16.4 ± 2.2^{AYc}	9.1 ± 0.8^{Ac}	9.1 ± 1.7^{Ac}	6.5 ± 0.5^{Bc}
		SR-3	22.9 ± 1.4^{Yb}	26.0 ± 6.2^{CYbc}	55.2 ± 3.1^{Ab}	51.0 ± 2.5^{Ab}	45.2 ± 1.5^{Bb}
		ST-3	28.9 ± 3.5^{Yb}	33.6 ± 4.9^{CYba}	52.1 ± 7.1^{Ab}	55.5 ± 8.4^{Ab}	47.1 ± 3.1^{Bb}
		SR-6	42.3 ± 3.2^{Ya}	43.9 ± 3.5^{CYa}	72.3 ± 10.1^{Aa}	68.8 ± 11.1^{Aa}	56.9 ± 2.6^{Ba}
		ST-6	40.2 ±3.6^{Ya}	41.0 ± 7.5^{CYa}	64.2 ± 9.6^{Aa}	63.3 ± 1.1^{Aa}	53.7 ± 7.3^{Ba}
FRAP (mg TE/ g d.w)	Free fraction	CS	0.69 ± 0.09^{Yc}	0.20 ± 0.01^{ZCc}	0.27 ± 0.03^{Ca}	0.77 ± 0.01^{Ba}	4.98 ± 0.19^{Ab}
		SR-3	1.05 ± 0.10^{Ybc}	0.42 ± 0.09^{ZCb}	0.32 ± 0.02^{Ca}	1.24 ± 0.06^{Ba}	5.21 ± 0.74^{Ab}
		ST-3	1.29 ± 0.14^{Yab}	0.37 ±0.05^{ZCab}	0.36 ± 0.09^{Ca}	1.17 ± 0.08^{Ba}	6.18 ± 0.76^{Aab}
		SR-6	1.52 ± 0.15^{Ya}	0.52 ± 0.07^{ZCa}	0.47 ± 0.07^{Ca}	1.52 ± 0.25^{Ba}	5.83 ± 0.44^{Ab}
		ST-6	1.79 ± 0.13^{Ya}	0.40 ± 0.05^{ZCa}	0.37 ± 0.01^{Ca}	1.49 ± 0.17^{Ba}	7.79 ± 0.95^{Aa}
	Bound fraction	CS	0.14 ± 0.02^{Yc}	0.16 ± 0.04^{YBc}	0.25 ± 0.02^{Ab}	0.21 ± 0.06^{ABb}	0.15 ± 0.03^{Bb}
		SR-3	0.49 ± 0.04^{Yb}	0.55 ± 0.14^{YAb}	0.49 ± 0.03^{ABab}	0.49 ± 0.03^{ABa}	0.32 ± 0.05^{Bab}
		ST-3	0.69 ± 0.09^{Yb}	0.75 ± 0.16^{YAab}	0.51 ± 0.03^{ABa}	0.52 ± 0.03^{ABa}	0.46 ± 0.01^{Ba}
		SR-6	1.12 ± 0.09^{Ya}	1.07 ± 0.03^{YAa}	0.49 ± 0.03^{Bab}	0.50 ± 0.02^{Ba}	0.52 ± 0.00^{Ba}
		ST-6	1.10 ± 0.22^{Ya}	1.00 ± 0.09^{YAa}	0.48 ± 0.02^{Bab}	0.54 ± 0.01^{Ba}	0.52 ± 0.01^{Ba}

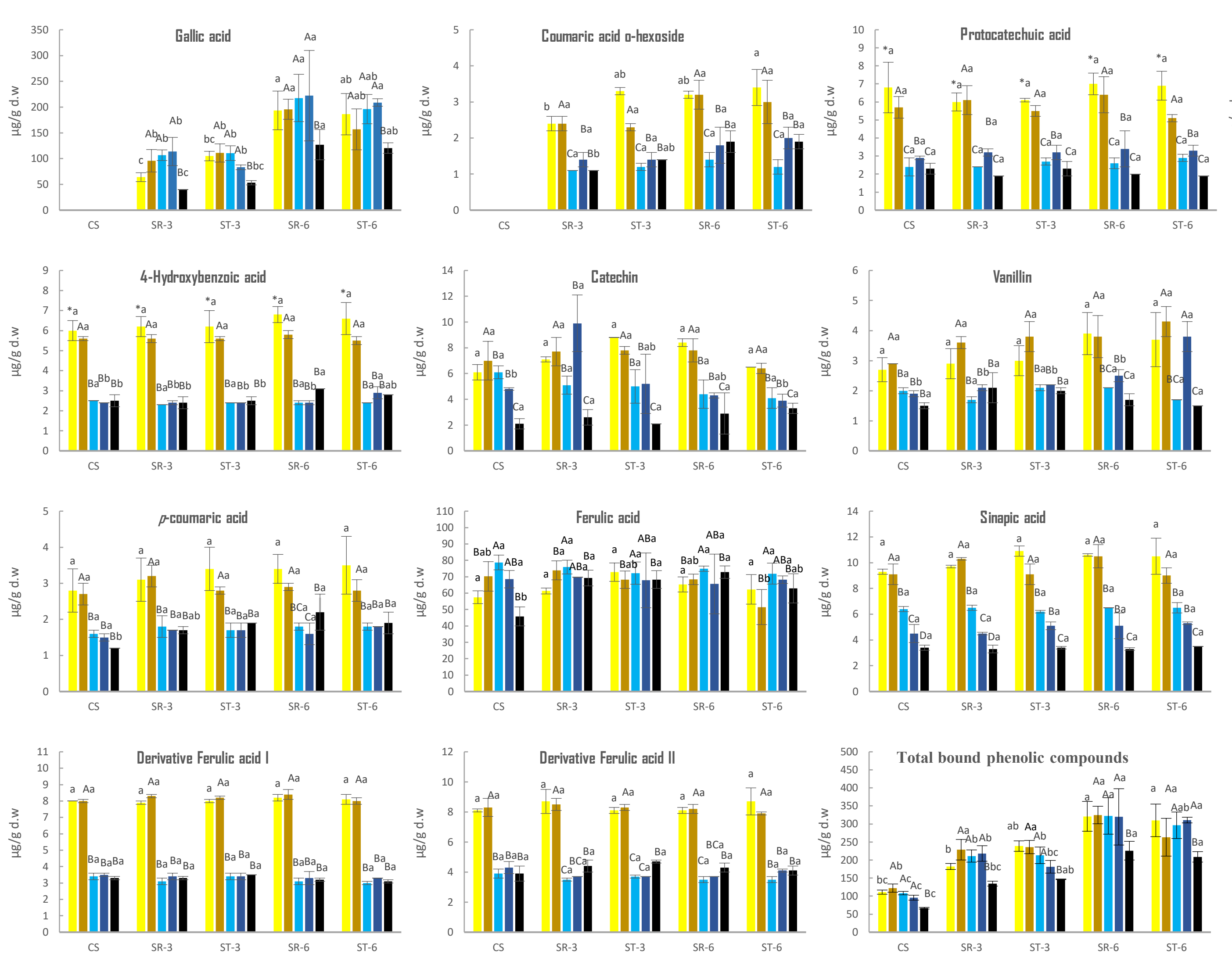
Values were expressed as mean ± standard deviation. Different capital letters ranged Y-Z expressed significant differences between crude and cooked values in the same row. Capital letters A-D expressed significant differences between cooked and the three digested phases (oral, gastric and intestinal) in the same row. Different case lower letters (a-e) in the same columns for each antioxidant method and fraction indicate significant differences. Statistically significant differences were considered when $p<0.05$ after Tukey's post hoc test. Control spaghetti (CS), spaghetti with 3 % of Rojo Brillante (SR-3), spaghetti with 3% of Triumph (ST-3), spaghetti with 6% of Rojo Brillante (SR-6) and spaghetti with 6% of Triumph (ST-6).

Table 2.- Total starch content (TS), total starch hydrolyzed at 120 and 180 min (SH_{120} and SH_{180}), kinetic constant (k), area under de curve (AUC), hydrolysis index (HI) and predicted glycemic index (pGI).

	TS (%)	SH_{180} (%)	SH_{120} (%)	k_{120}	AUC_{120}	HI_{120}	pGI_{120}
CS	74.5 ± 1.0^{a}	85.6 ± 4.3^{ab}	78.0 ± 9.0^{ab}	0.036 ± 0.001	7239 ± 889	82.5 ± 10.1^{ab}	85.0 ± 5.6^{ab}
SR-3	73.4 ± 0.5^{a}	76.6 ± 1.3^{b}	68.7 ± 1.5^{b}	0.035 ± 0.000	6326 ± 146	72.1 ± 1.7^{bc}	79.3 ± 0.9^{b}
ST-3	72.7 ± 0.7^{ab}	76.6 ± 6.7^{b}	70.1 ± 3.6^{b}	0.035 ± 0.000	6462 ± 354	73.7 ± 4.0^{c}	80.2 ± 2.2^{b}
SR-6	68.4 ± 2.7^{bc}	88.5 ± 0.2^{ab}	87.6 ± 0.8^{a}	0.037 ± 0.000	887 ± 78	93.3 ± 0.9^{a}	91.0 ± 0.5^{a}
ST-6	65.3 ± 1.1^{c}	91.7 ± 4.1^{a}	89.0 ± 1.3^{a}	0.037 ± 0.000	8324 ± 29	94.9 ± 1.5^{a}	91.8 ± 0.8^{a}
WB	65.2 ± 2.1	93.5 ± 2.0	93.5 ± 2.0	0.038 ± 0.000	877 ± 196	100	-

Different case lower letters (a-e) in the same columns indicated significant differences. Statistically significant differences were considered when $p<0.05$ after Tukey's post hoc test. Control spaghetti (CS), spaghetti with 3 % of Rojo Brillante (SR-3), spaghetti with 3% of Triumph (ST-3), spaghetti with 6% of Rojo Brillante (SR-6) and spaghetti with 6% of Triumph (ST-6).

Figure 1.- Bound polyphenol profile of crude, cooked and digested spaghetti (after the oral, gastric and intestinal phase).



Crude Cooked
Oral Gastric Intestinal

Values were expressed as mean ± standard deviation (µg/g dry weight).
* expressed significant differences between crude and cooked values. Capital letters A-D expressed significant differences between cooked and the three digested phases (oral, gastric and intestinal). Different case lower letters (a-e) for each individual phenolic compound indicate significant differences between the five studied samples. Statistically significant differences were considered when $p<0.05$ after Tukey's post hoc test. Control spaghetti (CS), spaghetti with 3 % of Rojo Brillante (SR-3), spaghetti with 3% of Triumph (ST-3), spaghetti with 6% of Rojo Brillante (SR-6) and spaghetti with 6% of Triumph (ST-6).

Figure 2.- Compounds detected in chyme soluble fraction of crude, cooked and digested spaghetti (after the oral, gastric and intestinal phase). A. Free polyphenol profile. B. Aromatic aminoacid.

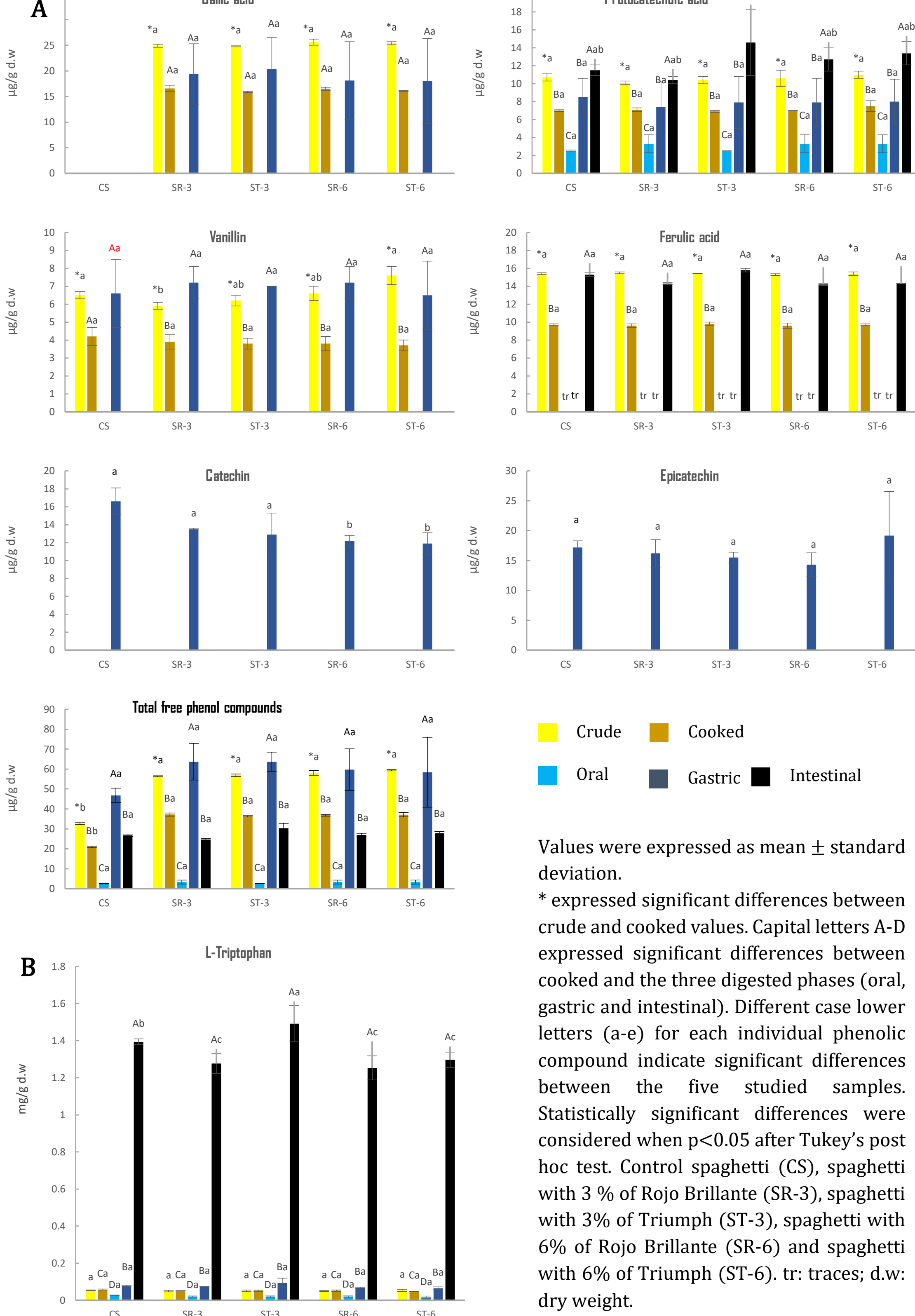
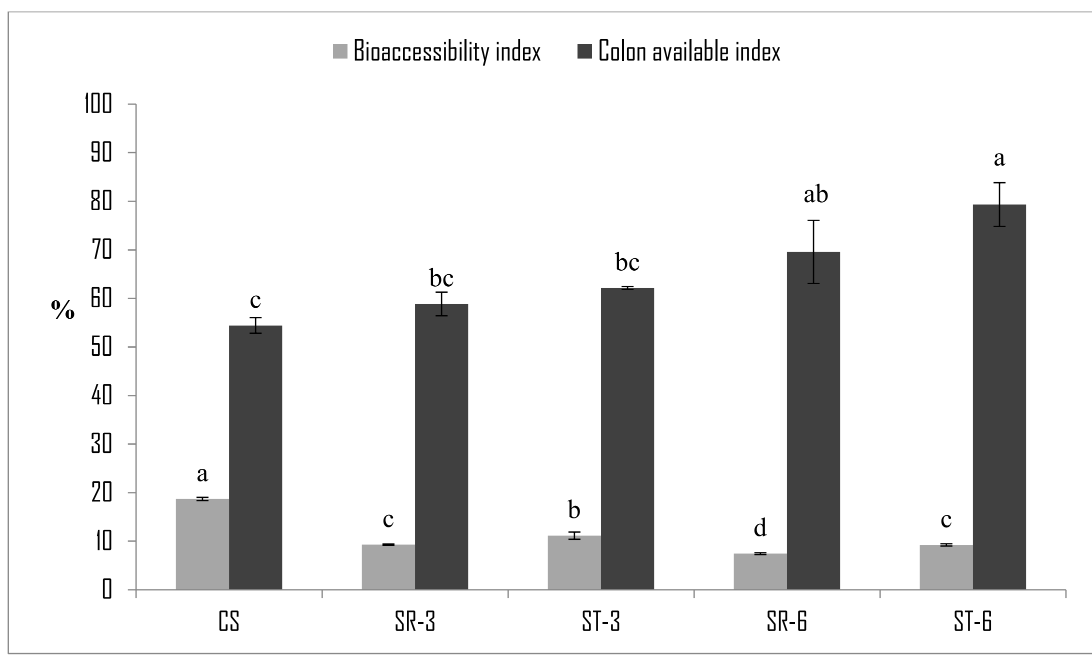


Figure 3.- Bioaccessibility index and colon available index of studied spaghetti.



Values are expressed as mean ± standard deviation (%). Different case lower letters (a-e) for each studied parameter indicate significant differences. Statistically significances were considered when $p<0.05$ after Tukey`s post hoc test. Control spaghetti (CS), spaghetti with 3 % of Rojo Brillante (SR-3), spaghetti with 3% of Triumph (ST-3), spaghetti with 6% of Rojo Brillante (SR-6) and spaghetti with 6% of Triumph (ST-6).

Figure 4.- Total starch hydrolysis rate of spaghetti during gastrointestinal digestion.

Different case lower letters (a-e) indicated significant differences. Statistically significant differences were considered when $p<0.05$ after Tukey's post hoc test. Control spaghetti (CS), spaghetti with 3 % of Rojo Brillante (SR-3), spaghetti with 3% of Triumph (ST-3), spaghetti with 6% of Rojo Brillante (SR-6) and spaghetti with 6% of Triumph (ST-6).

7.8.- PUBLICACIÓN 8

Título: Pork liver pâté enriched with persimmon coproducts: Effect of *in vitro* gastrointestinal digestion on its fatty acid and polyphenol profile stability

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Article

Pork Liver Pâté Enriched with Persimmon Coproducts: Effect of In Vitro Gastrointestinal Digestion on Its Fatty Acid and Polyphenol Profile Stability

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Abstract: Agrofood coproducts are used to enrich meat products to reduce harmful compounds and contribute to fiber and polyphenol enrichment. Pork liver pâtés with added persimmon coproducts (3 and 6%; PR-3 and PR-6, respectively) were developed. Therefore, the aim was to study the effect of their in vitro gastrointestinal digestion on: the free and bound polyphenol profile (HPLC) and their colon-available index; the lipid oxidation (TBARs); and the stability of the fatty acid profile (GC). Furthermore, the effect of lipolysis was investigated using two pancreatins with different lipase activity. Forty-two polyphenols were detected in persimmon flour, which were revealed as a good source of bound polyphenols in pâtés, especially gallic acid (164.3 µg/g d.w. in PR-3 and 631.8 µg/g d.w. in PR-6). After gastrointestinal digestion, the colon-available index in enriched pâté ranged from 88.73 to 195.78%. The different lipase activity in the intestinal phase caused significant differences in bound polyphenols' stability, contributing to increased lipid oxidation. The fatty acids profile in pâté samples was stable, and surprisingly their PUFA content was raised. In conclusion, rich fatty foods, such as pâté, are excellent vehicles to preserve bound polyphenols, which can reach the colon intact and be metabolized by the intestinal microbiome.

Keywords: lipolysis; coproducts; Rojo Brillante; *Diospyros kaki*; in vitro digestion; plant-food; UV spectra

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1. Introduction

Pork liver pâté is a widespread meat product with high nutritional and density value related to protein, hem-iron, and vitamin content [1]. Furthermore, for some sectors of the population, like children, the elderly, or people with swallowing problems, pork liver pâté can provide an excellent alternative to fresh meat, since it provides high-value nutrients in a small portion and does not require effort in chewing. Nevertheless, given the evidence linking daily meat products consumption with colorectal cancer [2,3], their consumption must be reduced to one or two portions per month. This association between meat products and cancer has been attributed, firstly, to an excessive consumption (50 g per day) and, secondly, to the presence of harmful substances [2]. In pork liver pâté, the most probably dangerous substances are N-nitrous compounds and lipolysis oxidative products. Most manufactured pork liver pâtés have the preservative sodium nitrite, which prevents the proliferation of *Clostridium botulinum* and has antioxidant and colorants effects. However, free form (residual nitrite) can react with hemo-pigments and generate N-nitrous compounds such as nitrosamines [4]. Furthermore, all rich fat foods are susceptible to lipid oxidation, above all, if they are rich in iron and are undergoing the mincing and high-temperature process [5,6].

Diverse strategies to reformulate healthy meat products have been studied and developed by both researchers and the meat industry [7–10]. One of them is the use of

agrofood coproducts, such as antioxidants, nitrite reducing agents, and fat replacers, for their fortification with fiber and bioactive compounds [4,11–13].

Recently, many investigations have pointed out that polyphenols, especially in their insoluble-bound form, which remains covalent joining the cell wall, could modulate the intestinal microbiome's composition [14,15]. Furthermore, some authors pointed out the potential of polyphenol-biofortified food as a novel tool for preventing human gut diseases [16]. Many authors have shown that polyphenol mitigates the early stage of colon inflammation [17,18]. In this line, Direito et al. [19] showed that persimmon phenols inhibit colitis and colon cancer cell proliferation in rats. Persimmon consumption has also been linked with reducing the atherogenic process due to, mainly, their ability to bind bile acids and protect plasma lipid from oxidation [20–22]. Regarding persimmon coproducts, they are rich in polyphenols, carotenoids, and fiber [23–25]. Previous works showed their ability to reduce residual nitrite in pork liver pâté [26]. Moreover, the use of agro-food coproducts stimulates food industry sustainability. However, during digestion, the nutrients' behavior related to their digestibility, stability, and absorption can change when the food matrix where it is embedded is modified or new compounds are added; consequently, the postprandial response is also expected to be changed [27]. Accordingly, as foods are more than the sum of their parts, it is essential to investigate the interactions and effects of the matrix on the final biological activity of nutrients, because more is not always the better. For this purpose, in vitro digestion methods are useful screening methods to evaluate the effects of food reformulations or fortifications.

In the last decade, many researchers have evaluated the digestive process's repercussion in developing lipid oxidation and fatty acids bioaccessibility in fresh meat and fish and their derived products [5,28–33] and polyphenols' stability after in vitro digestion [34–38]. However, most research have not considered the recommendations carried out by the harmonized, static in vitro digestion protocol proposed by the Cost-Infogest network about the increase in lipase activity (2000 UL/mL) and the addition of colipase in the intestinal phase when rich fatty foods are digested [39]. Recently, the Cost network has also recommended the use of gastric lipase [40]. The high cost of individual enzymes and colipase vs. pancreatin could explain these facts. Another alternative to achieve high lipase activity, cheaper than the use of individual enzymes and colipase, is to use pancreatin to avoid exocrine pancreatic insufficiency, previously proposed by Calvo-Lerma et al. [41]. Tullberg et al. [42] pointed out that lipolysis activity can be a critical factor in promoting lipid oxidation and releasing fatty acids after in vitro digestion.

Therefore, the aims of the current work were: (a) to determine the stability of free and bound polyphenols in pork liver pâtés enriched with persimmon (cv. "Rojo Brillante") flour (3 and 6%) obtained from juice coproducts; (b) to evaluate the stability of the free and bound polyphenol profile and their colon available index, the lipid oxidation (TBARs), and the stability of the fatty acid profile during in vitro gastrointestinal digestion of pork liver pâtés enriched with persimmon flour; and (c) to assess the effect of lipolysis in the mentioned determinations (polyphenol and fatty acid and lipid oxidation) using two pancreatins with different lipase activity.

2. Materials and Methods

2.1. Reagents

Individual polyphenol standard, green tea catechin mix (G-016): ((+)—Catechin, (−)—Catechin-3-gallate, (−)—Epicatechin, (−)—Epicatechin-3-gallate, (−)—Epigallocatechin-3-gallate, (−)—Gallocatechin, (−)—Gallocatechin-3-gallate), pancreatin from porcine pancreas (P1750), pepsin from porcine gastric mucosa (P6887), and bile from bovine and ovine (B8381) were purchased from Merck (Darmstadt, Germany). Methanol, ethyl acetate, acetone, hexane, and acetonitrile with HPLC grade were purchased from PanReac ApliChem (Barcelona, Spain). Kreon 25,000 U was purchased in a pharmacy with the corresponding permission. Six monoglycosides mixture (pelargonidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside, delphinidin 3-glucoside, petunidin 3-glucoside,

and malvidin 3-glucoside), malvidin, and malvidin 3,5-diglucoside were purchased from Biolink Group-Polyphenols AS (Sandnes, Norway). All other reagents were purchased from PanReac ApliChem (Barcelona, Spain).

2.2. Sample Preparation

Three pork liver pâté formulations were made following the indications described by Lucas-González et al. [26]: control pâté, without persimmon flour (CP); enriched pâté with 3% persimmon flour obtained from persimmon (cv. Rojo Brillante) juice coproducts (PR-3); and enriched pâté with 6% persimmon flour (PR-6)]. Two different batches of each studied pork liver pâté formulation (1.5 Kg) were made. Chemical composition of the three pork liver pâtés has been previously published by Lucas-González et al. [26].

2.3. In Vitro Gastrointestinal Digestion

In vitro digestion method was carried out following the standardized procedure described by Minekus et al. [39] and taking into account the recommendations of Brodkorb et al. [40] about not including amylase in the digestion of rich fatty foods, keeping enzyme solutions in ice, and starting the count of the incubation time when the enzyme was added to samples. In addition, the recommendations to achieve 2000 lipase units per mL (LU/mL) in intestinal fluid instead of using individual pancreatic enzymes and colipase were applied [39]. The addition of pancreatin (Kreon 25,000 U) to treat exocrine pancreatic insufficiency, previously used to study lipolysis by Calvo-Lerma et al. [41], was also applied.

Before digestion, pork liver pâté was left for 10 min at room temperature and then was smashed to obtain a paste. Carefully, 5.000 ± 0.005 g of sample was weighed into a 50 mL falcon tube. Stock digested solutions (oral, gastric, and intestinal) were prepared with the same saline concentration and pH described by Minekus et al. [39] and then were put in a water bath to temper. Then, 5 mL of simulated oral fluid without salivary amylase was added to sample vortex 5 s and put in a bath with holistic agitation (tubes were put in horizontal positions). The sample was incubated for 2 min at 37 °C and constant agitation. After the oral phase, 7.5 mL of gastric stock solution and 0.2 mL of HCl 0.2 M were added to the oral digested sample; when sample pH was 3.00 ± 0.05, 0.5 mL of pepsin (2000 U/mL) and 5 µL of $CaCl_2$ were added and made up to 20 mL with water. Then, samples were left for 2 h in the agitation bath.

Two intestinal conditions (C1 and C2) were simulated to study the probable effect on fatty acids, polyphenols stability, and on lipid oxidation, due to the lipase activity of pancreatin: (C1) using a pancreatin with high lipase activity (Kreon 25,000) vs. (C2) pancreatin from porcine pancreas (P1750) with low lipase activity. Figure 1 shows the final activity of lipase in each simulated intestinal phase. In both simulated intestinal conditions, intestinal stock solution, bile acid (10 mM), and $ClCa_2$ were prepared and added similarly. The only differences were related to the type of enzyme used and procedure. Therefore, to simulate intestinal condition 1, 11 mL of stock intestinal solution, 0.2 mL of NaOH (2 M), and 2.5 mL of bile solution (dissolved in water) were added to the gastric sample; for adjusting pH, NaOH was added drop by drop until a pH of 7.00 ± 0.05. Then $CaCl_2$, pancreatin solution (5 mL/ 100 UP/mL), and water were added. For condition 2, the simulate intestinal fluid was prepared using 3.2 capsules of pancreatin Kreon (to achieve 2000 UL/mL). Therefore, for 10 samples, 32 capsules were mixed with 160 mL to intestinal stock solution for preparing intestinal simulation. In brief, the capsule was opened carefully and all content was added into a glass bottle and mixed with 100 mL of intestinal stock solution. The bottle was closed and put in magnetic agitation to ambient temperature. Approximately, it took 30 min to completely dissolve. When the enzyme was dissolved, it was transferred to a test tube and filled up to 160 mL with the intestinal stock solution (pH 7.00). After the gastric phase, 0.2 mL of NaOH was added to digested gastric samples, followed by bile acid and $CaCl_2$. Then, 16 mL of simulate intestinal fluid (2000 UL/mL) was added and pH was adjusted to 7.00 ± 0.05. Water was added to make up 40 mL. Both intestinal conditions were incubated in agitation for 2 h at 37 °C. A blank for each digestive phase (oral, gastric,

and both intestinal phases: C1 and C2) was made by replacing the pâté sample for distilled water. For carrying out all determinations, three independent digestions for each pâté formulation were carried out. Figure 1 shows the in vitro gastrointestinal digestion process and the specific treatment of each sample, according to each determination.

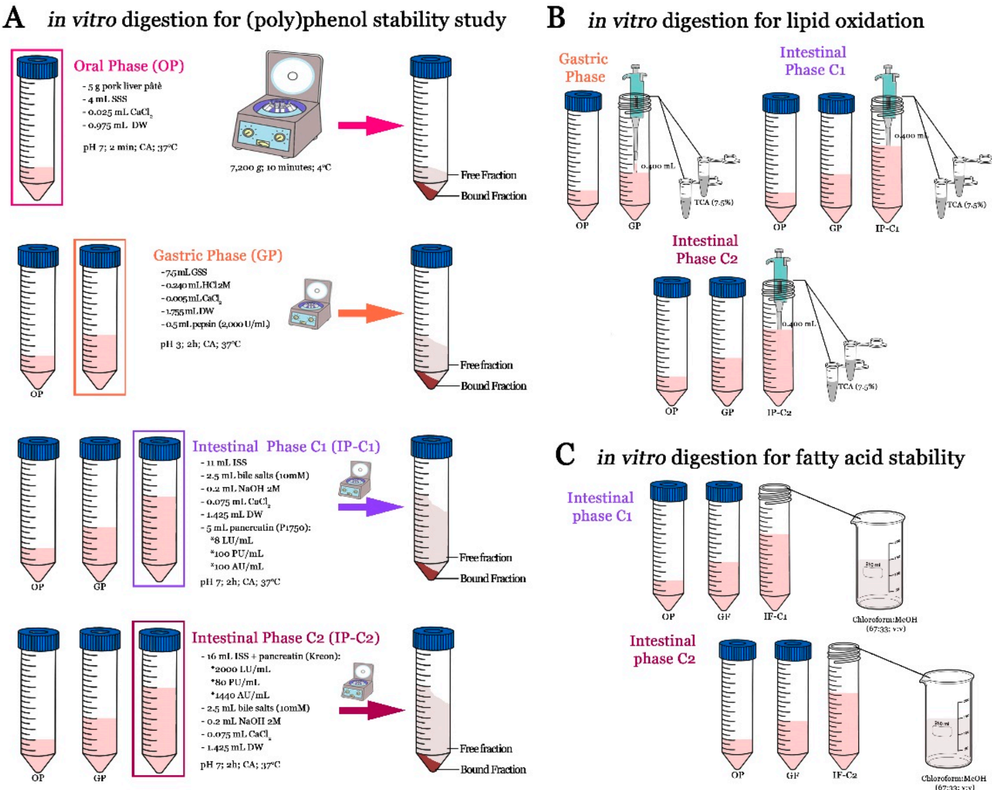

Figure 1. Schematic representation of the in vitro gastrointestinal digestion assays carried out to evaluate (**A**) polyphenol stability; (**B**) lipid oxidation; and (**C**) fatty acid stability in liver pork pâté samples. Imagen caption: SSS: salivary stock solution; GSS: gastric stock solution; ISS: Intestinal stock solution; LU: Lipase units; PU: Protease units; AU: amylase units. TCA: Trichloroacetic acid; DW: Distilled water; CA: Constant agitation.

2.4. Extraction, Identification, and Quantification of Bound and Free Polyphenol Fractions of Both Persimmon Flour and Pork Liver Pâté

2.4.1. Persimmon Flour and Undigested Pork Liver Pâté

Two polyphenolic compound fractions were studied both in Rojo Brillante flour and in undigested and digested pork liver pâté samples. For the undigested samples, the free polyphenol fraction was extracted as described by Pellegrini et al. [43] with some modifications. In brief, 2 g of sample (flour or pâté) were extracted two times, first with 20 mL of aqueous methanol (20:80; v:v) and then with 20 mL of aqueous acetone (30:70; v:v). After the sonication process (30 min), samples were centrifugated (4 °C; 7200× *g*; 10 min), and both supernatants were mixed and evaporated under vacuum. The sample was resuspended in distilled water (5 mL) and passed through a C-18 Sep-Pak cartridge previously activated (water; MeOH; HCl 0,01N). Sugar and other soluble compounds were discarded, and the polyphenols were collected in acidified methanol (MeOH: formic acid; 99:1; v:v) (1.5 mL for pâté and 3 mL for persimmon flour). The pellet was extracted following the procedure described by Mpofu et al. [44] to achieve the bound-insoluble polyphenol fraction. In brief, 50 mL of NaOH 4 M was mixed with the pellet sample and left in the dark for 4 h. Then, the sample was transferred to a beaker, and 25 mL of cold HCl 6 M was added. The pH of samples was monitored, and a HCl 6 M solution was added to the sample until a pH of 2.00 was achieved. After this, samples were

centrifugated for 20 min (4 °C; 8000× g). Then, 25 mL of ethyl acetate was added to a separatory funnel, and consecutively all supernatant was added. The mixture was shaken for 2 min and was left overnight to ambient temperature. After recovering the upper phase (ethyl acetate + polyphenol compounds), the supernatant was washed twice with ethyl acetate; in total, 70 mL was used. The ethyl acetate was evaporated under vacuum, and the residue was resuspended in methanol (2 mL for pâté and 5 mL for persimmon flour). At the end, samples were passed through a nylon filter (0.45 µL). For detection, twenty microliters of both fractions (free and bound) were injected in a Hewlett-Packard HPLC series 1200 coupled with a C18 column (Mediterranean Sea18, 25× 0.4 cm, particle size 5 µm; Teknokroma, Barcelona, Spain). The HPLC conditions were the same reported by Genskowsky et al. [45]. Gallic acid, 4-hydroxycinnamic acid, ellagic acid, protecatechuic acid, sinapic acid, syringic acid, vanillic acid, vanillin, ferulic acid, caffeic acid, *p*-Coumaric acid, chlorogenic, rosmarinic acid, catechin, epicatechin, gallocatechin gallate, gallocatechin-e-gallate, catechin-3-gallate, epicatechin-3-gallate, epigallocatechin-3-gallate, quercetin, kaempferol, myricetin, rutin, apigenin, luteolin, luteolin-7-glucoside, naringin, hesperidin, neorecitrin, neohesperidin, malvidin, malvidin 3,5-O-di-β-glucopiranoside pelargonidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside, delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside, L-tryptophan were injected in HPLC under the same conditions reported for samples. Retention time and UV spectra were used to identify the polyphenols in samples by comparing them with the standard. A calibrate curve of each mentioned standard was used to quantify polyphenols in samples.

2.4.2. Digested Pork Liver Pâté

In the case of the digested pork liver pâté samples, after each phase (oral, gastric, and both intestinal conditions), samples were put in ice and immediately were centrifugated (4 °C; 7200× g; 10 min) (Figure 1). For free fraction, supernatant was passed through a previously activated C-18 Sep-Pak cartridge (water; MeOH; HCl 0,01N), and for bound fraction the pellet was used, which was previously lyophilized. Both samples (supernatant and pellet) were processed as previously described in Section 2.4.1.

Colon available index (%) (CAI) was calculated as previously reported by Lucas-González et al. [38] following the Equation (1):

$$\%\ Colonavailable\ index = \frac{PF_i}{BP_c} \times 100 \quad (1)$$

where:

- PF_i: polyphenols (individual or total) in bound fraction after intestinal phase (µg/g d.w).
- BP_C: Bound polyphenol (individual or total) in undigested enriched pâté (µg /g d.w).

2.5. Fatty Acid Profile

2.5.1. Fat Extraction

The Folch method was used for fat extraction both in undigested and digested pâté samples [46]. For undigested samples, 5 g were used, while for digested samples (only after both intestinal simulated phase), the full sample was extracted, as recommended by Brodkorb et al. [40]. Therefore, after the in vitro intestinal phases (C1 and C2), 150 mL of chloroform: MeOH was added to 40 mL of the intestinal sample. After 20 min under magnetic agitation, the mixture was put into a separatory funnel, and 20 mL of NaCl (0.9% p/v) was added. The separatory funnel was vigorously shaken for 1 min, and the sample was left under ambient temperature overnight to achieve complete phase separation. Then, the chloroform phase was collected and evaporated under vacuum. If emulsion was formed after the repose time, the sample was centrifuged for 10 min at 6000× g.

2.5.2. Methylation of Fatty Acids

Extracted fat (100 µg) was used for the methylation process. In brief, the fat sample was mixed with sodium methoxide (1 mL) and dichloromethane (0.1 mL), and then it was mixed and heated for 10 min in a bath at 90 °C. After cooling in an ice bath, boron trifluoride-methanol solution (14% in methanol) (1 mL) was added and again vortexed and heated under the same conditions previously described. After cooling, water (1 mL) and hexane (0.6 mL) were added to the sample; then, it was heavily shaken. In the end, the upper phase (hexane + fatty acids) was carefully separated, and it was injected in a gas Gas-Chromatographer HP-6890 (Woldbronn, Germany) equipped with a flame ionization detector (FID) and a Suprawax 280 capillary column (30 m × 0.25 µm film thickness × 0.25 mm i.d.; Tecknokroma Barcelona, Spain), following the same conditions reported by Botella-Martínez et al. [47]. The response factors were calculated with fatty acids standards, and they were compared with the retention times of the FAMEs (Supelco 37 component FAME Mix, Bellefonte, PA, USA). All the samples were analyzed in triplicate and the results were expressed as mg fatty acid/100 g fatty acids. The variation of unsaturated fatty acid after gastrointestinal digestion acids was calculated following the Equation (2):

$$\%\ Fatty\ acid\ variation = \frac{FA_{id} - FA_u}{FA_u} \times 10 \qquad (2)$$

where:

- FA_u: Fatty acid content in undigested pâté samples (mg/100 g fatty acid).
- FA_{id}: Fatty acid content after intestinal digestion (mg/100 g fatty acid).

2.6. Lipid Oxidation (TBARs)

Lipid oxidation in undigested and digested pâté (gastric and intestinal phases) was determined following the spectrophotometric TBARs (thiobarbituric acid reactive substances) procedure described by Sobral et al. [32]. For each digested test tube, two aliquots of 400 µL were taken (Figure 1). In total, 6 determinations were analyzed for each sample. A blank for each digestive phase (oral, gastric, and both intestinal phases (C1 and C2)) was made by replacing the digested sample for distilled water. The absorbance was read at 532 nm. Malonaldehyde acid (MDA) quantification was made using a standard curve with 1,1,3,3-tetramethoxypropane, and the results were expressed as µmol MDA per Kg of the sample. For calculating the increase in lipid oxidation, Equation (3) was used:

$$\%\ Lipid\ oxidation\ increase = \frac{TBARS_d - TBARS_u}{TBARS_u} \times 100 \qquad (3)$$

where:

- $TBARs_u$: TBARs values in undigested pâté samples (µmol MDA/Kg).
- $TBARs_d$: TBARs values in digested (gastric or intestinal phases) pâté samples (µmol MDA/Kg).

2.7. Statistical Analysis

Values were expressed as mean ±standard deviation. Two-way ANOVA statistical assays was carried out. The factors used were pâté type (CP, PR-3, and PR-6) and type of sample (undigested, oral digested, gastric digested, intestinal C1 digested, or intestinal C2 digested). Pearson correlation analysis was used to investigate the relations between the fatty acid profile and lipid oxidation values in undigested samples. Tukey's post host test was used to elucidate significant differences when the *p*-value was <0.05. Statistical analyses were performed using the STAT Graphic Program.

3. Results and Discussion

3.1. Bound and Free Phenolic Compounds in Persimmon Flour

Among 42 detected polyphenolic compounds in persimmon flour, 37 of them were identified (Table 1), and 12 were confirmed by standards (gallic acid, catechin, caffeic acid, epigallocatechin-3-gallate, gallocatechin-3-gallate, *p*-Coumaric acid, epicatechin-3-gallate, ellagic acid, ferulic acid, myricetin, quercetin, and kaempferol). The others were tentatively identified; we analyzed their absorbance spectrum, consulting bibliography, and considering that glycosylated polyphenols eluded earlier than their aglycone due to the hydroxyl groups' contribution of the sugar [48]. Regarding tentative identification, except for compounds No. 4, 18, and 33, all the others showed an identical absorbance spectrum to the family or aglycone assigned. Figure 2 shows the absorbance spectrum of some of these compounds. The compound identified as catechin glucoside (Figure 2C) could also be proanthocyanidin dimers, since some of them elude before catechin and present the same major absorbance peaks [49,50]. The absorbance spectrum of the compound identified as coumaric glucoside (No. 4) had differences with the absorbance spectrum of *p*-Coumaric acid (Figure 2A) but had the same maximum absorbance peak. Furthermore, Martínez-Las Heras et al. [50] found coumaric acid-O-hexoside in persimmon leaves, and Sentandreu et al. [51] detected a coumaric acid-pentoside-hexoside in Rojo Brillante flesh. Figure 2A shows the spectrum of compound No. 33, which has been identified as a coumaric acid derivative because it eluded after coumaric acid and presented an absorption peak around 400 nm. The compound No. 18 was identified as anthocyanin, since it presented a very close absorbance spectrum to anthocyanin family as can be observed in Figure 2E. Concerning the unknown polyphenols observed, two of them, No. 12 and 39, showed very close UV spectra to flavone compounds (luteolin and apigenin) and phenolic acids (Figure 2D,F). In the available bibliography, six flavone C-di-hexoxides in Rojo Brillante flesh have been detected by Sentandreu et al. [51]. However, due to the substantial differences in the shape and maximum absorbance spectrum, these compounds have not been identified nor quantified.

The most abundant polyphenol family found in persimmon flour was flavonoids, and their main subfamilies were flavonols and flavanols, with 11 and 10 compounds detected, respectively. Many authors have previously detected quercetin, kaempferol myricetin, and high diversity of their glycosides, being quercetin and kaempferol predominantly in persimmon fruit cv. Rojo Brillante and in other cultivars [25,48,52–54]. Except for quercetin glucoside III and kaempferol glucoside II, which were detected in both studied fractions, all flavonol compounds were found in persimmon flour in the free fraction. Conversely, all flavanols identified in the persimmon flour, except catechin, were shown in the bound fraction. These results were expected since persimmon is a fruit rich in condensed tannin compounds resulting from the polymerization of the flavan-3-ols units [22,55], which because of their highly polymerized grade could not be extracted sufficiently from the fruit by ethanol or acetone [56]. Catechin usually is found in persimmon samples in a free form, as has been reported by Jiménez-Sánchez et al. [48], who only found catechin in juice made from the Rojo Brillante persimmon. However, Chen et al. [57] reported the same amount of catechin in free and bound fractions from persimmon leaves. At the same time, Suzuki et al. [58] observed that the amounts of catechin compounds were higher in astringent persimmons (such as Rojo Brillante) than in non-astringent persimmons. Furthermore, other authors have detected other flavanols like catechin glucoside, gallocatechin, catechin gallate, epigallocatechin-3-gallate, procyanidin dimers, or prodelphinidin dimers in the leaves and flesh of persimmon cultivar Rojo Brillante and other astringent cultivars [50,53,59].

Table 1. Specification to polyphenol compounds detected in persimmon flour obtained from juice coproducts (Rojo Brillante cultivar).

No	Rt (min)	ʎmax (nm)	Tentative Identification	Fr.	Standard Use to Quantify
1	4.2	234/268	Gallic acid glucoside [b]	B	Gallic acid
2	6.9	234/270	Gallic acid [a]	B	Gallic acid
3	8.7	236/270	Gallocatechin glucoside [b]	B	Gallocatechin gallate
4	9.5	236/300	Coumaric acid glucoside [c]	B	*p*-Coumaric acid
5	10.2	236/288sh338	Flavanone glucoside I [b]	B	Hesperidin
6	11.2	236/sh274/344/sh456	Unknow	B	Not quantified
7	12.3	236/280	Catechin glucoside I [b]	B	Catechin
8	12.8	236/280	Catechin glucoside II [b]	B	Catechin
9	13.1	236/280	Catechin glucoside III [b]	B	Catechin
10	13.5	236/280	Catechin glucoside VI [b]	B	Catechin
11	14.0	236/280/330	Vanillin glucoside [b]	B	Vanillin
12	14.5	244sh286/334	Unknow	B	Not quantified
13	15.0	236/280	Catechin [a]	F	Catechin
14	15.2	236/284sh338	Flavanone glucoside II [b]	B	Hesperidin
15	16.9	236/278	Epigallocatechin-3-gallate glucoside [b]	B	Epigallocatechin-3-gallate
16	17.3	246 sh298 324	Caffeci acid [a]	B	Caffeic acid
17	17.8	236/278	Epigallocatechin-3-gallate [a]	B	Epigallocatechin-3-gallate
18	17.9	242/272/sh334/494	Anthocyanin [c]	B	Malvidin-3-O-glucose
19	18.5	236/278	Gallocatechin-3-gallate glucoside [b]	B	Gallocatechin-3-gallate
20	19.2	236/278	Gallocatechin-3-gallate [a]	B	Gallocatechin-3-gallate
21	19.9	236/288sh334	Flavanone glucoside III [b]	F	Hesperidin
22	20.7	252/358	Quercetin glucoside I [b]	F	Rutin
23	21.0	236/288sh334	Flavanone IV [b]	B	Hesperidin
24	21.2	264/360	Quercetin glucoside II [b]	F	Rutin
25	22.0	240/sh300/310	*p*-Coumaric acid [a]	B	*p*-Coumaric acid
26	22.6	238/286sh334	Flavanone glucoside V [b]	F	Hesperidin
27	22.6	236/278	Epicatechin-3-gallate [a]	B	Epicatechin-3-gallate
28	23.2	254/362	Ellagic acid [a]	F	Ellagic acid
29	23.5	258/358	Quercetin glucoside III [b]	F/B	Rutin
30	23.7	242/sh296/324	Ferulic acid [a]	B	Ferulic acid
31	24.0	262/358	Quercetin glucoside IV [b]	F	Rutin
32	25.1	266/348	Kaempferol glucoside I [b]	F	Kaempferol
33	25.5	240/sh290/308/400	*p*-Coumaric acid derivative [c]	B	*p*-Coumaric acid
34	25.9	266/350	Kaempferol glucoside II [b]	F/B	Kaempferol
35	26.4	268/350	Kaempferol glucoside III [b]	F	Kaempferol
36	26.6	246/260/300	Unknow	B	Not quantified
37	27.6	266/344	Kaempferol glucoside IV [b]	F	Kaempferol
38	28.5	254/372	Myrecetin [a]	F	Myrecetin
39	28.8	238sh302/346	Unknow	F/B	Not quantified
40	33.2	256/370	Quercetin [a]	F/B	Quercetin
41	37.1	254sh264/312sh374	Unknow	B	Not quantified
42	37.8	258/372	Kaempferol [a]	F	Kaempferol

rt: retention time; Fr.: Fraction; B: bound; F: free; [a] compound confirmed by standard; [b] Compund with same UV spectrum of tentative compound or family compound; [c] Compound with similar UV spectrum of tentative compound or family compound.

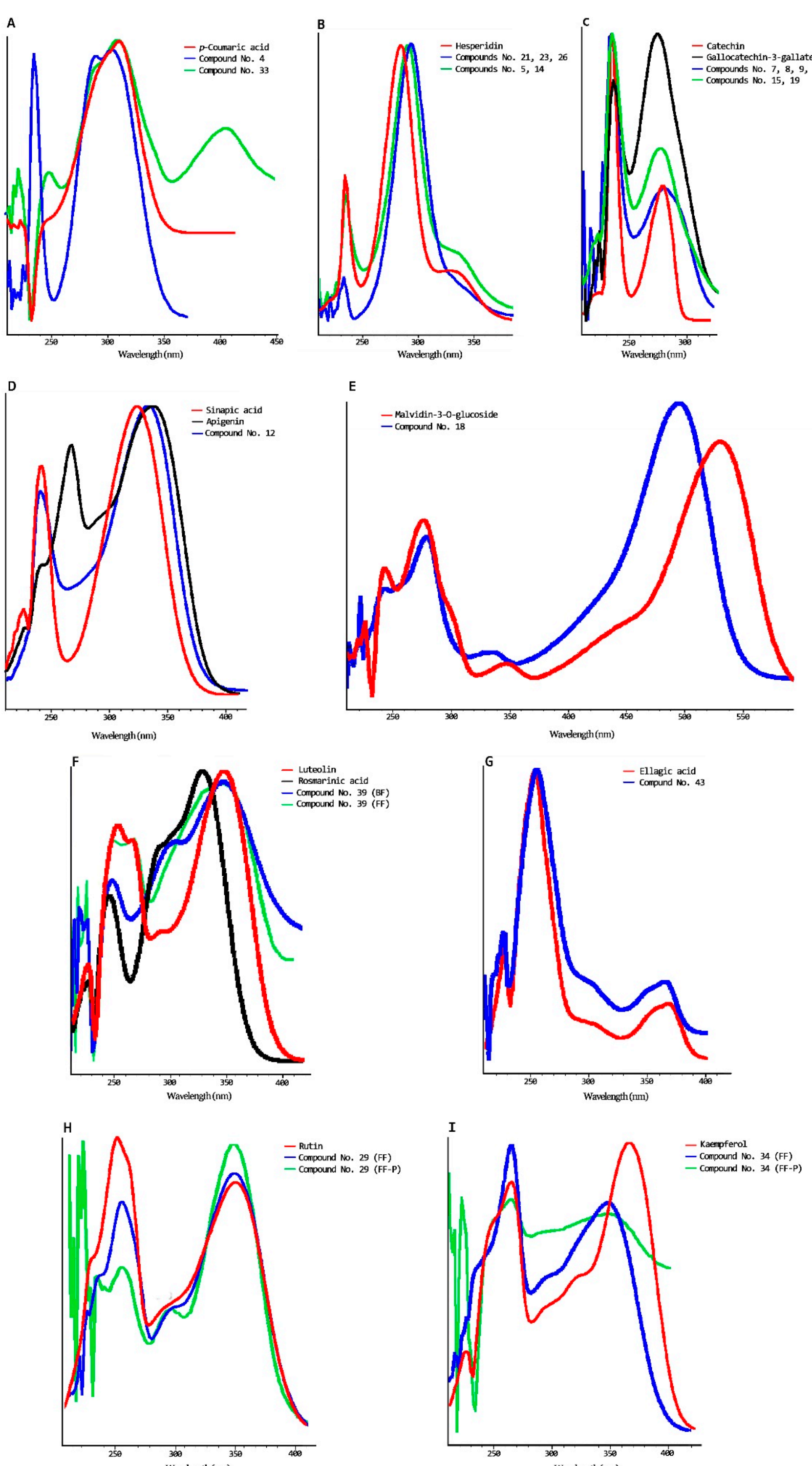

Figure 2. UV spectra of many standard and polyphenols detected in persimmon flour obtained from juice coproducts (cv. Rojo Brillante) and enriched pork liver pâté samples. Image caption: (**A**). *p*-coumaric acid and compounds No. 4 and 33; (**B**). Hesperidin and compounds No. 5, 14, 21, 23, 26; (**C**). Catechin, gallocatechin 3-gallate and compounds No. 7, 8, 9, 10, 15 and 19; (**D**). Sinapic acid, apigenin and compound No. 12; (**E**). Malvidin-3-O-glucose and compound No.18; (**F**). Luteolin, rosmarinic acid and compound No. 39 found in bound and free fractions; (**G**). Ellagic acid and compound No. 43. (**H**). Rutin and compound No. 29 found in persimmon flour and enriched pâté in the free fraction; (**I**). Kampferol and compound No 34 found in persimmon flour and enriched pâté in the free fraction.BF: Bound fraction; FF: free fraction; FF-P: polyphenol detected in the free fraction of the enriched pâtés.

The following abundant flavonoid subfamily was flavanone, with five detected compounds: three in the bound fraction and two in free forms. Flavanone compounds have never been detected in persimmon flour derived from juice coproducts; however, Jiménez-Sánchez et al. [48] identified naringin glucoside, hesperidin, and eriocitrin in different Rojo Brillante juices, and Martínez-Las Heras et al. [50] detected naringin in persimmon leaves.

As regard to phenolic acids, all of them were detected in bound form, with the exception of ellagic acid. These results could be expected since phenolic acids usually are found in vegetable foods bound to the cell wall [60]. Three of them were tentatively identified as glucosides. Monogalloyl-hexoside compounds have been mainly reported in persimmon fruit [48,51,54]. Furthermore, Maulidiani et al. [54] detected two vanillin glucosides (1-O-Vanilloyl-beta-D-glucose isomer and 1-O-Vanilloyl-beta-D-glucose) in different persimmon cultivars.

It is relevant to highlight that the main amount of polyphenols, both qualitatively and quantitatively, was found in the bound fraction of persimmon flours at 67% and 95%, respectively (Table 2). Although fruits are richer in free polyphenols than bound ones [60], and Chen et al. [57] in their study about the polyphenols fractions of persimmon leaves showed that the highest polyphenol amounts were in the free fraction followed by bound and conjugate fractions, this result could be expected since the persimmon flour was obtained from coproducts of the juice industry. The gallic acid showed the highest amounts in the bound fraction, representing 72% of the total amount followed by flavanone glucoside IV and catechin glucoside I. Previously the abundance of gallic acid in persimmon flour, fruits, and leaves has been reported [25,50,53,61]. In the free fraction, the predominant compounds were catechin followed by quercetin glucoside III and flavanone glucoside III. The sum of them represented around 50% of the free polyphenols quantified in the persimmon flour.

Table 2. Free and Bound polyphenolic compounds (µg/g dry weight) of the persimmon flour.

Family	No.	Compound	Free Fraction	Bound Fraction
Phenolic acids	16	Caffeic acid		23.80 ± 4.09
	25	*p*-Coumaric acid		39.40 ± 6.06
	4	Coumaric acid glucoside		77.35 ± 5.37
	33	Coumaric acid derivative		15.71 ± 3.93
	28	Ellagic acid	7.30 ± 1.88	
	30	Ferulic acid		21.08 ± 3.65
	2	Gallic acid		10,074.64 ± 1049.51
	1	Gallic acid glucoside		6.24 ± 0.56
	11	Vanillin glucoside		80.93 ± 25.09
Total phenolic acids			7.30 ± 1.88	10,339.15 ± 1098.26
Flavanones	5	Flavanone glucoside I		221.08± 50.98
	14	Flavanone glucoside II		222.41 ± 15.49
	21	Flavanone glucoside III	28.21 ± 6.74	
	23	Flavanone glucoside IV		1233.34 ± 104.81
	26	Flavanone glucoside V	23.65 ± 3.38	
Total flavanones			51.86 ± 10.12	1676.83 ± 171.29
Flavanols	13	Catechin	37.10 ± 5.42	
	7	Catechin glucoside I		540.52 ± 46.86
	8	Catechin glucoside II		122.69 ± 4.75
	9	Catechin glucoside III		158.00 ± 39.70
	10	Catechin glucoside IV		133.59 ± 32.04
	27	Epicatechin-3-gallate		30.84 ± 2.16
	17	Epigallocatechin-3-gallate		38.81 ± 6.37
	3	Gallocatechin glucoside		327.24 ± 1.23
	20	Gallocatechin-3-gallate		34.17 ± 8.85
	19	Gallocatechin-3-gallate glucoside		50.00 ± 9.49

Table 2. *Cont.*

Family	No.	Compound	Free Fraction	Bound Fraction
	15	Epigallocatechin-3-galato glucoside		173.24 ± 43.80
Total flavanols			37.10 ± 5.42	1628.47 ± 185.46
Flavonols	42	Kaempferol	2.96 ± 1.29	
	32	Kaempferol glucoside I	4.03 ± 0.34	
	34	Kaempferol glucoside II	11.84 ± 1.10	9.94 ± 2.19
	35	Kaempferol glucoside III	2.47 ± 0.48	
	37	Kaempferol glucoside IV	2.00 ± 0.64	
	40	Quercetin	7.40 ± 5.64	7.67 ± 1.24
	22	Quercetin glucoside I	6.07 ± 0.66	
	24	Quercetin glucoside II	6.30 ± 0.47	
	29	Quercetin glucoside III	29.76 ± 4.82	32.73 ± 1.02
	31	Quercetin glucoside IV	10.74 ± 0.11	
	38	Myricetin	3.61 ± 0.54	
Total flavonols			87.18 ± 13.09	50.34 ± 4.46
Anthocyanins	18	Anthocyanin		76.67 ± 6.59
Total flavonoids			176.14 ± 28.63	3432.31 ± 367.79
Total			183.44 ± 30.51	13,958.7 ± 1489.2

3.2. Bound and Free Phenolic Compounds in Undigested Enriched Pâté

In both undigested enriched pâté samples (PR-3 and PR-6), 21 bound polyphenols and 2 free, provided by persimmon flour since those compounds were not detected in control pâté, were found (Figure 3). Among 21 bound polyphenols compounds detected in enriched pâté samples, 16 could be quantified (Figure 4), and the others correspond to the unknown compounds detected in persimmon flours, compounds No. 6, 12, 36, 39, and 41. Interestingly, a compound identified as ellagic acid glucoside (No 43) (Figures 2G and 3) was detected in enriched pâtés but not in persimmon flour. This fact could be due to an overlap of the compounds since the ellagic acid glucoside elutes at the same retention time and wavelength as that of gallocatechin-3-gallate glucoside, which was detected in persimmon flour (Figure 3). Consequently, if this compound is to be identified in persimmon flour, the HPLC conditions should be changed to separate both compounds and modify their retention time. The ellagic acid was also detected in bound fraction instead of in free faction. This fact could also be due to that previously discussed.

The polyphenol amount quantified in each enriched pâté was in concordance with the theoretical prediction (Table 3) except for gallocatechin glucoside, which showed higher amounts than expected. The sum of both fractions in the PR-3 was 285.93 ± 14.60 µg/g d.w and 791.98 ± 121.64 µg/g d.w in the PR-6; the free fraction only contributed around 1%. Significant differences were observed in the polyphenol amount between both enriched pâtés ($p < 0.05$). As can be expected, in both enriched pâtés, gallic acid was the prominent compound quantified followed by flavanone I and gallocatechin gallate glucoside. However, flavanols were not stable in pâté samples, since from 10 bound flavanols detected in persimmon flour, only the gallocatechin glucoside was seen in pâté samples. These results were different from Ribas-Agustí et al. [49], who observed different flavanols (catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, and three procyanidin: C1, B1, and B2) in dry sausages enriched with extracts from cocoa and grape seeds. Regarding the pâté samples chromatographs (PR-3 and PR-6) for the bound fraction, we saw several peaks at the same retention time of many flavanols detected in the persimmon flours; however, their UV spectra were not available or were distorted. Gallic acid glucoside, quercetin, and kaempferol were not observed in pâté samples; this is probably due to the low concentration present in persimmon flour (Tables 2 and 3).

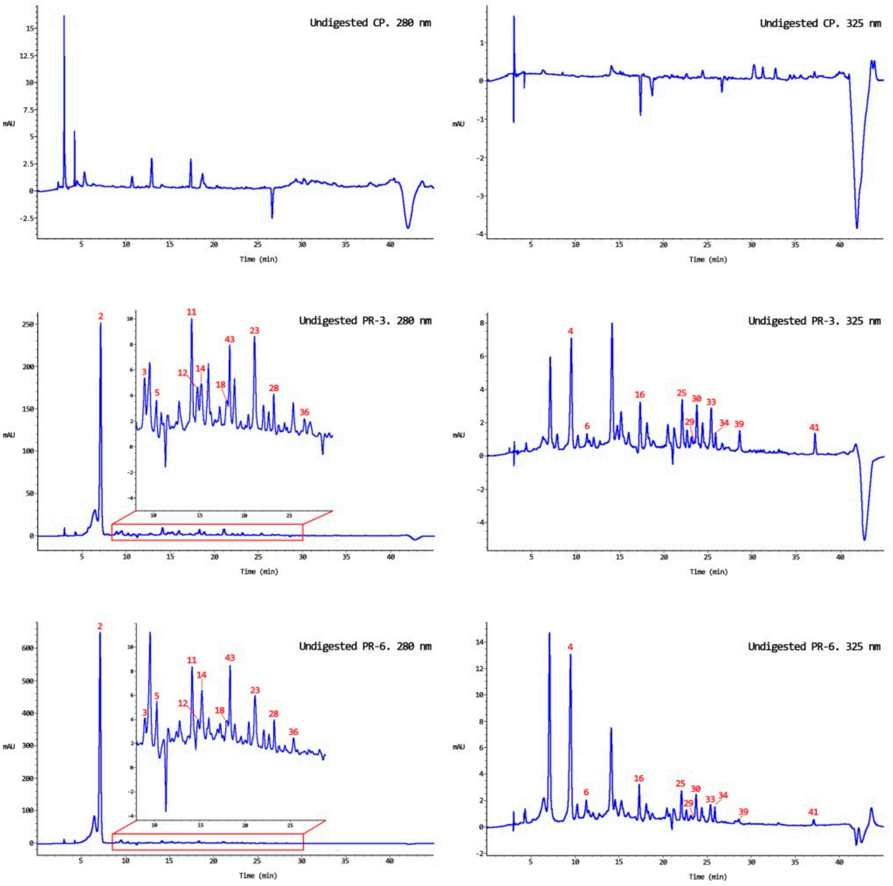

Figure 3. Chromatograms of bound polyphenols in the undigested pork liver pâté samples. CP: control pâté; PR-3 pâté with 3% persimmon flour (Rojo Brillante); PR-6 pâté with 6% persimmon flour (Rojo Brillante); mAU: milli-absorbance unit.

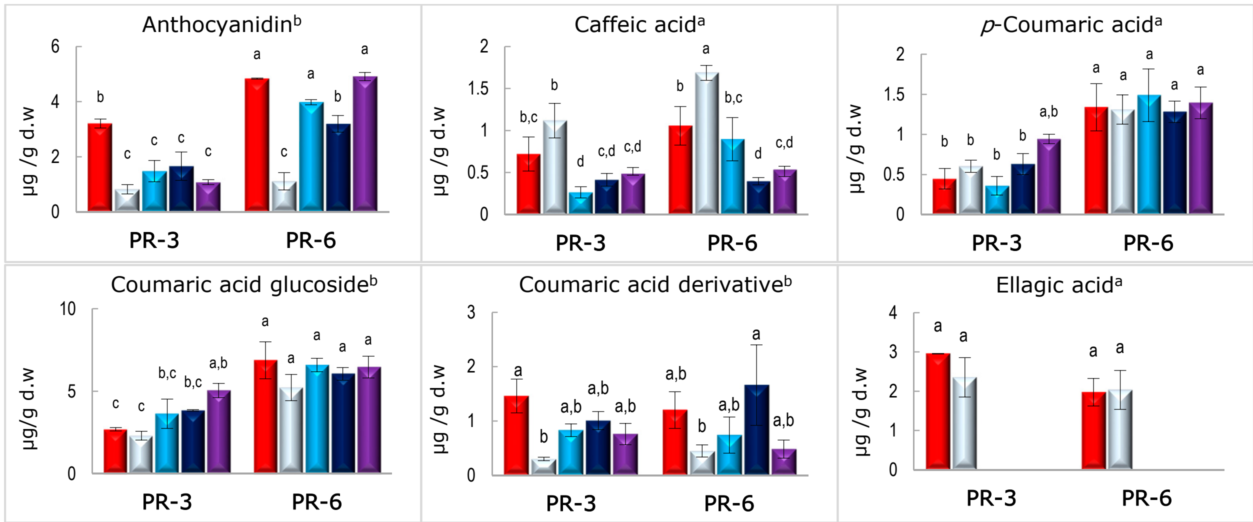

Figure 4. *Cont.*

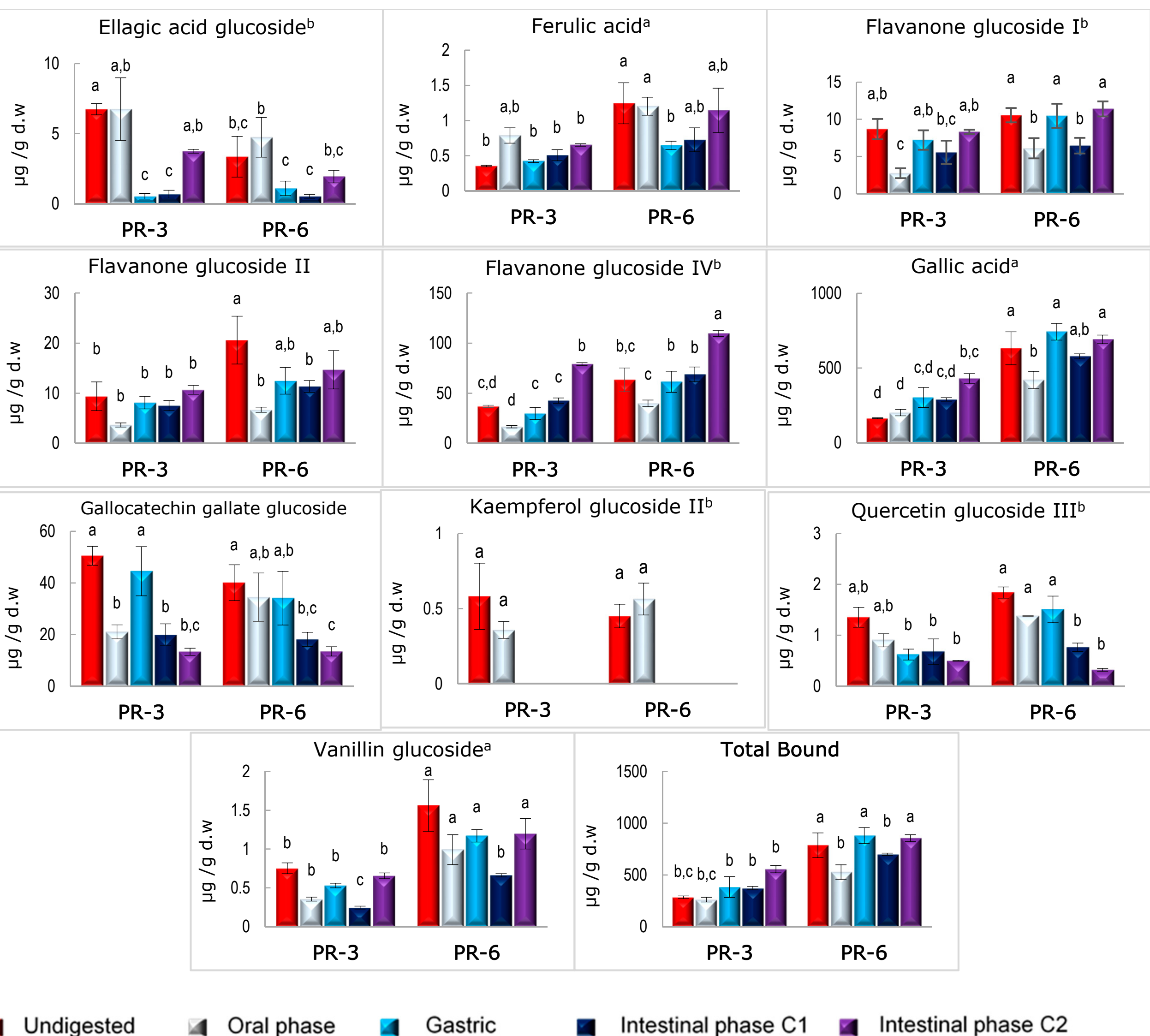

Figure 4. Bound polyphenolic compounds in undigested and digested enriched pork liver pâté. PR-3 pâté with 3% persimmon flour (Rojo Brillante); PR-6 pâté with 6% persimmon flour (Rojo Brillante). [a] compound confirmed by standard; [b] compound with same UV spectrum of tentative compound or family compound; values with different letter above the bars (**a**–**d**) indicates significant differences ($p < 0.05$) according to Tukey's post host test.

Regarding free fraction, only quercetin glucoside III was detected; furthermore, kaempferol glucoside II was also observed in pâté samples. Other authors have shown flavanone compounds in dry-cured sausages with other added fruit extracts [62]. The differences in the current work could be related to the initial polyphenol concentration and the different stability attributed to each compound. For example, the content of polyphenols in meat products with added herbal extract (e.g., *Hyssopus officinalis* and *Borago officinalis*) also was low; some polyphenols were not detected at all due to the lower concentrations (0.5% of meat amount) added [63]. The amount of quercetin glucoside III in the undigested PR-3 and PR-6 was 2.38 ± 1.39 µg/g d.w. and 4.64 ± 2.12 µg/g d.w, respectively. At the same time, the amount for kaempferol glucoside II was 0.44 ± 0.13 µg/g d.w. and 0.51 ± 0.20 µg/g d.w., respectively. The low amount of the other free polyphenols in persimmon flour (Table 2) could explain these results.

Given these results, persimmon flour could be considered a good source of insoluble bound polyphenols, especially gallic acid, to enrich meat products.

Table 3. Theoretical number of polyphenols in enriched pork liver pâté samples calculated based on their mean amount in persimmon flour. Values expressed as µg/g d.w.

Bound Polyphenols	PR-3	PR-6	Free Polyphenols	PR-3	PR-6
Gallic	302.2	604.5	Catechin	1.11	2.23
Flavanone glucoside IV	37.0	74.0	Quercetin glucoside III	0.89	1.79
Catechin glucoside I	16.2	32.4	Flavanone glucoside III	0.85	1.69
Gallocatechin glucoside	9.8	19.6	Flavanone glucoside V	0.71	1.42
Flavanone glucoside II	6.7	13.3	Kaempferol glucoside II	0.36	0.71
Flavanone glucoside I	6.6	13.3	Quercetin glucoside IV	0.32	0.64
Epigallocatechin-gallate glucoside	5.2	10.4	Ellagic acid	0.22	0.44
Catechin glucoside III	4.7	9.5	Hydroxycinnamic acid	0.09	0.18
Catechin glucoside IV	4.0	8.0	Quercetin	0.22	0.44
Catechin glucoside II	3.7	7.4	Quercetin glucoside II	0.19	0.38
Vanillin glucoside	2.4	4.9	Quercetin glucoside I	0.18	0.36
Coumaric acid glucoside	2.3	4.6	Kaempferol glucoside I	0.12	0.24
Anthocyanin	2.3	4.6	Myricetin	0.11	0.22
Gallocatechin-3-gallate glucoside	1.5	3.0	Kaempferol	0.09	0.18
p coumaric acid	1.2	2.4	Kaempferol glucoside III	0.07	0.15
Epigallocatechin-3-gallate	1.2	2.3	Kaempferol glucoside IV	0.06	0.12
Gallocatechin-3-gallate	1.0	2.1			
Quercetin glucoside III	1.0	2.0			
Epicatechin-3-gallate	0.9	1.9			
Caffeic acid	0.7	1.4			
Ferulic acid	0.6	1.3			
Coumaric acid derivative	0.5	0.9			
Kaempferol glucoside II	0.3	0.6			
Quercetin	0.2	0.5			
Gallic acid glucoside	0.2	0.4			

PR-3 pâté with 3% persimmon flour (Rojo Brillante); PR-6 pâté with 6% persimmon flour (Rojo Brillante).

3.3. Stability of Bound and Free Phenolic Compounds in Enriched Pâté after In Vitro Digestion

Pâté samples (PC, PR-3, and PR-6) after each in vitro digestion phase (oral, gastric, and both intestinal conditions: C1 and C2) were assessed for their content in soluble free and bound forms of polyphenols.

Digestion conditions strongly affected both free and bound flavanol content in enriched pâtés. After oral and gastric digestion, kaempferol glucoside II was not detected in any enriched pâté (PR-3 and PR-6). Quercetin glucoside III was detected, as trace, in PR-3 pâté after both oral and gastric digestion, while in PR-6 pâté, it was quantified at a level of 1.05 ± 0.28 µg/g d.w. after oral digestion and at 1.09 ± 0.03 µg/g d.w after gastric digestion. Both flavonols have been previously observed in digested samples of persimmon flours from Rojo Brillante coproducts [25] in both oral and gastric phases. Furthermore, kaempferol glucoside was lost even more than quercetin glucoside. Other authors have reported lower content of free rutin and isoquercetrin in digested carob flour in the gastric step than in the oral step [36]. In contrast, different quercetin glucosides as quercetin-galloyl-hexoside, quercetin-3-galoctidase, quercetin-3-glucoside, and quercetin-3-xyloside present in lyophilized maqui showed higher amounts in gastric step than oral step [34]. Therefore, the gastric medium can compromise glycosylated flavonols' stability or increase their content probably due to the food matrix's release under acid conditions. The intestinal phase data from the free fraction are not available due to problems in obtaining the sample. The high amount of intestinal supernatant, with lots of suspension compounds as fatty acids and proteins, presented problems with passing the supernatant through the column due to column obturation and consequently sample loss. Another procedure needs to be developed to study free fractions on rich fatty and protein foods and low in free polyphenols. Besides, amino acids generate interferences in the chromatograph since many amino acids, especially aromatic ones, have their maximum absorbance at 280 nm, making it challenging to identify polyphenols. The interference in the free fraction

of aromatic amino acid after in vitro digestion was recorded by Lucas-González et al. [37] and Podio et al. [38] in cereal-based foods. For this reason, other procedures based on protein precipitation (with TCA or phosphoric acid) were tried. After centrifuging the sample, the supernatant was filtered, and the pellet was hydrolyzed to break down the possible bond between polyphenols and proteins. However, due to the work and resources required to carry out the methodology and the need to improve it, the preliminary results are not shown.

About bound fraction, after the oral phase, the quantified bound compounds in enriched pâté samples (PR-3 and PR-6) showed two different tendencies. On the one hand, some polyphenols (caffeic acid, *p*-Coumaric acid, coumaric acid glucoside, gallic acid (only in PR-3%), ellagic acid, ellagic acid glucoside, vanillin glucoside, kaempferol glucoside II, and quercetin glucoside II) did not show variations with respect to the correspondent undigested sample. On the other hand, the rest of the polyphenols (the three glycosylated flavanones (I, II, and IV), the gallic acid (only in PR-6), the coumaric acid derivative, and the anthocyanin) showed a significant decrease with respect to their initial content ($p < 0.05$). Some of these polyphenols, since the gastric digestion returned them to their initial amount (referred to the undigested sample), therefore showed significant differences with oral values ($p < 0.05$).

The gastric phase caused a dramatic effect on ellagic acid and kaempferol glucoside II, which were not detected after this phase, nor after both intestinal conditions studied (C1 and C2). These results agree with those shown by Chait et al. [36], who also did not see insoluble, bound forms of kaempferol and other flavonols like myricetin after gastric and intestinal digestion in carob flours. Nevertheless, Gullón et al. [35] detected ellagic acid after gastric digestion in pellet fraction of pomegranate peel flour, and their content was higher than in the oral phase. In this line, Colantuono et al. [64] showed higher amount of bound ferulic acid after duodenal phase than after gastric phase in pomegranate peel-enriched cookies. However, considering that pomegranate is rich in ellagitannins [35,64], this increase could be due to the breakdown of ellagic polymers [65]. The content of caffeic acid and ferulic acid was also significantly reduced regarding oral steps in both studied pâtés (PR-3 and PR-6).

Furthermore, these phenolic acids after C1 intestinal phase showed a slight increase concerning gastric phase but not higher than oral or undigested value. The behavior observed in bound ferulic acid was observed in durum wheat spaghetti samples without and with persimmon flours and in carob flour [36,38]. Regarding bound caffeic acid, Juániz et al. [66] reported different behaviors after the intestinal step, depending on the type of treatment carried out on the pepper: dramatic losses in raw and fried (in olive oil) pepper and a small reduction in fried (in sunflower oil) and griddled pepper.

Among 14 polyphenols observed after the intestinal phase in both enriched pâtés (PR-3 and PR-6), 4 of them, flavanone glucoside I and IV, vanillin glucoside, and ellagic acid glucoside, showed higher values after C2 intestinal phase than after C1 intestinal phase ($p < 0.05$). Furthermore, the amounts of gallic acid in both enriched pâtés, of *p*-Coumaric and coumaric acid glucoside in the PR-3 and of caffeic acid and flavanone glucoside II in the PR-6, were similar after both intestinal phases (C1 and C2), showing the highest values after C2 intestinal phase ($p < 0.05$). In the case of gallocatechin glucoside, the behavior was the opposite, showing the highest values in both enriched pâtés after C1 intestinal phase ($p < 0.05$). Therefore, only caffeic acid, ferulic acid, and quercetin glucoside II showed the same quantity after both intestinal conditions ($p > 0.05$).

Given these results, it could seem that C2 intestinal phase, with high lipase activity, is more suitable for recovering polyphenolic compounds after digestion than C2 intestinal phase. This fact could be associated with a high level of free fatty acids on the digestive medium, which could have a protective effect on polyphenols. In this line, results reported by other authors would seem to support this hypothesis. Juániz et al. [66] showed that the presence of oil (olive or sunflower) decreased the loss of bound polyphenols in fried green pepper after in vitro digestion in greater amounts compared with crude and grilled green

pepper. Pineda-Vadillo et al. [67] hypothesized that the higher stability of anthocyanin in pancakes and omelets than in milkshakes and custard desserts was related to fatty acid release and the consequent reduction in the pH of the medium. Furthermore, McClements et al. [68] denoted that added fatty acids to the food matrix improved polyphenol bioaccessibility, and Guo et al. [69] showed more bioavailability of quercetin in rich fat diets, in a human study. Thus, although the differences between intestinal conditions 1 and 2 were not observed in all detected polyphenols (Figure 4), they were probably due to the different polarity of polyphenols compounds founded. With these results, high lipase activity (2000 UL/mL) would be recommended when rich fatty foods undergo in vitro digestion to study their polyphenols' bioaccessibility.

Table 4 shows the colon available index (%) of enriched persimmon flour pâtés. Other authors call this index a bioaccessibility index or recovery index (the way to calculate is the same) [34,36,66,70,71]. However, considering that the polyphenols were observed in the bound fraction, which is not released to medium, they probably will arrive intact to the colon and some of them could be metabolized by the intestinal microbiome. For this reason, it is considered that this name is more appropriate for bound compounds that have been detected in food samples after in vitro digestion.

Table 4. Colon Available index (%) of bound polyphenolic compounds in enriched pork liver pâté samples.

	PR-3		PR-6	
	Colon Available Index (%)		Colon Available Index (%)	
Polyphenolic Compound	**IP-C1**	**IP-C2**	**IP-C1**	**IP-C2**
Caffeic acid	57.53 ± 11.25 [a]	67.30 ± 2.10 [a]	37.58 ± 4.09 [b]	44.47 ± 1.52 [b]
p-Coumaric acid	140.7 ± 29.0 [b]	210.6 ± 13.2 [a]	95.74 ± 9.99 [b]	104.06 ± 14.73 [b]
Coumaric acid glucoside	198.75 ± 2.31 [b]	268.09 ± 24.94 [a]	95.26 ± 6.35 [a]	102.20 ± 11.03 [a]
Coumaric acid derivative	68.90 ± 11.43 [b]	52.08 ± 13.43 [b]	138.36 ± 61.75 [a]	40.12 ± 13.88 [b]
Ellagic acid	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ellagic acid glucoside	9.98 ± 4.15 [b]	55.44 ± 2.05 [a]	15.73 ± 4.08 [b]	57.73 ± 13.34 [a]
Ferulic acid	143.54 ± 22.05 [a]	184.50 ± 5.16 [a]	58.32 ± 13.66 [b]	91.87 ± 25.36 [b]
Gallic acid	176.25 ± 7.29 [b]	261.66 ± 20.04 [a]	91.56 ± 2.67 [c]	109.52 ± 4.58 [c]
Vanillin glucoside	32.54 ± 2.63 [b]	87.09 ± 4.90 [a]	42.34 ± 1.32 [b]	76.67 ± 12.59 [a]
Flavanone glucoside I	63.88 ± 18.17 [b]	95.84 ± 3.31 [a]	61.15 ± 9.97 [b]	108.08 ± 9.65 [a]
Flavanone glucoside II	80.20 ± 29.50 [a]	113.51 ± 9.32 [a]	55.13 ± 5.73 [a]	71.31 ± 18.62 [a]
Flavanone glucoside IV	115.5 ± 7.0 [c]	214.1 ± 3.9 [a]	108.68 ± 11.70 [c]	173.01 ± 4.46 [b]
Gallocatechin gallate glucoside	39.53 ± 8.22 [a]	26.38 ± 2.67 [a]	45.27 ± 6.83 [a]	33.59 ± 4.49 [a]
Quercetin glucoside III	50.283 ± 18.448 [a]	36.80 ± 0.57 [a]	41.51 ± 4.51 [a]	17.43 ± 1.49 [a]
Kaempferol glucoside II	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Anthocyanidin	51.8 ± 16.0 [bc]	33.7 ± 2.8 [c]	66.17 ± 6.26 [b]	101.61 ± 3.09 [a]
Total phenolic acids	170.25 ± 6.91 [b]	253.98 ± 19.03 [a]	91.15 ± 2.35 [c]	108.99 ± 4.35 [c]
Total flavonols	50.28 ± 18.448 [a]	36.80 ± 0.57 [a]	41.51 ± 4.51 [a]	17.43 ± 1.49 [a]
Total flavanone	105.80 ± 10.04 [c]	194.96 ± 1.28 [a]	91.70 ± 7.57 [c]	139.57 ± 4.82 [b]
Total flavan-3-ols	39.53 ± 8.22 [a]	26.38 ± 2.67 [a]	45.27 ± 6.83 [a]	33.59 ± 4.49 [a]
Total anthocyanidins	51.8 ± 16.0 [bc]	33.7 ± 2.8 [c]	66.17 ± 6.26 [b]	101.61 ± 3.09 [a]
Total polyphenols	131.39 ± 5.49 [b]	195.78 ± 12.46 [a]	88.73 ± 1.55 [d]	108.74 ± 4.08 [c]

IP-C1 intestinal phase C1; IP-C2 intestinal phase C2PR-3 pâté with 3% persimmon flour (Rojo Brillante); PR-6 pâté with 6% persimmon flour (Rojo Brillante). Values with different letter in the same row indicate significant differences ($p < 0.05$), according to Tukey's post host test.

The following compounds were released from the matrix in both enriched pâtés (PR-3 and PR-6) after intestinal digestion (independently of the intestinal condition used): caffeic acid, ellagic acid glucoside, vanillin glucoside, and gallocatechin glucoside. The lower CAI value was shown for ellagic acid glucoside after C1 intestinal phase, with values around 10%. Other authors have reported similar values for caffeic acid, gallocatechin glucoside, or quercetin glucoside III (Table 4) but different values for other compounds like ferulic acid (27.1%), isoquercitrin (34%), protocatechuic acid (35.28%), or chlorogenic acid (36.8%) [36]. However, in the current work, the CAI of ferulic acid (except for PR-6 after intestinal phase)

was higher than 100%, indicating that more compounds were present in the food matrix than previously detected, and the intestinal medium would help to make their extraction easier. This increase was also shown in the gallic acid, *p*-Coumaric acid, coumaric acid glucoside, and flavanone glucoside IV.

These results agree with Adom and Liu's work [72] reporting that insoluble bound phenols can resist gastrointestinal digestion and reach the colon. Considering that gallic acid and flavanone glucoside IV were the principal polyphenols in enriched pâté samples, this could explain the total CAI % shown, which was upper to 100% except in the PR-6 sample after C1 intestinal digestion, which was around 90%. These results agreed with Huang et al. [71], who reported an increase in bound polyphenols in some seaweed species after in vitro digestion. In contrast, other authors showed a polyphenol bioaccessibility index lower than 100% [36,66,70]. Furthermore, in a previous work where persimmon flour was used to enrich durum wheat spaghetti at the same concentration that was used in the current work (3 and 6%), only two bound polyphenols contributed by persimmon flour (gallic acid and coumaric acid glucoside) were detected, although in lower amounts than in enriched pâté [38]. This could be denoting the effect of the manufacturing process and food matrix on their stability, release, and availability. Other authors have also reported great effects of the food matrix on polyphenol stability [67,73].

The high number of polyphenols in pâté samples resisting enzymatic digestion and remaining covalently joined to the cell wall could arrive to the colon and modulate the intestinal microbiome's composition [14,15]. Several works have pointed out the relevance of intestinal microbiota in generating polyphenol metabolites, which are better absorbed than the precursor polyphenols, showing more bioactivity and persisting longer in blood [74,75]. About gallic acid, Li et al. [76] demonstrated that in a microbiome-metabolomics analysis in rats with induced colitis and treated with gallic acid, gallic acid intervention attenuated colitis by improving body weight loss, hematochezia, epithelial integrity of colon tissue, oxidative stress, and inflammation in the colon. Furthermore, gallic acid increased beneficial bacteria and decreased pathogenic bacteria. Yang et al. [77], in a review about the impact of gallic acid in gut health, reveled the potential of this acid and its derivatives for the treatment and prevention of gastrointestinal diseases through interaction with the gut microbiome and modulation of the immune response. The modulation of gut microbiota and the immunology response also have been shown in an animal study where female mice with induced chronic inflammation were fed meat product with an added antioxidant extract [18].

3.4. Lipid Oxidation in Undigested and Digested Pâté Samples

The lipid oxidation values of three pâté formulations determined as TBARs in undigested and digested pâtés can be observed in Table 5.

Table 5. Lipid oxidation (µmoles MDA/Kg pâté) of the undigested and digested pork liver pâté samples.

	Undigested	Gastric Phase	Intestinal Phase C1	Intestinal Phase C2
CP	3.19 ± 0.33 ^{d}	2.54 ± 0.48 ^{d}	5.33 ± 0.68 ^{b}	7.80 ± 0.50 ^{a}
PR-3	4.20 ± 0.33 ^{c,d}	3.52 ± 0.40 ^{c,d}	5.53 ± 0.35 ^{b}	8.08 ± 0.88 ^{a}
PR-6	4.63 ± 0.42 ^{b,c}	4.86 ± 0.45 ^{b,c}	5.77 ± 0.58 ^{b}	8.66 ± 0.85 ^{a}

CP: control pâté; PR-3 pâté with 3% persimmon flour (Rojo Brillante); PR-6 pâté with 6% persimmon flour (Rojo Brillante). Values with different letter in the same row indicates significant differences ($p < 0.05$), according to Tukey's post host test.

These values are in accordance with that reported by Goethals et al. [31] in commercial liver pâté samples. In undigested pâtés, the highest TBARs values were shown in PR-6, followed by PR-3 and PC, showing significant differences ($p < 0.05$). The prooxidant effect of vegetable ingredients, especially paste date coproducts in pork liver pâté, was been previously established by Martín-Sánchez et al. [78]. The food matrix disruption in

pork liver pâté enriched with persimmon flour observed by Lucas-González et al. [26], probably due to fiber and sugar, could induce lipid oxidation. Although other authors have supported the antioxidant effect of some rich polyphenol extracts in the meat matrix [32,62,79,80], it seems that the extract composition and meat matrix could influence the antioxidant-prooxidant outcomes [78]. About the effect of digestion on lipid oxidation, results showed that the gastric phase did not have oxidant effect in the samples (Table 5); some values were even lower than in undigested pâté. Still, no significant differences were shown among undigested and gastric samples in pâté samples ($p > 0.05$). Although some authors considered gastric medium as a bioreactor to promote lipid oxidation [81], in these pâté samples, it was not observed. These results could be due to the antioxidant action of ascorbate and nitrate, which were added at 0.5% and 125 ppm, respectively, in pâté formulation but also due to the absence of gastric lipase. Other authors have observed lipid oxidation reduction due to antioxidants compounds. Sobral et al. [32] showed that 0.2% oregano in chicken burgers reduced its TBARs values after intestinal digestion values of 3 nmol/g. Besides, Martini et al. [30] demonstrated that extra-virgin olive oil in low amounts (2.5%) reduces lipid oxidation in grilled turkey breast meat after in vitro co-digestion of both foods. Furthermore, Steppeler et al. [5] reported lower MDA values in minced pork after gastric digestion (6.7 µmol/Kg) than in minced chicken and salmon, pointing out that the presence of polyunsaturated fatty acids was determinant in increasing lipid oxidation.

Although enriched pâtés presented more oxidative end products than the control after the gastric phase ($p < 0.05$), no differences in TBARs values between pâté samples after the study of both intestinal conditions (1 and 2) were shown ($p > 0.05$). This could be due to the antioxidants present in persimmon flours as polyphenols or carotenoids, which would be released from the food matrix under intestinal conditions. It would be supported by the high levels of bound polyphenols detected in enriched pâté samples (Table 4). However, after both intestinal digestions (C1 and C2), lipid oxidation increased significantly ($p < 0.05$) in all samples. These results were concordant with Goethals et al. [31], who subjected three different commercial liver pâtés to in vitro gastrointestinal digestion. This significant rise in lipid oxidation after the intestinal phase could be related to the increase in the lipolysis in the intestinal phase mediated by lipase. It is known that fatty acids are more susceptible to oxidation than triglyceride and can induce pro-oxidation reactions by attracting pro-oxidant metals and co-oxidizing triglycerides [82,83]. In the same way, TBARs values after intestinal phase C2 were higher than after intestinal phase C1 ($p < 0.05$). Therefore, it seems that the increase in lipase activity in the digestive medium significantly increases lipid oxidation. These results were in concordance with Tullberg et al. [42], who studied the effect of lipolysis on lipid oxidation using a lipase inhibitor (Orlistat). They reported that Orlistat significantly reduced lipolysis and MDA formation in marine oils during in vitro gastrointestinal digestion. Furthermore, they reported the increase of primary lipid oxidation products, such as 4-hydroxy-2-hexenal, through the action of gastric lipase. Larsson et al. [28] also reported a relation between lipolysis activity and increased lipid oxidation.

In addition to TBARs values, the behavior of lipid oxidation (comparing initial values: undigested pâté) after gastric and intestinal digestion can be seen in Figure 5. After gastric phase, discrepant values were shown, derived from the fact that similar values were shown among the three undigested pâtés and their respective after-gastric phase. However, after both intestinal phases (conditions 1 and 2), lipid oxidation increased: PR-6 < PR-3 < PC ($p < 0.05$). Regarding results shown by Larsson et al. [28] and Nieva-Echevarría et al. [84], who reported that oxidized oils showed more lipid oxidation than non-oxidized oil after in vitro gastrointestinal digestion, it could be expected that, after intestinal digestion, PR-6 showed the highest TBARs value. Although the initial TBRAs value of the undigested PR-6 was higher than undigested PC, after gastrointestinal digestion, pro-oxidation reactions were not induced by the presence of persimmon flour in pâté samples; on the contrary,

lipid oxidation reactions were reduced (Figure 5). Furthermore, the protective effect of persimmon flour on lipid oxidation was concentration-dependent ($p < 0.05$).

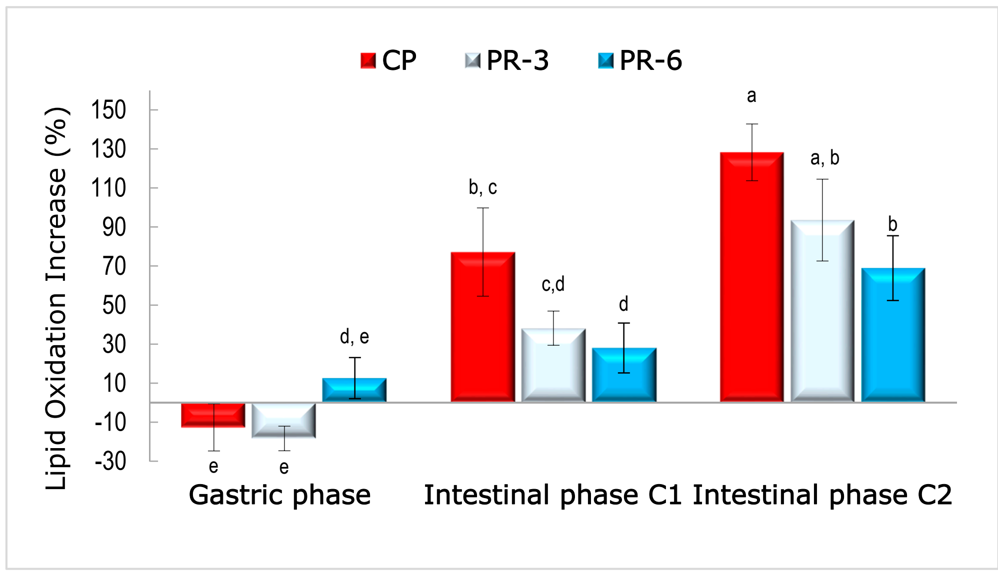

Figure 5. Lipid oxidation increase in pork liver pâté samples after gastric and both simulated intestinal digestions. CP: control pâté; PR-3 pâté with 3% persimmon flour (Rojo Brillante); PR-6 pâté with 6% persimmon flour (Rojo Brillante). Values with different letter indicates significant differences ($p < 0.05$), according to Tukey's post host test.

3.5. Fatty Acid Profile of Pâté and Its Stability after In Vitro Digestion

Fatty acid profile of undigested and digested pâté samples can be observed in Table 6. The main fatty acids in the three studied pâté formulations were oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2), and stearic acid (C18:0). These four fatty acids represent around 90% of total fatty acids in pâté samples. These results agree with the fatty acid profile of pork meat and pork liver pâté [5,85].

Table 6. Fatty acid profile of the undigested and digested pork liver pâté samples.

Fatty Acids (FA), g/100 g of FA	PC			PR-3			PR-6		
	Undigested	IP-C1	IP-C2	Undigested	IP-C1	IP-C2	Undigested	IP-C1	IP-C2
C10:0	0.07 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}	0.08 ± 0.00 ^{a}	0.07 ± 0.00 ^{a,b}	0.07 ± 0.01 ^{a}	0.08 ± 0.00 ^{a}	0.08 ± 0.00 ^{a}	0.08 ± 0.00 ^{a}
C12:0	0.10 ± 0.00 ^{a}	0.10 ± 0.00 ^{a}	0.10 ± 0.00 ^{a}	0.09 ± 0.00 ^{b}	0.09 ± 0.00 ^{b}	0.09 ± 0.00 ^{b}	0.09 ± 0.00 ^{b}	0.09 ± 0.00 ^{b}	0.09 ± 0.00 ^{b}
C14:0	1.31 ± 0.00 ^{a}	1.30 ± 0.00 ^{a}	1.28 ± 0.00 ^{b}	1.33 ± 0.01 ^{a}	1.32 ± 0.00 ^{a}	1.30 ± 0.02 ^{b}	1.30 ± 0.04 ^{a}	1.30 ± 0.00 ^{a}	1.28 ± 0.02 ^{b}
C15:0	0.07 ± 0.00 ^{c}	0.07 ± 0.00 ^{c}	0.07 ± 0.00 ^{c}	0.08 ± 0.00 ^{b}	0.08 ± 0.00 ^{b}	0.08 ± 0.00 ^{b}	0.09 ± 0.00 ^{a}	0.09 ± 0.00 ^{a}	0.08 ± 0.00 ^{a,b}
C16:0	22.59 ± 0.50 ^{a}	22.68 ± 0.04 ^{a}	22.40 ± 0.15 ^{a}	23.26 ± 0.39 ^{a}	22.59 ± 0.03 ^{a}	22.75 ± 0.07 ^{a}	22.62 ± 0.72 ^{a}	22.52 ± 0.10 ^{a}	22.53 ± 0.06 ^{a}
C16:1	2.22 ± 0.01 ^{b}	2.18 ± 0.00 ^{b}	2.16 ± 0.03 ^{b}	2.47 ± 0.03 ^{a}	2.45 ± 0.01 ^{a}	2.43 ± 0.01 ^{a}	2.42 ± 0.07 ^{a}	2.42 ± 0.01 ^{a}	2.39 ± 0.03 ^{a}
C17:0	0.39 ± 0.01 ^{c}	0.39 ± 0.00 ^{c}	0.38 ± 0.00 ^{c}	0.43 ± 0.01 ^{b}	0.42 ± 0.00 ^{b}	0.42 ± 0.00 ^{b}	0.46 ± 0.01 ^{a}	0.46 ± 0.00 ^{a}	0.46 ± 0.00 ^{a}
C18:0	11.98 ± 0.44 ^{a}	12.24 ± 0.03 ^{a}	11.94 ± 0.13 ^{a}	12.12 ± 0.47 ^{a}	11.61 ± 0.02 ^{a}	11.71 ± 0.12 ^{a}	11.57 ± 0.35 ^{a}	11.58 ± 0.10 ^{a}	11.78 ± 0.20 ^{a}
C18:1	41.16 ± 0.43 $^{b, c}$	39.82 ± 0.01 ^{b,c}	39.68 ± 0.29 ^{c}	42.45 ± 1.29 ^{a,b}	40.93 ± 0.18 ^{b,c}	42.02 ± 0.90 ^{a,b}	42.16 ± 1.92 ^{a}	41.06 ± 0.35 ^{b}	41.51 ± 0.10 ^{a,b}
C18:2 (n 6,9)	13.43 ± 0.36 ^{b}	13.99 ± 0.07 ^{a}	14.14 ± 0.28 $^{a, b}$	11.92 ± 0.15 ^{d}	12.92 ± 0.04 ^{c}	12.75 ± 0.24 ^{c}	11.49 ± 0.31 ^{d}	12.54 ± 0.09 ^{c}	12.59 ± 0.14 ^{c}
C18:3 (n 3,6,9)	0.71 ± 0.03 ^{b}	0.79 ± 0.01 ^{a}	0.75 ± 0.02 ^{b}	0.64 ± 0.01 ^{c}	0.76 ± 0.01 $^{a, b}$	0.72 ± 0.01 ^{b}	0.63 ± 0.02 ^{c}	0.77 ± 0.00 $^{a, b}$	0.75 ± 0.01 ^{b}
C20:0	0.16 ± 0.00 ^{b}	0.16 ± 0.00 ^{b}	0.17 ± 0.00 ^{b}	0.18 ± 0.01 ^{a}	0.17 ± 0.00 ^{a,b}	0.18 ± 0.01 ^{a}	0.19 ± 0.01 ^{a}	0.18 ± 0.00 ^{a}	0.20 ± 0.01 ^{a}
C20:1	0.92 ± 0.01 ^{a}	0.92 ± 0.00 ^{a}	0.91 ± 0.00 ^{a}	0.86 ± 0.01 ^{b}	0.89 ± 0.00 ^{a}	0.89 ± 0.01 ^{a,b}	0.85 ± 0.03 ^{b}	0.88 ± 0.00 ^{a,b}	0.90 ± 0.02 ^{a}
C20:2 (n 11,14)	0.62 ± 0.01 ^{a}	0.63 ± 0.01 ^{a}	0.61 ± 0.00 ^{a}	0.55 ± 0.00 ^{c}	0.58 ± 0.00 ^{b}	0.57 ± 0.01 ^{b,c}	0.52 ± 0.01 ^{c}	0.56 ± 0.01 ^{b}	0.56 ± 0.01 ^{b}
C20:3 (n 8,14,17)	0.57 ± 0.05 ^{c}	0.82 ± 0.01 ^{a}	0.74 ± 0.03 ^{a}	0.52 ± 0.02 ^{c}	0.81 ± 0.01 ^{a}	0.71 ± 0.02 ^{b}	0.51 ± 0.03 ^{c}	0.83 ± 0.01 ^{a}	0.76 ± 0.03 ^{a}
Others	3.71 ± 0.24 ^{a}	3.86 ± 0.16 ^{a}	4.62 ± 0.74 ^{a}	3.01 ± 0.94 ^{a}	4.30 ± 0.27 ^{a}	3.30 ± 0.79 ^{a}	2.69 ± 0.99 ^{a}	4.66 ± 0.69 ^{a}	4.03 ± 8.03 ^{a}
SFA	36.66 ± 0.97 ^{a}	37.00 ± 0.07 ^{a}	36.38 ± 0.30 ^{a}	37.57 ± 0.88 ^{a}	36.36 ± 0.06 ^{a}	36.61 ± 0.22 ^{a}	36.39 ± 1.14 ^{a}	36.29 ± 0.21 ^{a}	36.50 ± 0.29 ^{a}
UFA	59.62 ± 0.91 ^{a}	59.15 ± 0.12 ^{a}	58.99 ± 0.67 ^{a}	59.42 ± 1.51 ^{a}	59.34 ± 0.25 ^{a}	60.09 ± 1.21 ^{a}	58.60 ± 2.38 ^{a}	59.05 ± 0.48 ^{a}	59.47 ± 0.34 ^{a}
MFA	44.30 ± 0.45 ^{b}	42.92 ± 0.02 ^{c}	42.75 ± 0.33 ^{c}	45.78 ± 1.33 ^{a}	44.27 ± 0.19 ^{b}	45.34 ± 0.93 ^{b}	45.44 ± 2.02 ^{a}	44.36 ± 0.36 ^{b}	44.81 ± 0.15 ^{a}
PFA	15.32 ± 0.46 ^{b}	16.23 ± 0.10 ^{a}	16.25 ± 0.34 ^{a}	13.64 ± 0.18 ^{c}	15.07 ± 0.06 ^{b}	14.75 ± 0.28 ^{b}	13.16 ± 0.37 ^{c}	14.69 ± 0.11 ^{b}	14.66 ± 0.19 ^{b}

CP: control pâtè; PR-3 pâtè with 3% of persimmon flour (Rojo Brillante); PR-6 pâtè with 6% of persimmon flour (Rojo Brillante). IP: Intestinal phase. SFA: saturated fatty acid; UFA: Unsaturated fatty acid; MFA: Monounsaturated fatty acid; PFA: Polyunsaturated fatty acid; Values with different letter in the same row (a–c) indicates significant differences ($p < 0.05$) according to Tukey's Multiple Range Test.

The undigested PC showed the highest values regarding the four observed polyunsaturated fatty acids (PUFAs): linoleic (C18:2), linolenic (C18:3), cis-11,14-eicosadienoic acid (C20:2), and cis-8,11,14-eicosatrienoic acid (C20:3). The monounsaturated fatty acid C20:1 and the saturated fatty acid C12:0 were detected in higher amount in CP than in enriched

pâtés (PR-3 and PR-6) ($p < 0.05$). In contrast, both pâtés with persimmon flours (PR-3 and PR-6) showed the most significant quantities of the saturated fatty acids, C10:0, C15:0, and C17:0, and the monounsaturated fatty acids palmitoleic acid (C16:1) and oleic acid (C18:1). Considering that PUFAS are more sensitive to oxidation due to their high double bounds [5,86], it could explain the differences in lipid oxidation shown among pâtés. In this line, a positive and statistic correlation was demonstrated between the amount of the fatty acid C20:3 in undigested pâté samples and their lipid oxidation (R2 = 0.74; $p < 0.015$).

Table 6 shows the fatty acid profile of pâté samples after intestinal digestion. This profile was not qualitatively modified after the digestion process. PUFAs content in the three pâté samples was increased after digestion. The fatty acids C18:3 and C20:3 reported the highest variations with respect to undigested pâté samples (Figure 6). It was an unexpected result since, as mentioned before, gastrointestinal conditions promote lipid oxidation [81,87], and the greater the number of double bonds, the greater the oxidation [86]. Therefore, the expected result would have been a decrease in their content, as was reported by Sobral et al. [32] in digested chicken meat burgers and Liu et al. [33] in digested mushroom *Oudemansiella radicata*. However, both authors reported an increase in PUFA after the cooking process. Liu et al. [33] attributed this effect to the fact that PUFAs are part of the cell membrane and could resist oxidation derived from high temperature. Zhu et al. [29], in a study about the release of fatty acids from emulsified lipids during in vitro digestion, pointed out that fatty acid release is dependent on the structure of triglycerides and on the length of the carbon chain. The tendency observed was the longer the carbon chain, the greater the time needed to release. In another study, Costa et al. [88] showed a lower PUFAs bioaccessibility in grilled salmon than in crude salmon, probably due to the fact that PUFAs are prone to attach to the protein aggregates formed by a result of cross-linking reactions induced by grilling. Therefore, considering these works, it could be hypothesized that part of the PUFA content remains attached to pâté matrix after the extraction process but could be released during the digestion process.

Comparing both intestinal conditions (C1 and C2), significant differences in the amount of some fatty acids were found. In all pâtés (PC, PR-3, and PR-6), the number of fatty acids C18:3 and C14 after C1 intestinal phase was higher than after C2 intestinal phase ($p < 0.05$). In addition, the highest value of C20:3 was found after the C1 intestinal phase, but these differences only were significant in PR-3 ($p < 0.05$). However, fatty acid variations (Figure 6) showed a significant difference among intestinal conditions in all studied pâtés regarding the fatty acid C20:3 ($p < 0.05$). We suppose that these differences between methods are related to different lipolysis activity. The highest lipase activity in C2 intestinal phase promotes the rapid release of fatty acids; consequently, the fatty acids were more time-exposed to the intestinal medium and underwent more lipid oxidation than the fatty acid release under C1 intestinal condition. In contrast, enriched pâtés showed the lowest percentage of C18:1 in C1 intestinal condition ($p < 0.05$). However, although significant differences in the amount of specific fatty acids have been shown between undigested and digested samples, in general, the fatty acid profile in pâté samples after digestion was preserved due to the antioxidant activity of antioxidants used in the formulation, as mentioned before.

Given these results, more studies are needed to understand the mechanism and behavior of fatty acids after digestion as well as food matrix, antioxidants, and lipase activity implications in the oxidation process and their stability in order to generate formulation strategies in rich fatty foods with high nutritional value after the digestive process.

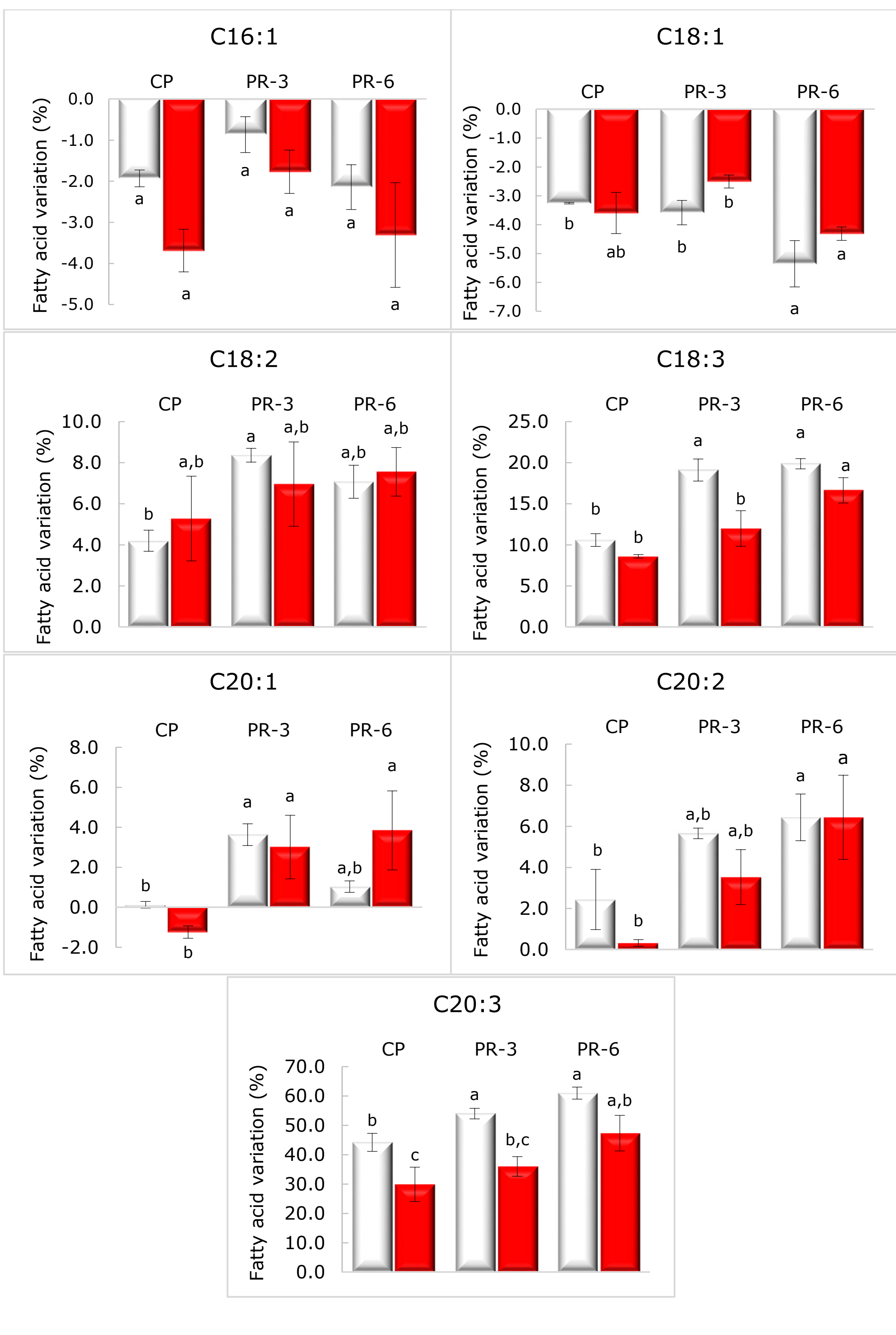

Figure 6. Unsaturated fatty acid variations of pork liver pâté samples. CP: Control pork liver pâté; PR-3: Pork liver pâté with 3% Rojo Brillante flour; PR-6: Pork liver pâté with 6% Rojo Brillante flour after digestion. Values with different letter indicates significant differences ($p < 0.05$), according to Tukey's post host test.

4. Conclusions

To the best of our knowledge, this is the first time that the bound and free polyphenolic compounds' stability has been studied in enriched meat products (with extracts rich in polyphenols) after in vitro gastrointestinal digestion.

Persimmon flour is a good source of bound polyphenols, especially gallic acid and flavanone glucosides, and can be successfully used to enrich pork liver pâté. Rich fatty foods such as pâté are excellent vehicles to preserve bound polyphenols, which could arrive at the colon intact and so be metabolized by the intestinal microbiome. However, PUFAs stability is negatively affected, inducing their oxidation, especially when added at the highest concentrations (6%). The use of two pancreatins with different lipase activity (8 UL/mL vs. 2000 UL/mL) considerably affects both the stability of bound polyphenol compounds and lipid oxidation. The highest number of bound polyphenols and TBARs values was reached after C2 intestinal phase: the higher the rate of lipolysis, the higher the number of fatty acids in the medium, which induced protection of polyphenols against degradation and lipid oxidation. Low variations were shown among fatty acid profiles between undigested and digested pâté samples. Surprisingly, their PUFA content increased after both intestinal phases probably due to the fact that this digestion phase improved their extractability. Lipid oxidation was reduced in pâtés in a dose-dependent way by persimmon flour after both intestinal phases (C1 and C2). Therefore, although the R-6 pâté showed higher oxidation than the control, it was not increased after digestion. Therefore, it could be concluded that lipase activity is an important factor that must be taken into account in the intestinal phase of the in vitro digestion process.

Nevertheless, more studies, both in vitro and in vivo, are needed as well as increasing knowledge about in vitro bile holding ability, lipid digestibility, colonic fermentation, and polyphenols transformations in order to achieve a complete vision of the health implications that could be useful to reinforce the suitability of meat product enrichment with persimmon flour coproducts.

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