Universitat de Lleida

In vitro bioaccessibility of health-related compounds from beverages based on fruit juice, milk or soymilk: Influence of food matrix and processing

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Dipòsit Legal: L.853-2014
http://hdl.handle.net/10803/146285

UNIVERSITAT DE LLEIDA
Escola Tècnica Superior d'Enginyeria Agrària
Departament de Tecnologia d'Aliments

Doctoral Thesis

In vitro bioaccessibility of health-related compounds from beverages based on fruit juice, milk or soymilk: Influence of food matrix and processing

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Lleida, 2014

The present research work was carried out in the laboratory of New Technologies on Food Processing, Department of Food Technology of the University of Lleida, Spain, under the supervision of Prof. Olga Martín Belloso and Prof. Pedro Elez Martínez.

This work was developed as part of the proyect “Mejora de la calidad nutricional de bebidas mixtas de zumos de frutas, leche y soja mediante la aplicacion de tecnologias no termicas” (AGL2006-12758-C02-02/ALI and AGL2006-12758-C02-01/ALI) supported by the Ministerio de Ciencia e Innovación (Spain).

The PhD candidate was financially suported by a predoctoral grant from the Agència de Gestió d’Ajuts Universitaris i de Recerca (AGAUR) funded by the Comissionat per a Universitats i Recerca, del Departament d’Innovació, Universitats i Empresa de la Generalitat de Catalunya and European Social Fund. A complementary grant from the Secretaría de Educación Pública de México (SEP) was also obtained.

The studies of food processing by high-pressure were carried out in the Department of Characterization, Quality and Safety, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain.

Dedicado a:

Mi ESPOSO e HIJOS,

Gracias por todo su amor y comprensión

en cada instante de esta etapa,

LOS AMO

Mis PADRES Y HERMANOS,

A pesar de la distancia siempre

han sabido estar cerca

AGRADECIMIENTOS

En primer lugar me gustaría agradecer a mis directores de tesis Dra. Olga Martín Belloso y Dr. Pedro Elez Martínez. Gracias por brindarme la oportunidad de realizar este trabajo bajo su dirección. Su apoyo y confianza, así como su capacidad para guiar mis ideas han sido muy importantes no solo en el desarrollo de esta tesis, sino también en mi formación como investigador.

Alejandra Rojas, muchas gracias por tu apoyo y participación activa en el desarrollo de esta tesis. Sin lugar a duda tus consejos han enriquecido este trabajo.

Al personal técnico del departamento, Manel, Gemma y Magda. Gracias por que también han aportado su granito de arena en el desarrollo de esta tesis, especialmente te agradezco a ti Magda, por todo tu apoyo en el laboratorio y sobre todo por tu amistad.

A mis compañeros de doctorado…. los que están y los que se han ido (Esther, Cyndia, Guillermo, Blanca, Karla, Martha, Laura, Ingrid, Mariana…). Gracias por todos los momentos que hemos compartido juntos…. risas, lágrimas, cafés, pica-pica, consejos, etc…. Por ayudarme a crecer, por los días felices y porque en los días difíciles me han extendido su hombro para llorar… simplemente gracias por su amistad.

Quiero expresar mi más sincero agradecimiento a María y Rubén, por su invaluable apoyo sobre todo en los momentos más difíciles. De verdad que no tengo palabras para expresarles mi gratitud. Son unos grandes amigos.

A los estudiantes de practicum, Isaac, Clara, Pilar, Marta, Alba, Maiara y Benedetta, gracias por su apoyo, porque juntos aprendimos muchas cosas en el laboratorio y también compartimos momentos inolvidables.

A las personas que he conocido en Lleida y que sin lugar a duda son un gran ejemplo a seguir, Maria Luisa, Andreu y Merced, Ramón y Elena, y demás familias que forman parte del splai, los llevaré siempre en mis pensamientos. Adriana, gracias también a ti por tu amistad.

A mis padres y hermanos, gracias porque a pesar de la inmensa distancia que nos ha separado todos estos años, siempre han estado cerca. Mamá, de ti he aprendido a luchar ante la adversidad, a levantarme después de cada tropiezo, a tener fe y esperanza…. que

diferente se ven las cosas desde este punto de vista!!. Papá, me enseñaste a ser perseverante, dedicada y a no rendirme hasta alcanzar mis sueños. Siempre he sabido cuanto se han esforzado porque pudiera alcanzar mis metas, por eso gran parte de este logro es también de ustedes…. A mis hermanos, quienes son mis amigos, cómplices y guías… hemos vivido tantas cosas juntos que sólo puedo agradecerles por compartir conmigo todos esos momentos y aligerar la carga en los momentos difíciles.

A mi compañero de vida, quien ha sabido darme lo mejor de sí, su amor, su alegría y dulzura, sobre todo que ha sabido estar ahí cuando más lo he necesitado, con la mano extendida y el corazón abierto. Rogelio, gracias porque me contagiaste y motivaste para seguir preparándome y venir a Lleida, porque sin tu apoyo no estaría donde estoy. Eres un hombre excelente, un excelente padre y un excelente esposo.

A mis hijos… Damián, has tenido que pasar largas horas de trabajo en "mi cole", teniendo que sacrificar paseos y juegos en el parque. A pesar de ello, siempre me has regalado una maravillosa sonrisa o una conversación agradable, eres un niño maravilloso, tú me llenas de ánimo y fuerzas para seguir luchando…A mi hermosa princesita, no puedo describir la ilusión que siento de tenerte en mi vientre, y de saber que pronto tendré la dicha de tenerte entre mis brazos. Gracias por existir y por llenar de ilusión y alegría mi vida…. A ustedes dedico todo este esfuerzo, por ustedes y para ustedes…. son el motor de mi vida y siempre lo serán.

Especialmente dedico estas últimas palabras a Dios quien me ha permitido vivir esta inolvidable experiencia, por todas las bendiciones recibidas hasta este momento, porque nunca he estados sola, siempre has puesto en mi camino a personas que me han guiado y apoyado en todo momento…. Infinitamente… ¡gracias!

ABSTRACT

Functional foods are receiving increasing consumer attention due to their relation with health, well-being, and the reduction of the risk of several diseases. The bioavailability and bioaccessibility of compounds such as vitamin C, phenolic compounds, isoflavones, and carotenoids give food and beverages their functional and biological activities.

Several factors are known to affect the bioaccessibility of bioactive compounds from foods and beverages, such as the food matrix and processing. Comprehension of the bioaccessibility of beverage constituents is an important challenge due to the complexity of the food matrix in which these compounds are contained. In addition, the extent to which food processing could modify their bioaccessibility needs to be determined. Through a better understanding of the stability and bioaccessibility of these constituents, it is possible to obtain beverages with higher nutritional value and functional quality.

For this reason, this doctoral thesis was focused on evaluating the *in vitro* digestibility and bioaccessibility of hydrophilic (vitamin C, phenolic compounds and isoflavones) and lipophilic bioactive compounds (carotenoids) of fruit juice-, milk- or soymilk-based beverages; and on determining the influence of food matrix and processing on the bioaccessibility of these constituents.

To achieve this main objective, the evaluation of the changes in the concentration of hydrophilic and lipophilic compounds from soymilk (SM), milk (M), blended fruit juices (BFJ_1, containing orange, kiwi and pineapple juices; and BFJ_2, containing orange, kiwi, pineapple and mango juices) and blended beverages (BFJ_1-SMB and BFJ_2-MB) during *in vitro* gastrointestinal digestion was studied, as well as determining the bioaccessibility of these food constituents. Additionally, it was evaluated the influence of the food matrix (water, milk and soymilk) and food processing (high-intensity pulsed electric fields [HIPEF]; high-pressure processing [HPP]; and thermal treatment [TT]) on the bioaccessibility of hydrophilic and lipophilic constituents from blended fruit juice-based beverages.

Results showed that *in vitro* gastrointestinal digestion exerted a significant influence on the concentration of bioactive compounds from beverages. Gastric digestion improved the concentration of some bioactive constituents, such as phenolic compounds and isoflavones. However, significant losses in the concentration of all the analyzed

substances during the small intestinal digestion, mainly in the dialyzed and micellar fractions, were observed.

Generally, the bioaccessibility of hydrophilic compounds was higher (with bioaccessibilities in the range of 11% to 42%) than that of lipophilics (bioaccessibilities between 9% and 27%), with the exception of milk-based beverages, where the lipophilic compounds were the most bioaccessible (bioaccessibilities up to 56%).

The bioaccessibility of hydrophilic and lipophilic constituents from beverages was modulated by both the food matrix and food processing. Hydrophilic bioactive compounds showed their highest bioaccessibility in matrices containing water and soymilk (WB, water-fruit juice beverage; and SB, soymilk-fruit juice beverage), while a milk matrix (MB, milk-fruit juice beverage) improved the bioaccessibility of lipophilic substances. Non-thermal technologies (HIPEF and HPP) were more effective than TT in preserving the concentration and bioaccessibility of most of the analyzed compounds from WB, MB and SB. Therefore, HIPEF and HPP could be considered promising technologies to obtain highly nutritional and functional beverages.

In vitro gastrointestinal digestion can be considered a useful tool for estimating the release, stability and bioaccessibility of bioactive compounds from functional beverages, as well as for determining the influence of the food matrix and processing on the bioaccessibility of these substances. Thus, information obtained through *in vitro* studies can be applied in the design of future *in vivo* studies.

RESUMEN

Los alimentos funcionales están recibiendo una creciente atención por parte de los consumidores debido a su relación con la salud, el bienestar y la reducción en el riesgo de padecer diversas enfermedades. La biodisponibilidad y bioaccesibilidad de compuestos bioactivos como vitamina C, compuestos fenólicos, isoflavonas y carotenoides, proporcionan a los alimentos y bebidas sus características biológicas y funcionales.

Diversos factores pueden afectar a la bioaccesibilidad de los compuestos bioactivos presentes en alimentos y bebidas, destacándose entre ellos la matriz alimentaria y el procesado. La comprensión de la bioaccesibilidad de los constituyentes de las bebidas representa un reto importante debido a la complejidad de la matriz alimentaria en la que estos compuestos están contenidos. Además, se debe determinar en qué grado el procesado de los alimentos puede modificar la bioaccesibilidad de dichos compuestos. La obtención de bebidas con un mayor valor nutricional y calidad funcional es posible a través de una mejor comprensión de la estabilidad y bioaccesibilidad de estos constituyentes bioactivos.

Por esta razón, la presente tesis doctoral se ha centrado en evaluar la digestibilidad y la bioaccesibilidad *in vitro* de compuestos bioactivos hidrofílicos (vitamina C, compuestos fenólicos e isoflavonas) y lipofílicos (carotenoides) en bebidas elaboradas a base de zumos de frutas, leche o leche de soja; así como en determinar la influencia de la matriz alimentaria y del procesado sobre la bioaccesibilidad de estos compuestos.

Para alcanzar el objetivo principal se estudiaron los cambios en la concentración de los compuestos bioactivos hidrofílicos y lipofílicos durante la digestión gastrointestinal *in vitro* de leche de soja (SM), leche de vaca (M), mezclas de zumos de frutas (BFJ_1, conteniendo zumos de naranja, kiwi y piña; y BFJ_2, conteniendo zumos de naranja, kiwi, piña y mango) y bebidas mixtas (BFJ_1-SMB y BFJ_2-MB), así como también se determinó la bioaccesibilidad de los constituyentes bioactivos de estos alimentos. Adicionalmente, se estudió la influencia de la matriz alimentaria (agua, leche y leche de soja) y del procesado de alimentos (pulsos eléctricos de alta intensidad de campo [HIPEF]; altas presiones hydrostáticas [HPP]; y tratamiento térmico [TT] sobre la bioaccesibilidad de los compuestos bioactivos hidrofílicos y lipofílicos de bebidas mixtas elaboradas a base de mezclas de zumos de frutas.

Los resultados mostraron que la digestión gastrointestinal *in vitro* ejerció una influencia significativa sobre la concentración de los compuestos bioactivos en las bebidas. La digestión gástrica mejoró la concentración de algunos constituyentes bioactivos, tales como compuestos fenólicos e isoflavonas. Sin embargo, se observaron pérdidas significativas en todos los compuestos bioactivos analizados durante la digestión intestinal, principalmente en las fracciones dializada y micelar.

Generalmente, la bioaccesibilidad de compuestos hidrofílicos fue mayor (con bioaccesibilidades entre 11% y 42%) que la de los compuestos lipofílicos (con bioaccesibilidades entre 9% y 27%), excepto en las bebidas a base de leche, donde los compuestos lipofílicos fueron los más bioaccesibles (hasta un 56%).

La bioaccesibilidad de los constituyentes hidrofílicos y lipofílicos de las bebidas fue modulada tanto por la matriz alimentaria como por el procesado. Los compuestos bioactivos hidrofílicos mostraron su mayor bioaccesibilidad en matrices conteniendo agua y leche de soja (WB, bebida a base de agua-zumo de frutas; y SB, bebida a base leche de soja-zumo de frutas), mientras que la matriz de leche (MB, bebida a base de leche-zumo de frutas) mejoró la bioaccesibilidad de los compuestos lipofílicos. Las tecnologías no térmicas (HIPEF y HPP) fueron más efectivas que el TT para preservar la concentración y bioaccesibilidad de la mayoría de los compuestos bioactivos analizados de WB, MB o SB. Por lo tanto, HIPEF y HPP podrían considerarse como tecnologías prometedoras para la obtención de bebidas altamente nutritivas y funcionales.

La digestión gastrointestinal *in vitro* puede ser considerada como una herramienta útil para la estimación de la liberación, la estabilidad y la bioaccesibilidad de compuestos bioactivos de las bebidas funcionales, así como para evaluar la influencia de la matriz alimentaria y del procesado sobre la bioaccesibilidad de estas sustancias. Así, la información obtenida a través de los estudios *in vitro* puede aplicarse en el diseño de futuros estudios *in vivo.*

RESUM

Els aliments funcionals estan rebent una atenció creixent per part dels consumidors degut a la seva relació amb la salut, el benestar i la reducció del risc de patir diverses malalties. La biodisponibilitat i bioaccessibilitat de compostos bioactius com la vitamina C, compostos fenòlics, isoflavones i carotenoids, proporcionen als aliments i les begudes les seves característiques biològiques i funcionals.

Diversos factors poden afectar a la bioaccessibilitat dels compostos fenòlics presents en aliments i begudes, destacant entre ells la matriu alimentària i el processat. La comprensió de la bioaccessibilitat dels constituents de les begudes representa un repte important degut a la complexitat de la matriu alimentaria en la què aquests compostos estan continguts. A més, s'ha de determinar en quin grau el processat dels aliments pot modificar la bioaccessibilitat d'aquests compostos. L'obtenció de begudes amb major valor nutricional i qualitat funcional és possible a través d'una millor comprensió de l'estabilitat i la bioaccessibilitat d'aquests constituents bioactius.

Per aquesta raó, la present tesi doctoral s'ha centrat en avaluar la digestibilitat i la bioaccessibilitat *in vitro* de compostos bioactius hidròfils (vitamina C, compostos fenòlics i isoflavones) i lipòfils (carotenoids) en begudes elaborades a base de sucs de fruites, llet o llet de soja, així com en la determinació de la influència de la matriu alimentaria i del processat sobre la bioaccessibilitat d'aquests compostos.

Per aconseguir l'objectiu principal s'han estudiat els canvis en la concentració dels compostos bioactius hidròfils i lipòfils durant la digestió gastrointestinal *in vitro* de la llet de soja (SM), llet de vaca (M), barreges de sucs de fruites (BFJ_1, que contenia sucs de taronja, kiwi i pinya; i BFJ_2, que contenia sucs de taronja, kiwi, pinya i mango) i begudes mixtes (BFJ_1-SMB y BFJ_2-MB), així com s'ha determinat la bioaccessibilitat dels constituents bioactius d'aquests aliments. Addicionalment, s'ha estudiat la influència de la matriu alimentària (aigua, llet i llet de soja) i del processat dels aliments (pulsos elèctrics d'alta intensitat de camp (HIPEF), altes pressions de processat (HPP) i tractament tèrmic (TT)) sobre la bioaccessibilitat dels compostos bioactius hidròfils i lipòfils de les begudes mixtes elaborades a base de barreges de sucs de fruites.

Els resultats han demostrat que la digestió gastrointestinal *in vitro* exerceix una influència significativa en la concentració dels compostos bioactius en les begudes. La digestió gàstrica va millorar la concentració d'alguns dels constituents bioactius, com els compostos fenòlics i les isoflavones. No obstant, es van observar pèrdues

significatives en tots els components bioactius analitzats durant la digestió intestinal, principalment en les fraccions dialitzada i micel·lar.

En general, la bioaccessibilitat dels compostos hidròfils va ser més gran (amb bioaccessibilitats entre 11% i 42%) que la dels compostos lipòfils (amb bioaccessibilitats entre 9% i 27%), excepte en les begudes a base de llet, en les què els compostos lipòfils van ser els més bioaccessibles (fins un 56%).

La bioaccessibilitat dels constituents hidròfils i lipòfils de les begudes va ser modulada tant per la matriu alimentaria com pel processat. Els compostos bioactius hidròfils van ser més bioaccessibles en les matrius que contenien aigua i llet de soja (WB, beguda a base d'aigua-sucs de fruites; i SB, a base de llet de soja-suc de fruites), mentre que la matriu de llet (MB, beguda a base de llet-sucs de fruites) va millorar la bioaccessibilitat dels compostos lipòfils. Les tecnologies no tèrmiques (HIPEF i HPP) van ser més efectives que el TT per preservar la concentració i la bioaccessibilitat de la majoria dels compostos bioactius analitzats de WB, MB o SB. Per tant, HIPEF i HPP poden considerar-se com tecnologies prometedores per l'obtenció de begudes altament nutritives i funcionals.

La digestió gastrointestinal *in vitro* pot ser considerada com una eina útil per l'estimació de l'alliberació, l'estabilitat i la bioaccessibilitat de compostos bioactius de les begudes funcionals, així com per avaluar la influència de la matriu alimentària i del processat sobre la bioaccessibilitat d'aquestes substàncies. Per tant, la informació obtinguda de estudis *in vitro* pot ser utilitzada pel disseny de futurs estudis *in vivo*.

CONTENT

1. INTRODUCTION

1.1. Bioactive compounds and antioxidant activity of foods

Bioactive compound is defined as a component of food that influences physiological or cellular activities, resulting in a beneficial health effect (Kris-Etherton, et al. 2004). These compounds are not nutrients because they are not essential for life, and they typically occur in small amounts in food. Nonetheless, diverse epidemiological studies suggest that bioactive compounds contribute to reduce the risk of several diseases (Kris-Etherton, et al. 2002).

Food constituents with biological properties are derived from the plant kingdom and from animal sources in major and minor proportions, respectively (Rein et al., 2013). In this sense, fruits and vegetables are considered as the main dietary sources of bioactive compounds, such as vitamins, phenolic compounds and carotenoids, which are linked with decreased incidence of cardiovascular and neurodegenerative diseases, as well as some cancer types (Daly, Jiwan, O'Brien, & Aherne, 2010; Harrison & May, 2009; Scalbert & Williamson, 2000). On the other hand, products from animal origin, such as milk, possess anticarcinogenic compounds such as vitamins (i.e. A and D), carotenoids, peptides, lactoferrin, α-lactoglobulin and fatty acids (Özer & Kirmaci, 2010).

1.1.1. Vitamin C

Vitamin C is one of the most important water soluble antioxidants from food. This compound mainly occurs in citrus fruits, green peppers, cabbage, strawberries, and green leafy vegetables (Kris-Etherton, et al. 2004). It is found in two L-isomeric forms: ascorbic acid (Vitamin C) in the reduced state and dehydroascorbic acid in the oxidized state (Figure 1). The physiological and biochemical functions of vitamin C are given by their ability as electron donor. In fact, this compounds acts as antioxidant by donating its electrons and thus, preventing the oxidation of other compounds (Padayatty et al., 2003). Although the antioxidant activity of vitamin C is mainly achieved in water-soluble environments, it also plays a protective effect in lipids through the regeneration of fat-soluble vitamin E to its active form α-tocopherol (Schlueter & Johnston, 2011).

In addition, vitamin C helps in the synthesis of the amino acids carnitine and catecholamine, which regulates the nervous system, as well as in the formation of neurotransmitters. It also reduces the cholesterol in blood and increases the absorption

of iron in the gut (Ball, 2006; McRae, 2006). On the other hand, the deficiency of this vitamin can bring scurvy, infections, poor wound healing, bleeding gums, capillary hemorrhaging, anemia, muscle degeneration, atherosclerotic plaques, and neurotic disturbances, among others (Schlueter & Johnston, 2011).

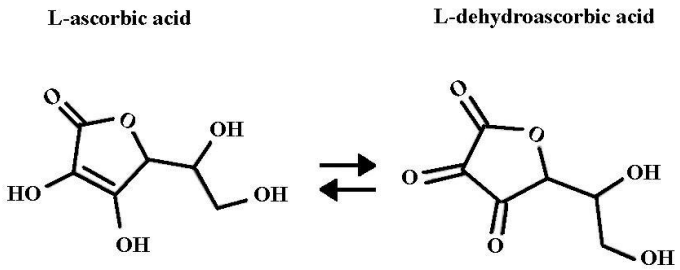

Figure 1. L-isomeric forms of ascorbic acid: L-ascorbic acid (PM= 176.13) and L-dehydroascorbic acid (PM= 174.11). Adapted from Chemical Book (www.chemicalbook.com)

Digestion, absorption and metabolism of vitamin C. Vitamin C is ingested in both reduced and oxidized forms. There is scarce information about the digestibility of vitamin C through the gastrointestinal tract. However, it has been established that reduced and oxidized forms are absorbed throughout the small intestinal epithelium by means of different mechanisms. Ascorbic acid is absorbed via sodium-dependent active transporter (SVCT1) mainly in the ileum and jejunum. Dehydroascorbic acid is absorbed by facilitated diffusion in the duodenum and jejunum with the aid of glucose transporters (Malo & Wilson, 2000; Fujita et al., 2000).

Vitamin C circulates free (unbounded) and is available as a reductant agent in blood and intestinal fluids. Dehydroascorbic acid is transported into cells (i.e. erythrocytes, leukocytes, and insulin-sensitive tissues) through glucose transporters and is quickly recycled to vitamin C (Paddayatty & Levine, 2001). Thus, glucose is a competitive inhibitor of dehydroascorbic acid transport. Vitamin C is transported into tissues via sodium-dependent transporter (SVCT2) (Kuo, MacLean, McCormick, & Wilson, 2004).

Dehydroascorbic acid has a short half-life (<2 minutes) and it is metabolized to excretory products (mainly oxalic acid) if it is not taken up by cells (Schlueter & Johnston, 2011).

1.1.2. Phenolic compounds

Phenolic compounds are secondary metabolites of plants and contribute to organoleptic and nutritive quality of food in terms of color, taste, aroma, and flavor (Karakaya, 2004). The main dietary sources of phenolic compounds are fruit and vegetables, but they are also found in legumes, cereals, nuts, medicinal plants and spices (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010).

The most abundant phenolic compounds in the diet are phenolic acids and flavonoids (Figures 2 and 3). Phenolic acids can be classified in two classes: benzoic and cinnamic acids. They are found in different forms in plants, such as aglycones (free phenolic acids), esters, glycosides, and/or bound complexes. On the other hand, flavonoids can be contained in food mainly as glucosides although some are found as aglycones. The most important classes of flavonoids are: flavones, flavonols, flavanones, flavanols (catechins), anthocyanidins (its glycoside is called anthocyanin), and isoflavones (Weichselbaum & Buttriss, 2010). Isoflavones occurs almost exclusively in legumes, being soybeans and soy-derived products the richest sources of these compounds (Larkin, Price, & Astheimer, 2008). Isoflavones are found as glycosides (daidzin, genistin, and glycitin) or aglycones (daidzein, genistein, and gycitein) and their molecular structure is represented in Figure 4.

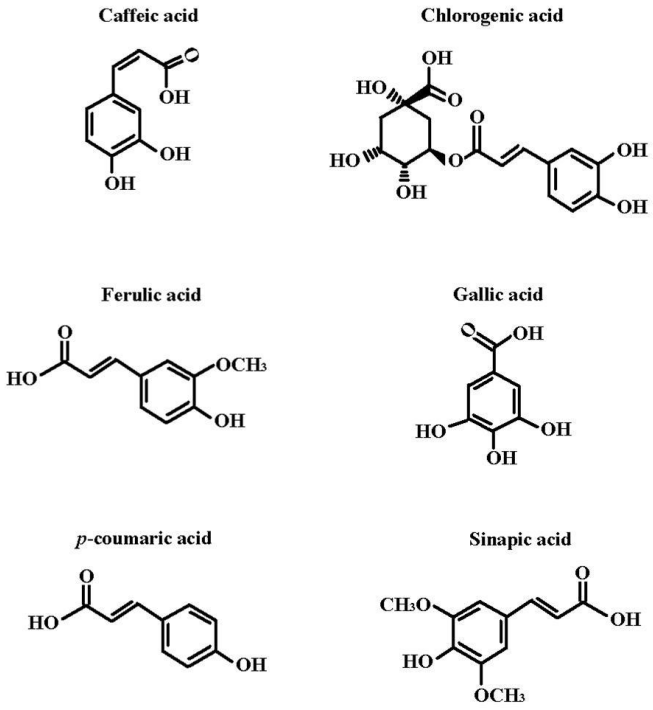

Figure 2. Structures of phenolic acids. Adapted from Lafay & Gil-Izquierdo (2008).

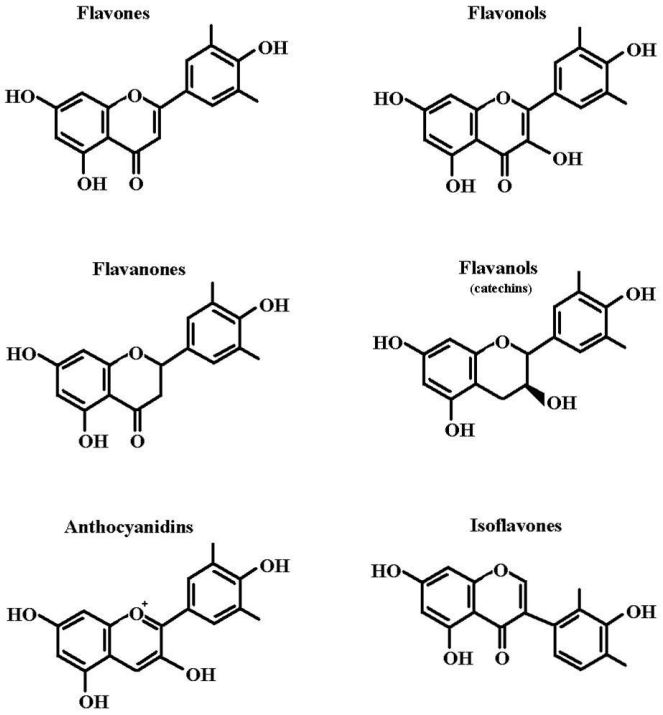

Figure 3. Main structures of flavonoids. Adapted from Karakaya (2004).

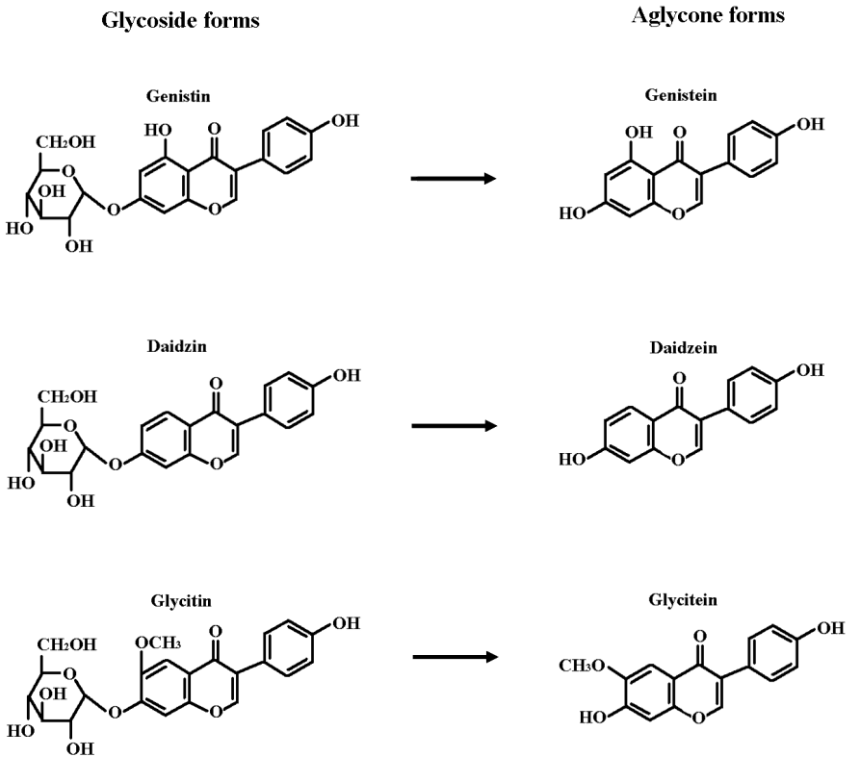

Figure 4. Glycoside and aglycone forms of isoflavones. Adapted from Cederroth & Nef (2009).

Epidemiological studies showed that diets rich in phenolic compounds could inhibit the development of cardiovascular and inflammatory diseases, as well as some cancer types (Scalbert & Williamson, 2000). These compounds could provide health benefits by scavenging free-radicals, protecting and/or regenerating other dietary compounds (i.e. vitamin E), and chelating metal ions (Garcia-Salas et al., 2010). Healthy properties of phenolic compounds are closely related to their structure and concentration. Their structure determines the antioxidant and metal-chelating activities. In addition, if these compounds are contained in food at low concentration, they may act as antioxidant and protect foods from oxidative deterioration but at high concentration they may interact with proteins, carbohydrates and minerals (Karakaya, 2004). Isoflavones possess hormonal and nonhormonal properties that contribute to their biological activity. Among the hormonal attributes of isoflavones are the reduced risk of heart disease, osteoporosis, and hot flashes in menopausal women (Messina, Ho, & Alekel, 2004; Nestel, 2003). Nonhormonal properties have been linked to chemopreventive activity from both hormone-dependent and independent cancer types (Messina, Kucuk, & Lampe, 2006; Sarkar & Li, 2003).

Digestion, absorption and metabolism of phenolic compounds. Chemical structure of phenolic compounds greatly affects their absorption because it determines the reactivity and conjugation reactions with glucuronide, methyl and sulphate groups that are involved in absorption reactions.

Phenolic acids are absorbed in the upper part of gastrointestinal tract if they are in aglycone form (Lafay & Gil-Izquierdo, 2008). The stomach is an active absorption site of several phenolic acids such as gallic, caffeic, ferulic, coumaric and chlorogenic acids (Konishi, Zhao, & Shimizu, 2006; Lafay, Gil-Izquierdo, et al., 2006). The small intestine constitutes another absorption site. Aglycones can be absorbed in the range of 19.1% (for caffeic acid) and 56.1% (for ferulic acid). However, the absorption of esterified phenolic acids reached only 0.3 and 0.4% from the initial intake because the intestinal enzymes are not so efficient to hydrolyse the ester bonds (Adam et al., 2002; Lafay, Morand, Manach, Besson, & Scalbert, 2006). Phenolic acids that reach the colon are metabolized by the microflora. The resultant microbial metabolites are the most abundant in plasma and urine, although free and conjugated phenolic acids (with

glucuronide, sulphate or sulfoglucuronide) are also found (Lafay & Gil-Izquierdo, 2008).

Flavonoids exist predominantly as glycosides, with the exception of flavan-3-ols. Absorption of most of the dietary flavonoids generally occurs in the small intestine (Donovan, Manach, & Faulks, 2006) following hydrolysis and release of aglycone forms. It has been postulated two ways by which glucoside conjugates are hydrolysed to aglycones: a) lactase phloridizin hydrolase/diffusion, and b) transport/cytosolic β-glucosidase. In the brush border of the small intestine, the enzyme lactase phloridizin hydrolase exhibits substrate specificity for flavonoid O-β-D-glucosides. The released aglycones enter into epithelial cells by passive diffusion as a result of their increased lipophilicity and their proximity to cellular membrane (Day et al., 2000). In contrast, the cytosolic β-glucosidase acts on polar glucosides within the epithelial cells. It is possible that polar glucosides are transported into epithelial cells through the active Na-dependent GLUT-1 (Gee et al., 2000) but a recent study showed that flavonoid glycosides were not transported by Na^+/glucose transporter and that glucosylated flavonoids, and some aglycones inhibit GLUT (Kottra & Daniel, 2007). Aglycones are metabolized due to the action of sulphotransferases, UDP-glycoronosyltransferases and catechol-*O*-methyltransferases forming sulphate, glucoronide and methylated metabolites, respectively (Del Rio, Borges, & Crozier, 2010). Some flavonoids and their metabolites are not absorbed in the small intestine but they can be absorbed in the large intestine through the action of colonic microflora.

Isoflavones are mainly ingested as a complex mixture of glucoside conjugates (Setchell et al., 2002). Following ingestion, isoflavone aglycones are absorbed from the stomach but their glucoside form must be deglycosylated to aglycones prior to absorption in the gut (Karakaya, 2004). Both the small intestinal tissue and bacterial β-glucosidase are capable to hydrolyze glucosides. Thus, the released aglycones are either absorbed directly in the duodenum or further metabolized in the large intestine into other metabolites (i.e. equol) by the intestinal microflora (Murota et al., 2002).

1.1.3. Carotenoids

Carotenoids are a widespread family of fat-soluble plant pigments with more than 700 naturally occurring pigments synthesized by plants, algae, and photosynthetic bacteria. However, fifty of them can be absorbed and metabolized by the human body

and only six (β-carotene, α-carotene, lycopene, β-cryptoxanthin, zeaxanthin, and lutein) represent more than 95% of total blood carotenoids (Figure 5) (Maiani et al., 2009).

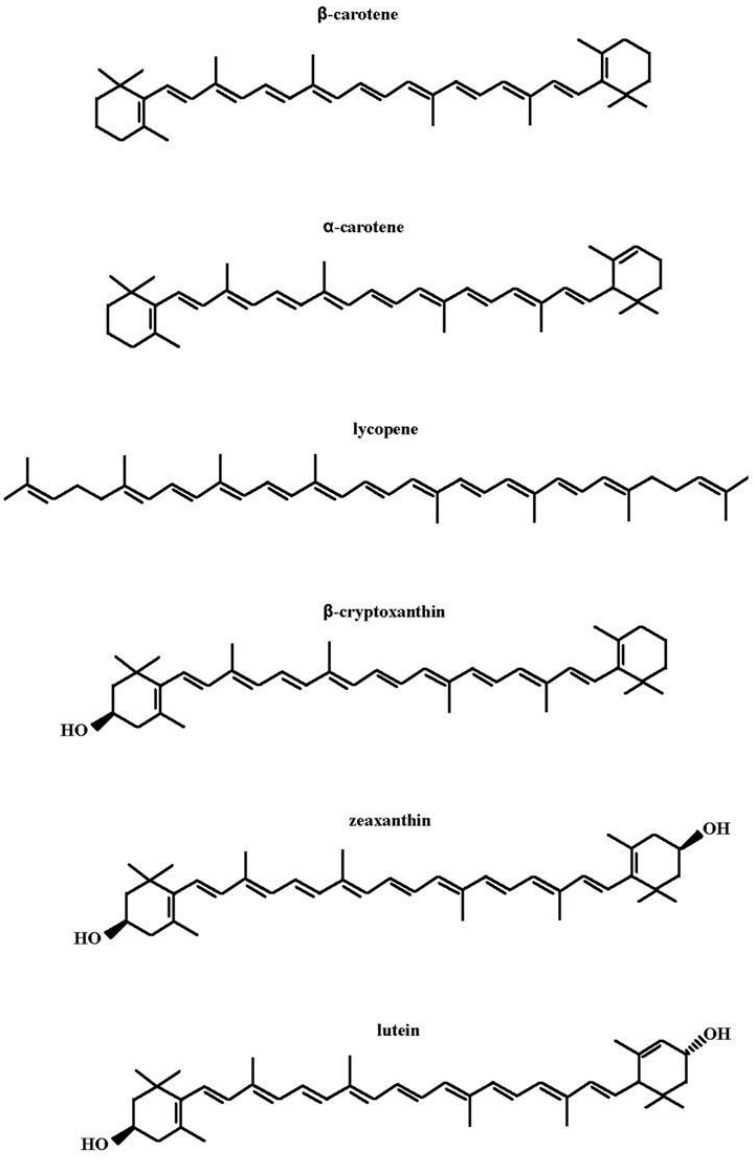

Figure 5. Main structures of carotenoids. Adapted from Krinsky & Johnson (2005).

Carotenoids can be classified into carotenes or xanthophylls in basis of their chemical composition. Carotenes are composed only of carbon and hydrogen atoms, being β-carotene, α-carotene, and lycopene the main carotenes. On the other hand, xanthophylls contain at least one oxygen atom and zeaxanthin, lutein, α- and β-cryptoxanthin, canthaxanthin and astaxanthin are the major xanthophylls (Stahl & Sies, 2005).

These compounds have shown to play an important role in human health by their powerful antioxidant potential and because some of them possess provitamin A activity. Carotenoids with provitamin A activity (they can be converted to retinol in the body) are β-carotene, α-carotene, and β-cryptoxanthin. Lutein, zeaxanthin, and lycopene do not have vitamin A activity. Carotenoids have been associated with anti-aging, anti-inflammation, anti-ulcer, and anticancer properties; as well as with immune system enhancement (Fernández-García et al., 2012). The main sources of carotenoids are yellow and orange fruits, dark green vegetables and dairy products (Maiani et al., 2009).

Digestion, absorption and metabolism of carotenoids. Carotenoids must be first released from the food matrix and incorporated into mixed micelles before being available for absorption. Micelles are molecular aggregates formed by carotenoids, acylglycerols, cholesterol and phospholipids, which transport liposoluble material to the intestinal epithelium (Fernández-García et al., 2012) . Therefore, the formation of mixed micelles is one of the critical factors that affect the bioaccessibility of carotenoids.

Digestibility and absorption of carotenoids depends on several factors related to the food matrix (i.e. presence of other carotenoids, dietary fat and fiber, food processing), co-ingested food, nutrient status and genetic profile of the host (Van Het Hof, West, Weststrate, & Hautvast, 2000). Fat solubility of individual carotenoids also affects their micellization and it depends on their structural features. Dietary fiber reduces the micellization of carotenoids because biliary fluids become soluble in the gel formed during gastric digestion. Esterification of xanthophylls with fatty acids modifies the fat solubility of these compounds because they are more liposoluble than either carotenes or their corresponding free xanthophylls (Fernández-García, Mínguez-Mosquera, & Pérez-Gálvez, 2007). Bioaccessibility of carotenoids is generally low in animals and humans because their chemical structure affords interactions with other constituents within the food matrix (Van Het Hof et al., 2000).

1.1.4. Antioxidant activity of food

Recently, it has been stated that the beneficial effect of foods on human health comes from the antioxidant activity of bioactive compounds contained in these products (Gülçin, 2012).

Oxidation is the transfer of electrons from one atom to another and it is a normal part of aerobic life. Nevertheless, this process generates free radicals when the electron flow becomes uncoupled (transfer of impaired single electrons). Free radicals are continuosly produced during normal physiological events but they are capable to damage important biomolecules, such as proteins, lipids and DNA (Liu, 2003).

Fortunately, aerobic organisms have antioxidant defenses, including antioxidant enzymes and antioxidant compounds which repair the damaged molecules by free radicals. An antioxidant is a molecule that inhibits the oxidation of other molecules (Figure 6). Food antioxidant is any substance that inhibits the oxidation of an oxidizable substrate (Gülçin, 2012).

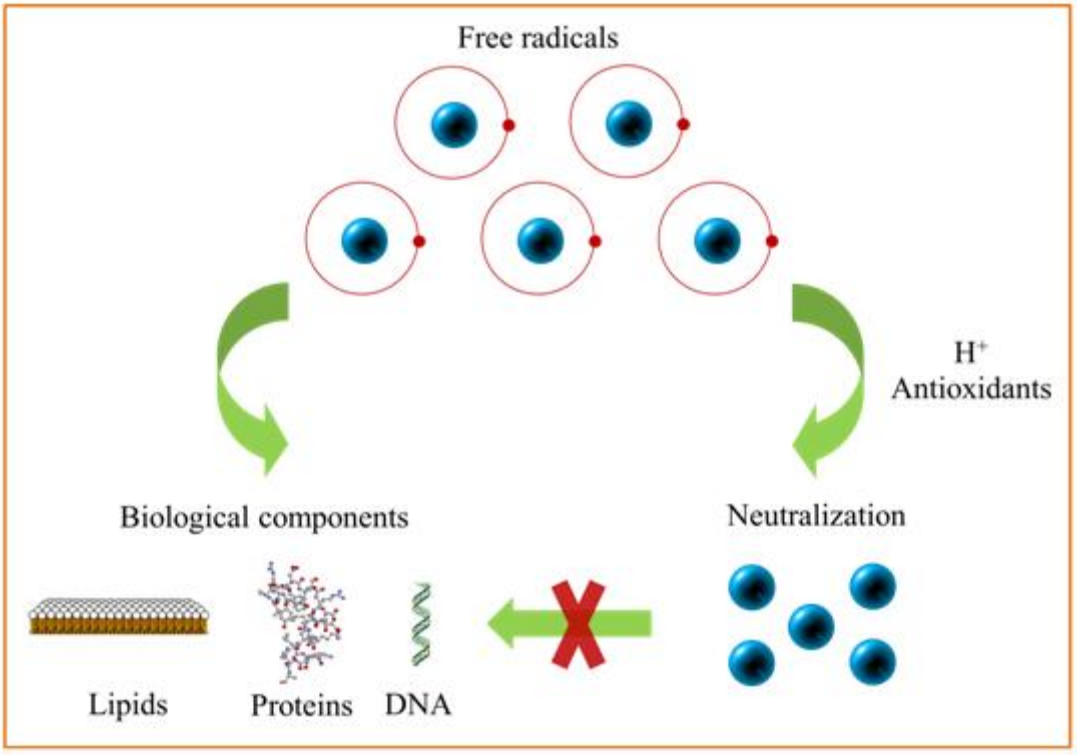

Figure 6. Radical scavenging activity of antioxidants for protecting biological components. Adapted from Rodríguez-Roque et al. (2012).

Antioxidants have the ability to scavenge free radicals, increasing the shelf life of food, retarding the oxidative reactions and thus, avoiding food deterioration.The main dietary antioxidants are vitamin C, phenolic compounds and carotenoids (Ryan & Prescott, 2010). A large diversity of methodologies for determining the antioxidant acitivity of food constituents is available in the literature, including radical scavenging assay (i.e. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical ($ABTS^{+}$)

scavenging; 1,1-diphenyl-2-picrylhydrazyl, ($DPPH^{\bullet}$) radical; superoxide anion radical, nitric oxide), non-radical reactive oxygen and nitrogen species scavenging (i.e. hydrogen peroxide, single oxigen, metal chelating, thiobarbituric acid), among others (Gülçin, 2012; Sánchez-Moreno, 2002)

1.2. Bioaccessibility of bioactive compounds from foods

Plant-derived foods are rich sources of bioactive compounds. The nutritional value of food is generally given by their content in bioactive compounds. However, not much attention has been focussed in assessing how much of these compounds is available to exert their biological function in the human body. Therefore, the information about the concentration of bioactive compounds reaching the bioavailable and bioaccessible fractions is a more important data than the concentration of these compounds in the corresponding food.

Bioavailability, defined as the fraction of ingested nutrient or bioactive compounds that is digested, absorbed, and metabolized through normal pathways (Figure 7), is an essential parameter to determine the amount of active compounds present in food that are available to reach the relevant sites of the body in appropriate concentrations and active form (Rein et al., 2013; Wood, 2005).

The fraction of bioactive compound released from the food matrix following digestion that is solubilised into the gut for intestinal uptake is usually known as bioaccessible fraction (Figure 7) (Ferruzzi, 2010). Thus, gastrointestinal digestion is an essential first step that allows a better understanding of the biological activity of bioactive compounds contained in foods. Bioactive compounds must be in a molecular dispersed state (i.e. aqueous solution), in a colloidal form, or in a micellar system (for hydrophobic compounds) to be bioaccessible (Duchateau & Klaffke, 2008). The incorporation of bioactive substances from the food matrix into an aqueous or micellar system depends on several factors related to the bioactive compounds and the food matrix in which these compounds are immersed (Table 1) (Duchateau & Klaffke, 2008).

Different *in vivo* and *in vitro* methodologies have been utilized to assess the bioavailability and bioaccessibility of bioactive compounds from food, all of them showed advantages and disadvantages.

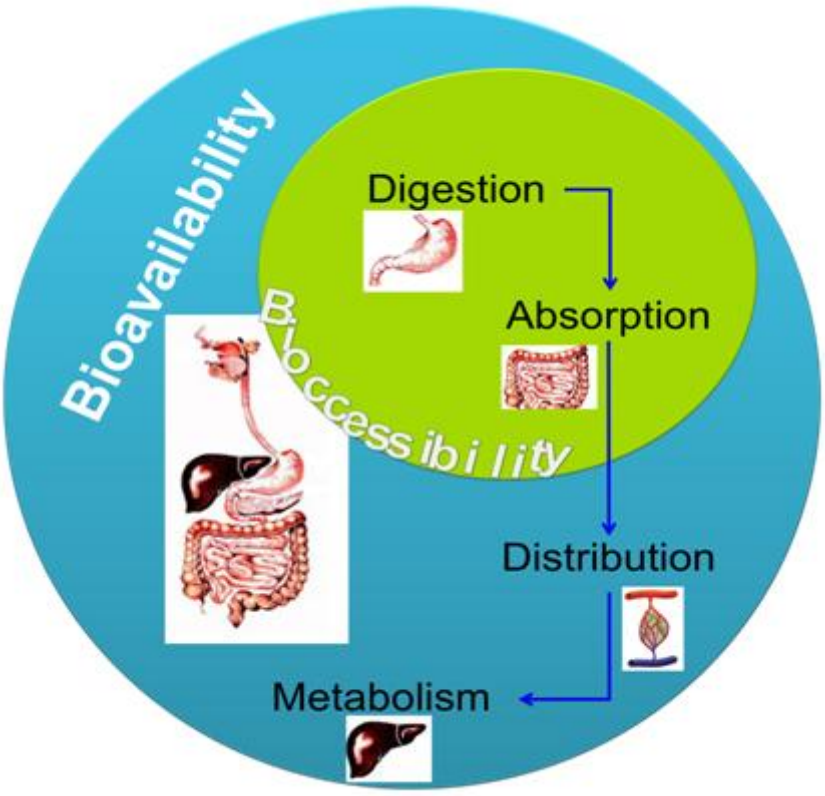

Figure 7. Bioaccessibility and bioavailability steps.

Table 1. Factors affecting the bioaccessibility bioactive compounds.[a]

Factors	Description or characteristic
Bioactive compounds	Hydrophobicity
	Molecular weigh
	Physicochemical features
	Proton donor/aceptor
	Solubility
Food matrix	Composition
	pH
	Processing
	Structure
	Type of product
	Viscosity

[a] Adapted from Duchateau & Klaffke (2008).

In overall, bioavailability of bioactive substances is evaluated through *in vivo* studies carried out with humans or animals. Although *in vivo* studies, like human intervention studies, provide more specific information about the bioavailability of bioactive compounds, these studies are unaffordable because they are expensive and time-

consuming trials. Animal studies are generally less expensive than human intervention studies but the main drawbacks of these studies are the differences in the metabolism between animals and humans, making difficult the extrapolation of results (Wienk, Marx, & Beynen, 1999).

As alternative, *in vitro* methodologies have been developed as an earliest and fast approach to *in vivo* studies and they are considered simple, cheap and reproducible tools for assessing the bioaccessibility and bioavailability of different food constituents (Failla & Chitchumroonchokchai, 2005). One such approach is the *in vitro* gastrointestinal digestion.

1.2.1. *In vitro* gastrointestinal digestion

In vitro gastrointestinal digestion is a valuable and useful methodology for the estimation of pre-absorptive events as stability and bioaccessibility of nutrients and bioactive compounds from food.

For this reason, several *in vitro* gastrointestinal digestion methods have been developed. Most of these studies have been reviewed by Hur, Lim, Decker, & McClements (2011). These authors surveyed more than 80 *in vitro* digestion models and reported significant differences in their operation: number and type of digestive phases (i.e. mouth, stomach, small intestine, and large intestine), composition and concentration of digestive fluids (i.e. enzymes, salts, buffers, biological polymers, and surface-active components), and the length of the incubation times of samples in each digestive stage. The time of digestion, the concentration and composition of enzymes must be adjusted according to sample features.

Foods are usually digested through two *in vitro* steps: gastric and small intestinal digestions (Figure 8) with pepsin and pancreatin enzymes, respectively. The initial steps of food breakdown are achieved during gastric digestion. Acidic conditions of gastric digestion help to break most of the polymeric and oligomeric structures such as proteins and carbohydrates (Wood, 2005).

During intestinal digestion a significant increase in the pH occurs, which change the activity of gastric enzymes (Duchateau & Klaffke, 2008). Bile flow and the release of pancreatic enzymes reduce the intragastric surface tension and thus facilitate the emulsification of lipids into mixed micelles (Fennel-Evans & Wennerström, 1994; Kalantzi et al., 2006). The micellar fraction is usually obtained by low-speed

centrifugation or ultracentrifugation of intestinal digesta and affords a complete experimental tool for the analysis of liposoluble compounds such as carotenoids (Fernández-García et al., 2012). Only lipids incorporated into micelles can be taken up by intestinal cells in significant amounts (Trevaskis, Charman, & Porter, 2008).

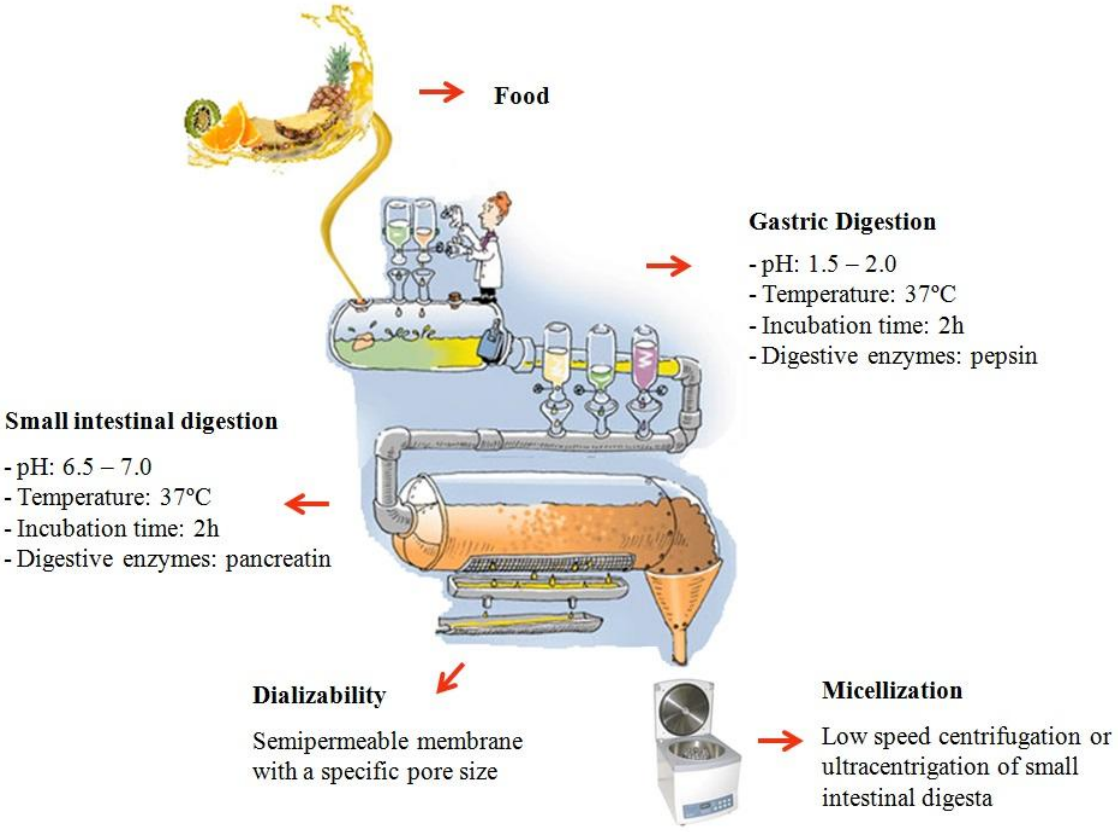

Figure 8. *In vitro* gastrointestinal digestion scheme

In vitro gastrointestinal digestion followed by the determination of bioactive compounds which are capable to cross a semipermeable membrane with a specific pore size (dializability), simulates the passive diffusion of these compounds through the intestinal epithelium. Dializability methods have been shown to be useful for assessing the bioaccessibility of food compounds such as phenolic acids, flavonoids, anthocyainins, vitamin C, and minerals (Bouayed, Deußer, Hoffmann, & Bohn, 2012; Bouayed, Hoffmann, & Bohn, 2011; Pérez-Vicente et al., 2002; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004).

1.3. Functional beverages

The modern lifestyle along with other factors such as industrialization and globalization has changed the eating habits of population. The reduced time for preparing food has increased the intake of ready-to-eat food. At the same time, the daily consumption of fruit and vegetables in Spain, among other countries only reaches 1.7 portions per day (Dembitsky et al., 2011). Because of these factors, chronic and degenerative diseases have increased considerably in recent years. In fact, the incidence of disease will increase from 46% to 57% in 2020 according to data compiled by the Food Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (FAO/WHO, 2003).

Fortunately, consumers are becoming aware of the role of food on health, and they are demanding products that beyond their nutritional value, improve health and well-being, reducing the risk of disease. These products are usually known as functional food (Howlett, 2008) and their potential market is continuously growing with sales of US$16 billion in the world in 2005 (Leatherhead Food International 2006), US$30 billion in 2007 (Bech-Larsen & Scholderer, 2007) and an increase in sales from 8.5% to 20% in 2009 (PricewaterhouseCoopers, 2009).

Nowadays, the food industry is attracting the consumer attention through functional foods and beverages that besides being highly nutritious and healthy, they are easy to prepare and consume (Wootton-Beard, Moran, & Ryan, 2011). Originality, convenience and quality are considered as important marketing tools in the food industry to increase sales (Marsellés-Fontanet, Elez-Martínez, & Martín-Belloso, 2012). A great variety of functional foods are available in the market to suit different lifestyles of consumers, as well as to satisfy their preferences for tasty, nutritious, healthy and convenient products. Functional beverages stand out among these foods and their consumption increased 30% in Europe in 2007 (Zulueta, Esteve, Frasquet, & Frígola, 2007).

In overall, functional beverages are made from fruits, vegetables and herbs in combination or not with dairy and/or soy-derived products, which naturally provide great amounts of health-promoting compounds (Prado, Parada, Pandey & Soccol, 2008; Zulueta, Esteve, Frasquet, et al., 2007). Because of the wide range of possibilities for developing functional beverages, there is a great interest to elucidate the health benefits

of these products in order to design new foods and beverages with better functional properties.

In Table 2 some functional beverages available in the market at this time are shown.

1.3.1. Fruit juice-based beverages

According to the legislation of the European Union (EU) (UE1050/2003), fruit juice is defined as the product obtained from healthy and ripe fruits from one or more species, which are fresh or preserved by chilling, and keep the color, aroma and flavor from the fruits which they come from.

Health benefits of fruit juices are attributed to the wide range of compounds with biological activity contained in these beverages. The major bioactive compounds of fruit juices are vitamins, phenolic compounds, carotenoids, and micronutrients (fatty acids, amino acids, minerals, among others) (Ryan & Prescott, 2010; Seeram et al., 2008; Wootton-Beard et al., 2011).

In recent years, several researches have evaluated the bioactive substances from fruits and their derived products, such as fruit juices. Results showed that diets rich in these foods may reduce the risk of certain types of diseases (Gardner, White, McPhail & Duthie, 2000). For this reason, the FAO and WHO recommend to eat at least 400 g or five portions of fruit and vegetables per day for preventing chronic diseases, such as heart disease, cancer, diabetes and obesity (FAO/WHO, 2003). In this sense, fruit juices represent an easy and convenient way of consumption in order to include the bioactive compounds from fruits in the diet.

Currently, the potential market of fruit juices is growing and some beverage industries have focused on the design and development of new fruit-derived products. Among them, blended fruit juices stand out to enhance the sensorial and nutritional characteristics of fruits. In addition, mixing different fruit juices allow increasing the concentration of bioactive compounds, adding new nutrients or improving the flavor and appearance of these beverages. However, interactions among bioactive compounds and other food constituents could occur in complexes matrices, adversely modifying the quality of food (Gaonkar & McPherson, 2006).

Table 2. Some functional beverages available in the market.

Brand	Product	Description	Bioactive ingredients
Alpro, UK	Alpro's Soya & Fruity	Fruit juices + soymilk	Antioxidants and proteins
Chadwick Bay, USA	Chadwick juices-smoothies	100% Fruit juices	Enriched with vitamins (A, C, D and E)
Chiquita, USA	Chiquita-smoothies	Frozen fruit smoothies	Antioxidants
Coca-Cola, USA	Minut maid antiox	Blend of fruit juices	Enriched with antioxidants
Coca-Cola, USA	Minut maid duofrutas	Blend of fruit juices	Antioxidants
Danone, France	Activia drink	Yogurt with probiotics + fruit juices	Antioxidants and probiotics
Del Valle, Mexico	Del Valle Soya	Soy drink + fruit juices	Isoflavones and antioxidants
Don Simon, Spain	Don Simón-antioxidante	Fruit beverage	Antioxidants
Don Simon, Spain	Funciona Max	Fresh fruit juices + milk	Antioxidants and proteins
Fuze Company, USA	Fuze	Fruit beverage	Enriched with vitamins
Hérdez, Mexico	Ocean Spray	Blend of fruit juices	Antioxidants
Ingman Food, Finland	Rela	Juice + milk with probiotics	Antioxidants, proteins and probiotics
Juver, Spain	OFF (Relaxing beverage)	Pineapple and kiwi juices + tila and melissa	Antioxidants, tila and melissa
Juver, Spain	ONN (Stimulant beverage)	Orange and mango juices + guarana and gingseng	Antioxidants, guarana and gingseng
Juver, Spain	Juvital	Fruit juices + milk	Antioxidants and proteins
Juver, Spain	Fruit & Fibra	Fruit juices + prebiotic	Antioxidants and fiber
National Foods, Australia	Mildura sunrise	Exotic fruit juices	Antioxidants
Pascual, Spain	Bifrutas	Fruit juices + milk	Antioxidants and proteins
Pascual, Spain	ViveSoy	Flavored soy beverages	Isoflavones
Pascual, Spain	Zumosol	Blend of fruit juices	Antioxidants
Pepsico, USA	Trop50 (Tropicana)	Orange juice + calcium + Vitamin D	Calcium and vitamins (C and D)
Skane mejerier , Sweden	Proviva	Fruit beverage + yogurt with probiotics	Antioxidants and *L. plantarum*
Spes, Chile	Omega W-bond forte 1300	Orange juice + fatty acids	Vitamins and omega-3
Unilever, USA	Ades	Soy beverage + fruit juice	Antioxidants and isoflavones

De Sousa, Maia, de Azeredo, Ramos, & de Figueiredo (2010) developed a tropical fruit (cashed apple, papaya, guava, and passion fruit) mixed nectar with addition of caffeine. The resulted beverage was microbiologically stable with a high concentration of vitamin C and good sensorial acceptance. De Carvalho, Maia, De Figueiredo, De Brito, & Rodrigues (2007) studied the physicochemical, microbiological and sensory stability of a beverage containing coconut water and clarified cashed apple juice.

1.3.2. Milk-based beverages

Milk is a colloidal complex formed by fat globules suspended in aqueous medium which contains important amounts of proteins (essential amino acids), fat (unsaturated fatty acids such as omega-6 and omega-3-fatty acids, conjugated linoleic acid), vitamins (i.e. A and D) and minerals (i.e. calcium and phosphorus) (Claeys et al., 2013; Givens & Kliem, 2009; Özer & Kirmaci, 2010). The major contributors to the antioxidant activity of whole milk and whey proteins are casein and albumin, respectively (Zulueta et al., 2009). The vitamin E, carotenoids and ubiquinol can act as radical scavengers in the lipid phase of milk; the vitamin C is the main antioxidant of the aqueous phase; while some flavonoids have the ability to scavenge radicals in both phases (lipid and aqueous phases) (Lindmark-Månsson & Åkesson, 2000).

Milk is a product consumed in all over the word and is one of the most important vehicles for delivering bioactive substances to consumers (Shukla, Sharma, & Singh, 2003). A number of milk-based beverages are marketed in the United States and the European markets (Özer & Kirmaci, 2010; Zulueta, Esteve, Frasquet, & Frígola, 2007).

Different studies have been carried out to evaluate the stability and healthy properties of these products. Shukla et al. (2003) developed delicious and nutritious beverages by blending juice/pulp of apples, bananas, guavas, litchis and mangos with milk products. The vitamin C, vitamin A, phenolic compounds and antioxidant activity of a fruit juice and skim milk beverage was evaluated by Zulueta, Esteve, Frasquet, & Frígola (2007). The carotenoid composition and color of fruit juice and milk beverages were also studied (Zulueta, Esteve, & Frígola, 2007). Salvia-Trujillo, Morales-De La Peña, Rojas-Graü, & Martín-Belloso (2011) evaluated the changes in water-soluble vitamins and antioxidant activity of fruit juice-milk beverages treated by high-intensity pulsed electric fields.

1.3.3. Soymilk-based beverages

Epidemiological evidence suggest that consumption of soy products have potential health benefits related to cardiovascular diseases, menopausal symptoms, osteoporosis, breast and prostate cancers because they are rich sources of bioactives as phenolic compounds (Devi, Gondi, Sakthivelu, Giridhar, Rajasekaran & Ravishankar, 2009). The popularity of soy-derived products was significantly growing with retail sales in U.S from $300 million in 1992 to more than $4 billion in 2008. Soymilk drove this growth

with retail sales from $2 million in 1980 to $1.06 billion in 2009 (Soyatech, Inc. and Spins 2009).

Basically, soymilk is an aqueous extract of whole soybeans and is an important source of protein, iron and niacin, but it contains low concentration of fat, carbohydrates and calcium compared to cow's and human milks (Jinapong, Suphantharika, & Jamnong, 2008). Moreover, soymilk is cholesterol and lactose free. For these reasons, soymilk is considered an excellent alternative to cow's milk for people with milk protein allergy, lactose intolerance, galactosemia (Xu & Chang, 2009) or for those who avoid eating animal products.

The major phenolic compounds contained in soy-derivates are flavonoids, specifically isoflavones in their glucoside (genistin, daidzin and glycitin) or aglycone forms (genistein, daidzein, and glycitein) (Sanz & Luyten, 2006), while their principal phenolic acids are syringic, chlorogenic, gallic, vanilic and ferulic (Tyug, Prasad, & Ismail, 2010).

Because of the attractive nutritional properties of soymilk, this product has been mixed with fruit juices to obtain beverages with high nutritional value and functional properties. Furthermore, the characteristic beany flavor of soymilk is masked when this product is combined with fruit juices (Potter, Dougherty, Halteman, & Camire, 2007). For these reasons, a great variety of soy-based beverages are commercially available in the market at this time. In fact, Rau de Almeida Callou, Sadigov, Lajolo, & Genovese (2010) analyzed the isoflavone content and the antioxidant activity of beverages available in the Brazilian marked, which contain soymilk and fruit juices (apple, pineapple, guava, passion fruit, mango, peach, orange, strawberry, grape, watermelon, pineapple/mint, peach/tangerine, Swiss lemonade, grape, mango, peach, orange, and papaya/orange). Similarly, Rostagno, Palma, & Barroso, (2007) determined the isoflavones content of soy beverages blended with fruit juices. Potter et al. (2007) reported that flavor of blueberries could mask the beany flavor of soy beverages. Oliveira et al. (2010) evaluated the consumer acceptance of an acai-soymilk beverage, while Valim et al. (2003) analyzed the sensory acceptance of a beverage based on orange juice and soymilk. Morales-De La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2010a; 2010b; 2011) mixed soymilk with a blend of fruit juices (orange, pineapple and kiwi) in order to obtain a beverage with good sensorial characteristics and

high concentration of bioactive compounds (phenolic compounds, isoflavones, vitamin C and carotenoids).

1.3.4. Blended beverages: advantages and disadvantages

Blended beverages are complexes systems that allow interactions between bioactive compounds, nutrients and/or other food constituents. These interactions significantly influence on the structural, textural, nutritional and organoleptic features of these products. Some interactions are desirable and may help in improving quality of beverages, but others are undesirable because adversely affect the quality of foods. For this reason, it is important to take into consideration the interactions among food ingredients when developing functional beverages in order to obtain food products with high nutritional and functional quality.

Advantages: Blended beverages represent a good option to increase the nutritional value of beverages due to the combination of two products with different profile of biologically active substances. Therefore, they could contribute to the intake of a wide range of health-promoting compounds, such as carotenoids, vitamins, phenolic compounds, and minerals (De Carvalho et al., 2007; Morales-de La Peña et al., 2011; Müller, Gnoyke, Popken, & Böhm, 2010; Torregrosa et al., 2006).

In addition, these products could improve the sensorial characteristics of beverages through the combination of different flavors and aromas (Zulueta, Esteve, Frasquet, & Frígola, 2007) or they could mask undesirable flavors, such as that of soymilk.

Another important benefit of blended beverages is the fact that they could provide a better surrounding environment where the less soluble compounds may increase their solubility and/or stability. For instance, it has been reported that riboflavin and folic acid increased their solubility in presence of food matrices containing niacinamide (Berry, 2009). Lipophilic compounds, such as carotenoids, are better solubilised into mixed micelles in food matrices containing certain amount of fat (i.e. whole milk) instead of that of low-fat content (i.e. skimmed milk or soymilk) (Cilla et al., 2012).

Recent researches also suggest that these products could allow synergistic interaction among bioactive compounds in blended fruit juices in comparison to those made with a single fruit. The *in vitro* bioaccessibility of vitamin C was 12.6% in beverages made with a blend of fruit juices and milk (Cilla et al., 2012), whereas in pomegranate juice

just showed 2.5% of bioaccessibility (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002). Moreover, other bioactive substances, such as phenolic compounds, carotenoids, and tocopherols from blended beverages were available for absorption after *in vitro* gastrointestinal digestion of blended beverages (Cilla et al., 2011, 2012; Cilla, González-Sarrías, Tomás-Barberán, Espín, & Barberá, 2009). These results were also corroborated by *in vivo* studies where an increase in the anti-inflamatori, antioxidant and immunologic properties due to the intake of mixed beverages were observed (Jensen et al., 2008; Watzl, Bub, Briviba, & Rechkemmer, 2003).

Disadvantages: Because of the complexity of the medium in which bioactive compounds of blended beverages are immersed, special care should be taken in order to avoid interactions that may cause the instability of these products.

It has been reported that the addition of high concentrations of vitamin C to beverages produces browning, off-flavor and loss of color (Ashurst, 2005). In addition, vitamin C can interact with other vitamins such as B_1, B_2 and B_{12}, yielding instability of beverages (Berry, 2009). Vitamin C is also easily oxidized in presence of metal ions and sulphites (Ball, 2006).

β-carotene is usually added to beverages to provide antioxidant potential and color to foods. However, this lipophilic compound must be incorporated in an emulsion, which may produce turbidity in the beverage (Ashurst, 2005). Similarly, the fiber is an ingredient that precipitates when is incorporated to beverages due to its physical properties. Both turbidity and precipitation affect the visual quality and consistence of beverages (Dikeman & Fahey, 2006; Sun-Waterhouse et al., 2010).

The incorporation of minerals to beverages is easily achieved but particular care must be taken because the high concentration of minerals may change the nutritional, physical and sensorial features of foods. Among these changes stand out laxatives effects, undesirable flavors (such as salty, metallic or astringent), or reduced absorption of nutrients (Ashurtst, 2009; Berry, 2009; Givens & Kliem, 2009). Moreover, if the pectin components of juices have been degraded, calcium may be linked to the residual pectin acids leading to the formation of a gel (Ashurst 2009).

1.4. Beverages processing

Consumers demand food products that provide variety, convenience, adequate shelf life, nutritional quality and fresh-like appearance. For this reason, food processing has become more sophisticated and diverse to meet such demands (Barbosa-Cánovas, Tapia, & Cano 2005).

Thermal processing is the commonest method to inactivate enzymes and destroy microorganisms from foods and beverages. However, this treatment may cause undesirable biochemical and nutritious changes to the final product (Ortega-Rivas & Salmerón-Ochoa, 2014). Non-thermal food preservation technologies have been developed as alternatives to heat treatments in order to obtain products that maintain their nutritional and sensorial attributes as unchanged as possible.

1.4.1. Thermal treatment (TT)

Food have been traditionally preserved by thermal treatment (TT) for a number of reasons, the main one being to avoid microorganism spoilage or contamination with pathogens that can cause severe toxinfections in humans who consume them (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013). It may also be important to inactivate enzymes, to avoid the browning of fruit juices by polyphenol oxidases and minimise flavour changes resulting from lipase and proteolytic activity.

Foods which are thermally treated can be either solid or liquid. Thermal processes vary considerably in their intensity, ranging from mild process (i.e. thermisation and pasteurisation) through to more severe conditions (sterilisation). Treatment conditions depend on factors such as the type of product, the pH and the sugar content, among others. Food should be heated and cooled as quickly as possible. The heating medium is usually saturated steam or hot water, whereas cooling is achieved using mains water, chilled water, or glycol solution (Lewis, 2006). The Food and Drug Administration (2004) suggests that thermal process requires treatments of at least 71.1ºC during 3 seconds for achieving five logarithmic reductions for *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*. Currently, fruit juices are thermally treated at temperatures from 90 to 95 ºC for 15 to 20 seconds (Aguiló-Aguayo, 2010).

The process of heating a food also induces physical changes and chemical reactions, which can degrade the flavor, color, volatile compounds (aroma), and nutritional value of foods (losses of water-soluble vitamins and carotenoids) to varying degrees (Hjelmqwist, 2005). At the same time, the high temperature increases the rate of non-enzymatic browning reactions (Millard reaction, sugar caramelization and ascorbic acid oxidative reaction), as well as the starch gelatinisation and protein denaturation, resulting in a decrease in the sensorial and nutritional features of beverages.

1.4.2. Non-thermal technologies

Non-thermal technologies are currently undergoing extensive research with the aim to produce food products that are better in quality than heat-treated foods, which either decrease the processing cost (they utilize less energy than thermal technology) or add value to the product (Barbosa-Cánovas, Swanson, San Martín, & Harte, 2005).

Food can be non-thermally treated by irradiation, high hydrostatic pressure, electrical methods (pulsed electric fields, light pulses and oscillating magnetic fields), ultrasound, micro and ultrafiltration, among others (Zhang et al., 2011). In these technologies the temperature is not the main factor for inactivating microorganism and enzymes. In fact, there is only a slight increase in the temperature in most of these processing methods (Raso & Barbosa-Cánovas, 2003). Each technology has specific applications depending on the type of food processed.

The most appreciated non-thermal technologies are high-intensity pulsed electric fields (HIPEF) and high-pressure processing (HPP) due to they inactivate microorganisms and enzymes without compromise the nutritional and sensorial quality of food (Odriozola-Serrano et al., 2013; Sánchez-Moreno, De Ancos, Plaza, Elez-Martinez, & Cano, 2009). For this reason, non-thermal food preservation technologies are considered as an alternative to thermal treatments for obtaining highly nutritional and functional foods and beverages.

1.4.2.1. High-intensity pulsed electric fields (HIPEF)

HIPEF is one of the most relevant non-thermal processing technologies available for food processing because of its potential to inactivate enzymes without compromise the organoleptic and nutritional features of food (Barbosa-Cánovas & Sepúlveda, 2005).

HIPEF is based on the properties of liquid food to conduct electricity due to their high concentration of ions and to their capability to transport electric charges. The application of an external electric field on biological cells (animal, plant or microbial) led to the disruption of cell membrane and this phenomenon is usually know as electroporation (permeabilisation of the cell membrane). Irreversible electroporation induces microbial inactivation, facilitates the extraction of certain constituents and/or increases drying rates. Additionally, HIPEF can also induce stress reactions in plant systems or cell cultures, resulting in an enhancement in the bioproduction of specific compounds (Soliva-Fortuny, Balasa, Knorr, & Martín-Belloso, 2009).

The effectiveness of HIPEF processing depends on factors coming from the parameters related to the treatment, as well as the intrinsic features of the product. Among the factors related to the treatment stand out pulse frequency, pulse width, polarity, treatment time, and field strength. On the other hand, electrical conductivity, pH, ionic strength, viscosity, content of nutrients, and suspended particles are among the intrinsic characteristic of the product that determine the effectiveness of HIPEF treatment.

HIPEF equipment consisted of a pulsed electric field generator unit, which is composed of a high voltage generator and a pulse generator, a treatment chamber, a product handling system, and a set of monitoring and controlling devices. HIPEF processing consists in the application of pulses at high voltage (above 20 kV/cm) and short duration (ms or μs) to liquid food placed in a treatment chamber confined between electrodes (Odriozola-Serrano et al., 2013).

1.4.2.2. High pressure processing (HPP)

HPP is based on the use of pressure to compress food located inside a pressure vessel. The applied pressure is isostatically transmitted by a fluid (Pascal's law), meaning that pressure is instantaneously and uniformly transmitted throughout the food regardless of the size or shape of the sample (Norton & Sun, 2008).

HPP is also based on both Le Chatelier's principles, which governed the effect of HPP on food chemistry and microbiology. This principle suggests that when a system at equilibrium is disturbed, the system then responds in a way that tends to minimize the disturbance (Leadly & Williams, 1997). Thus, pressure favors all structural reactions

and changes that involve a reduction of volume, but opposes reactions that involve an increase in volume (Kadam, Jadhav, Salve, & Machewad, 2012; Norton & Sun, 2008).

HPP can be generated by direct or indirect compression, or by heating the pressure medium. In overall, the pressure level ranges from 100 to 600 Mpa and the pressure-transmitting medium is usually water (Welti-Chanes et al. 2005). The treatment chamber is loaded with the product and closed, then the pressure-transmitting medium is placed and degassed and the pressure is generated through a pump. The presence of air in the food increases the time of pressurization. Although HPP is a non-thermal food preservation technology, the pressure increase led to a small adiabatic rise in temperature (Chawla, Patil, & Singh, 2011).

1.5. Food matrix and processing influence on the bioaccessibility of bioactive compounds from functional beverages

Food matrix and processing are among the main factors that are able to modify the bioaccessibility of the bioactive compounds contained in food (Parada & Aguilera, 2007; Rein et al., 2013). Therefore, these factors should be taken into consideration when new functional foods and beverages are designed and developed.

1.5.1. Food matrix

Food and beverages are complexes systems in which interactions among bioactive compounds and other food ingredients (i.e. pectins, minerals, proteins, fat, fiber) establish the structural, textural, organoleptic, nutritional and functional properties of foods. Some of these interactions could be desirable and improve quality of food but others could produce the adverse effect. Therefore, selection of the most suitable ingredients for improving the bioaccessibility and bioavailability of bioactive substances must be one of the main steps in the design and development of functional beverages.

The solubility of bioactive compounds in gastric and intestinal fluids influences their rate of absorption. Cassidy (2006) found that the absorption rate of isoflavones in soymilk was faster compared with solid soy food matrices likely because isoflavones in soymilk are mainly hydrophilic β-glycoside conjugates and thus, highly solubilized in

the intestine. In addition, the stomach-emptying of solid foods occurs later with respect to that of liquid food matrices. Zubik & Meydani (2003) reported that the peak serum concentration of daidzein and genistein occurred about 2 h later for solid soy food in comparison to soymilk. As a result, a liquid matrix yields a faster absorption rate and higher peak plasma concentration of isoflavones than solid matrices. It has been also reported that the intestinal absorption of fruit juices bioactive compounds is even better than that coming directly from fruits (Perales, Barberá, Lagarda, & Farré, 2008). Thus, the type of food matrix (solid or liquid) significantly influences the bioaccessibility and bioavailability of bioactive compounds.

García-Nebot, Alegría, Barberá, Clemente, & Romero (2010) performed a study to assess the influence of milk based fruit beverages (grape concentrate, orange concentrate and apricot puree) upon iron bioavailability. These authors found that the addition of milk to fruit beverages improved iron retention, transport and uptake versus fruit beverages (García-Nebot et al., 2010).

Recent researches suggest that the *in vitro* bioaccessibility of many bioactive compounds contained in blended beverages can be enhanced by changes in the food matrix. For instance, Cilla et al. (2012) reported that beverages based on whole milk and fruit beverages showed higher *in vitro* bioaccessibility of carotenoids (β-carotene, β-cryptoxanthin, zeinoxanthin, lutein and total carotenoid content) in comparison to those beverages containing soy or skimmed milk. On the other hand, *in vivo* studies reported an increase in the anti-inflammatory, antioxidant and immunologic properties due to the intake of mixed beverages (Jensen et al., 2008; Watzl et al., 2003), suggesting that these food matrices allow synergistic interactions among the bioactive compounds and food constituents.

1.5.2. Processing influence

The analysis of the effect of food processing on the concentration of bioactive compounds contained in beverages has been widely evaluated. However, studies focused on evaluating the influence of these technologies on the bioaccessibility of nutrients are really scarce.

Processing had a significant influence on the bioaccessibility of bioactive compounds from beverages (Maiani et al., 2009), mainly through changes in the natural matrix of food (i.e. pH, viscosity, etc.) or in their microstructure (i.e. cell walls rupture, release of

bounded compounds, changes in their solubilisation, etc.). These changes could modify the release, transformation and absorption of some nutrients during their digestion (Parada & Aguilera, 2007). As an example, the cell wall represents an important barrier for releasing bioactive compounds and it is resistant to degradation in the gastrointestinal tract but not to some treatments types (Parada & Aguilera, 2007).

Processing also may led interactions among food compounds, resulting in altered food quality, color and flavor changes, as well as it modify the functionality and bioavailability of certain compounds. For instance, processing induced interactions between proteins, resulting in the unavailability of certain amino acids. Processing of soy-derived foods improves its digestibility and destroys much of the inhibitors, but at the same time may lead to the formation of unnatural amino acids such as fructosyllysine, lysinoalanine and D-amino acids (Kilara, 2006).

Thermal treatment is a technology widely used to achieve microbial and enzymatic inactivation, as well as to extend the shelf life of juices, but this technology could promote the release of nutrients due to cell rupture and/or cell separation (Wollstonecroft, Ellis, Hillman, & Fuller, 2008). The cell rupture occurs when the plant tissue is disrupted by the breakage of the cell wall, exposing their content. Typically, crunchy fruits and uncooked vegetables where the cell-cell adhesion is very strong follow this pattern (cell rupture). On the other hand, cell separation occurs when cells become detached from each other because the cell-cell adhesion is weak and there is a depolymerisation and/or dissolution of the cell wall polymers, therefore the tissue is disrupted along the plane of the middle lamella. Cell separation is usually the result of the softening of plant tissue by thermal processing.

It has been suggested that thermal processing could enhance the bioavailability of carotenoids, thiamine, vitamin B-6, niacin, and folate by releasing them from the food matrix (Maiani et al., 2009). Thermal treatment, affects the subcellurar structure in which carotenoids are located, thus the release and solubilisation of these compounds could be improved (Paetau, Chen, Goh, & White, 1998). Nevertheless, it is not clear whether thermal processing could also favour the bioaccessibility of carotenoids in liquid food and beverages.

Because of the nutritional and sensorial properties of food may be affected by the high temperatures reached during thermal treatment. Non-thermal food preservation technologies, such as high-intensity pulsed electric fields (HIPEF) and high-pressure

processing (HPP) have been developed as alternatives to thermal treatments. Interestingly, really few studies concerning the bioavailability and bioaccessibility of bioactive compounds from processed beverages by non-thermal technologies have been done at this time. In this context, Sánchez-Moreno et al. (2003, 2004) evaluated the effect of the consumption of high-pressurized or pulsed electric fields-processed orange juice on plasma vitamin C, antioxidative status and inflammatory markers in healthy humans. Cilla et al. (2012) found that HPP diminished significantly the *in vitro* bioaccessibility of vitamin C in both milk and soymilk beverages, while it increased in TT beverages. In contrast, the same authors reported that the *in vitro* bioaccessibility of zeaxanthin, lutein, zeinoxanthin, and β-cryptoxanthin in HPP and TT beverages in whole and skimmed milk diminished in comparison to their respective control beverages.

1.6. Final remarks

Development of new functional products is generally driven either by consumer demand or by advances in science and technology (Sun-Waterhouse, 2011). A great deal of information is available in the literature concerning the bioactive composition of functional beverages. However, it is essential to take into consideration that functional attributes of food is not only a matter of nutritional composition. The most important feature is to know the proportion of these compounds that is available to exert their biological function because from the amount of ingested compound only a small fraction is absorbed and utilized by the human body. Thus, bioaccessibility could provide more specific information concerning dietary requirements of bioactive compounds to achieve health benefits beyond recommended dietary patterns reported at this time.

Bioaccessibility is an essential first step for better understanding the biological activity of compounds contained in foods and beverages. It is also important assessing the impact of factors, such as food matrix and food processing, on the bioaccessibility of these compounds to choose the most suitable matrices and processing technologies to get a better functionality of foods and beverages.

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2. OBJECTIVES

The general objective of the present doctoral thesis was to evaluate the *in vitro* bioaccessibility of health-related compounds from beverages based on fruit juices, milk or soymilk, as well as to analyze the influence of the food matrix and processing technology on the bioaccessibility of these compounds. Aiming to achieve the main goal, the following specific objectives were proposed:

1. Evaluation of the changes in the concentration of hydrophilic (vitamin C, phenolic compounds and isoflavones) and lipophilic compounds (carotenoids), as well as in the antioxidant activity of milk (M), soymilk (SM), blended fruit juices (BFJ_1 containing orange, kiwi and pineapple juices; and BFJ_2 containing orange, kiwi, pineapple and mango juices) and in blended beverages (BFJ_1-SMB and BFJ_2-MB) during an *in vitro* gastrointestinal digestion (gastric digesta, small intestinal digesta, dialyzed and micellar fractions).

2. Study of the bioaccessibility of hydrophilic and lipophilic compounds from milk (M), soymilk (SM), blended fruit juices (BFJ_1 and BFJ_2) and mixed beverages (BFJ_1-SMB and BFJ_2-MB).

3. Assessment of the effect of the food matrix (water, milk and soymilk) on the *in vitro* bioaccessibility of hydrophilic and lipophilic bioactive compounds from beverages based on a blend of fruit juices (orange, pineapple, kiwi and mango).

4. Evaluation of the influence of the food processing (high intensity pulsed electric fields (HIPEF); high pressure processing (HPP); and thermal treatment (TT)) on the *in vitro* bioaccessibility of hydrophilic and lipophilic bioactive compounds from beverages based on a blend of fruit juices (orange, pineapple, kiwi and mango) and water (WB), milk (MB) or soymilk (SB).

3. MATERIALS AND METHODS

The experimental design carried out in the present research is represented in Figure 1.

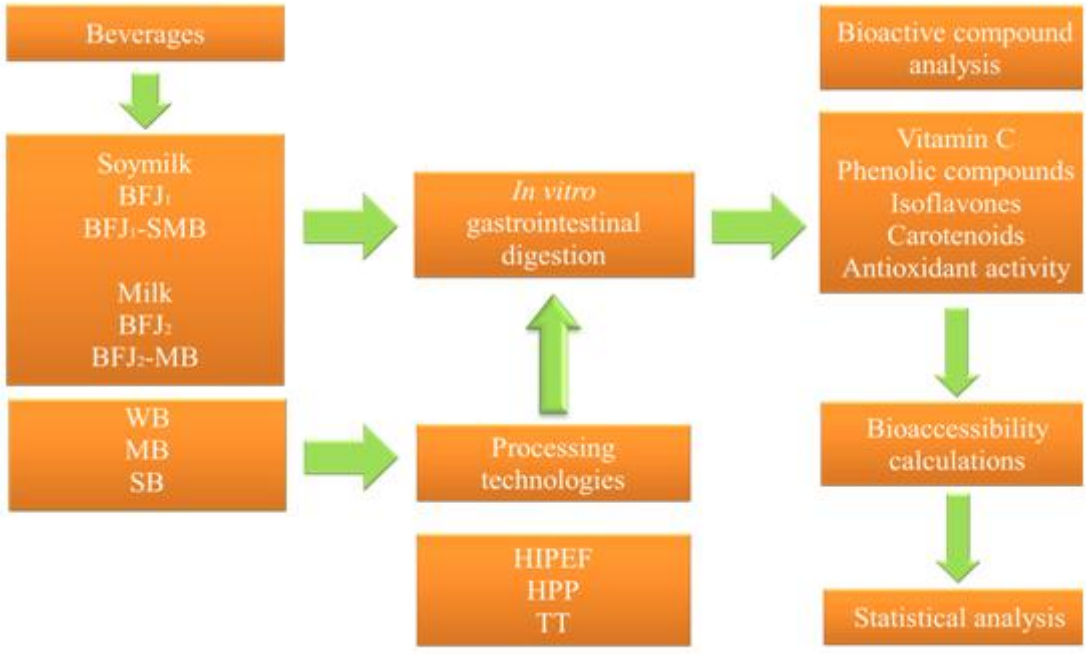

Figure 1. General scheme of experimental methodology. BFJ_1, blended fruit juice containing orange, kiwi and pineapple juices; BFJ_2, blended fruit juice containing orange, kiwi, pineapple and mango juices; BFJ_1-SMB, blended beverage made with a blend of fruit juices and soymilk; BFJ_2-MB, blended beverage made with a blend of fruit juices and milk. WB, water-fruit juice beverage; MB, milk-fruit juice beverage; SB, soymilk-fruit juice beverage. HIPEF, high-intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.

3.1. Materials

Pepsin from porcine stomach, pancreatin from porcine pancreas, bovine bile, DL-1,4-dithiothreitol, metaphosphoric acid, phenol standards (caffeic, chlorogenic, p-coumaric, ferulic, sinapic and 4-hydroxybenzoic acids; hesperidin, naringenin, rutin, quercetin and [+]-catechin), carotenoid standards (α-carotene, β-carotene, zeaxanthin, lutein, α-cryptoxanthin and β-cryptoxanthin), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and cellulose dialysis membrane (molecular weight cutoff of 12,000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid and Folin-Ciocalteu (F-C) reagent were acquired from Scharlau Chemie S.A. (Barcelona, Spain). Ultrafiltration devices (Amicon Ultra 3 K) and 0.2 μm membranes were bought from Millipore Iberica S.A. (Madrid, Spain).

3.2. Beverages

3.2.1. Soymilk and milk

Soymilk (Yosoy, Girona, Spain) and whole cow's milk (Hacendado, Córdoba, Spain) were acquired at a local supermarket. According to manufacturer, soymilk composition was 3.6 g of protein, 0.7 g of carbohydrates, 1.8 g of fat and 1 g of fiber in 100 mL; whereas milk contained 3.6% of fat, 3.0% of protein and 4.5% carbohydrates. The pH (Crison Instruments S.A., Alella, Barcelona, Spain) and the soluble solids content (Comecta S.A., Abrera, Barcelona, Spain) of each product were determined, resulting in 7.34 ± 0.02 and 5.83 ± 0.14 ºBrix, respectively, for soymilk; and 6.75 ± 0.06 and 11.83 ± 0.29 ºBrix for milk.

3.2.2. Blended fruit juices

Fruits (orange, kiwi, pineapple and mango) were purchased at commercial maturity in a local supermarket. Each fruit was washed, peeled and juice extracted. Afterwards, every juice was filtered with cheesecloth using a vacuum pump.

Two blended fruit juices (BFJ_1 and BFJ_2) were manufactured following the proportions displayed in Table 1. The pH (Crison Instruments S.A., Alella) was 3.40 ± 0.04 and 3.38 ± 0.04 for BFJ_1 and BFJ_2, respectively. The soluble solids content (Comecta S.A.) was 10.75 ± 0.01 ºBrix for BFJ_1 and 11.00 ± 0.10 ºBrix for BFJ_2.

Table 1. Proportions used to manufacture blended fruit juices.[a]

	BFJ_1 (%)	**BFJ_2 (%)**
Orange juice	50	40
Kiwi juice	36	33
Pineapple juice	14	13.5
Mango juice	-	13.5

[a] BFJ_1, blended fruit juice containing orange, kiwi and pineapple juices; BFJ_2, blended fruit juice made with orange, kiwi, pineapple and mango juices. % means the percent by weight of each fruit juice.

3.2.3. Blended fruit juices and soymilk or milk beverages

Two blended beverages based on soymilk (BFJ_1-SMB) or milk (BFJ_2-MB) were manufactured as follows:

The blended fruit juice-soymilk beverage (BFJ_1-SMB) was prepared by mixing 50.0% of BFJ_1 (see table 1), 42.5% of soymilk (SM) and 7.5% of sugar. Finally, the pH of the beverage was adjusted to 3.7 with citric acid (Crison Instruments SA). The soluble solids content was measured in a refractometer (Comecta S.A.), resulting in 15.0 ± 0.12 ºBrix. The formulation of this beverage was selected based on a previous study where the combination of these fruit juices with soymilk displayed high concentrations of isoflavones (Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2010).

The blended fruit juice-milk beverage (BFJ_2-MB) was manufactured by mixing 75% of BFJ_2 (see table 1), 17.5% of milk and 7.5% of sugar. The pH of BFJ_2-MB was adjusted to 3.3 ± 0.05 with citric acid if necessary, and the soluble solid content was assessed (17.5 ± 0.03 ºBrix). The formulation of the beverage was selected based on a previous study where the combination of these fruit juices with milk displayed a high concentration of vitamin C, as well as antioxidant activity and stability (Salvia-Trujillo, Morales-de la Peña, Rojas-Graü, & Martín-Belloso, 2011a).

3.2.4. Blended beverages

Three different beverages were prepared following the proportion reported in Table 2. The pH of each beverages was adjusted to 3.30 ± 0.20 (Crison Instruments S.A.) with citric acid and the soluble solids content was determined (Comecta S.A.), resulting in 18.0 ± 0.2, 19.3 ± 0.3, 18.5 ± 0.2, ºBrix for WB (water-fruit juice beverage), MB (milk-fruit juice beverage) and SB (soymilk-fruit juice beverage), respectively.

Table 2. Proportions used to manufacture blended beverages.[a]

	WB (%)	MB (%)	SB (%)
Orange juice	40	40	40
Kiwi juice	33	33	33
Pineapple juice	13.5	13.5	13.5
Mango juice	13.5	13.5	13.5
Distilled water	17.5	-	-
Milk	-	17.5	-
Soymilk	-	-	17.5
Sugar	7.5	7.5	7.5

[a] WB, water-fruit juice beverage; MB, milk-fruit juice beverage; SB, soymilk-fruit juice beverage. % means the percent by weight of each ingredient.

Beverages formulations were selected according to a previous study, in which a high concentration of bioactive compounds (mainly vitamin C) in a similar milk-based beverage was reached (Salvia-Trujillo et al., 2011a).

3.3. Processing technologies

3.3.1. High-intensity pulsed electric fields (HIPEF)

HIPEF treatment was performed in a continuous-flow bench scale system (OSU-4F, The Ohio State University, Colombus, OH, USA), using square-wave pulses. Eight collinear chambers serially connected were used as treatment system. Each chamber consisted of two stainless steel electrodes separated by a gap of 0.29 cm. The flow rate was adjusted to 60 mL/min and controlled by a variable speed pump (model 752210-25, Cole Palmer Instrument Company, Vermon Hills, IL, USA). Beverages were HIPEF treated at 35 kV/cm field strength in bipolar mode, 4-μs pulse width, 200 Hz pulse frequency and 1800 μs total treatment time. Temperature was kept below 35 ºC using a cooling coil connected before and after each pair of chambers and submerged in an ice-water shaking bath. HIPEF conditions were selected based on previous studies, where the nutritional and microbiological stability of similar beverages was accomplished (Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2011; Salvia-Trujillo, Morales-de la Peña, Rojas-Graü, & Martín-Belloso, 2011b).

3.3.2. High-Pressure processing (HPP)

Beverages were treated in a high hydrostatic pressure unit with a vessel of 2925 mL capacity, a maximum pressure of 900 MPa, and a maximum temperature of 100 ºC (High Pressure Iso-Lab System, Model FPG7100:9/2C, Stansted Fluid Power LTD., Essex, UK). Previously to HPP, all three beverages were vacuum packed in flexible Doypack bags (300 mL). Afterwards, they were introduced in the pressure unit filled with pressure medium (water). Beverages were processed at 400 MPa with a holding time of 5 min. The rates of compression and decompression were both 3 MPa/s. Because of adiabatic compression, the maximum temperature in the vessel was 40 ºC at 400 MPa. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the process. These conditions were selected

based on previous studies where the nutritional and microbiological stability of HPP fruit juices and similar beverages were achieved (Muñoz, De Ancos, Sánchez-Moreno, & Cano, 2007; Sánchez-Moreno et al., 2005).

3.3.3. Thermal Treatment (TT)

A tubular stainless-steel heat exchanger coil immersed in a hot water shaking bath was used to treat beverages by heat (University of Lleida, Spain). The flow rate of beverages was maintained through a gear pump. Beverages were thermally treated at 90 ºC for 60 s. Once processed, beverages were immediately cooled down to 5 ± 1 ºC in a heat exchanger coil immersed in an ice-water bath.

3.4. In vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion was carried out following the methodology described by Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera (2004), with some modifications. Beverages were digested in two sequential phases: gastric and small intestinal digestions with dialysis. Additionally, a micellar fraction was obtained from digesta following the completion of the small intestinal phase.

To monitor the changes in bioactive compounds concentration along the *in vitro* gastrointestinal digestion of beverages, aliquots were taken at the end of each digestive phase and immediately placed in a cold water bath during 10 min and frozen (-45 ºC) until analysis.

3.4.1. Gastric digestion

A portion of 200 mL of beverages and 0.2 g of pepsin were mixed in a beaker. Afterwards, the pH was immediately adjusted to 2 by addition of 12 M HCl and the mixture was incubated at 37 ºC and 90 rpm during 2 h (incubation chamber with orbital agitation Ovan, Badalona, Spain).

3.4.2. Small intestinal digestion with dialysis

Segments of cellulose dialysis membrane were cut at 12 cm of length and filled with 25 mL of water-$NaHCO_3$ (0.5 N) mixture. The required amount of $NaHCO_3$ (0.5 N) to

titrate the gastric digesta to pH 7.5 was that contained in the dialysis membrane. Every 20 mL of gastric digesta were placed into a polyethylene tube and a dialysis membrane was completely immersed until reaching pH 5.0. Later, 5 mL of pancreatin (4 g/L) and bile (25 g/L) mixture was added to each tube and incubated during 2 h at 37 ºC and 90 rpm.

The dialysis membrane was removed, rinsed with distilled water and weighed once the small intestinal digesta was over. Bioactive compounds of small intestinal digesta corresponded to those contained outside the dialysis membrane. Bioaccessible hydrophilic compounds (vitamin C, phenolic compounds and isoflavones) were obtained from the dialyzed fraction (inside the dialysis membrane).

3.4.3. Micellar fraction

Micellar fraction, containing the bioaccessible carotenoids, was obtained from the supernatants of the small intestinal digesta (Granado-Lorencio et al., 2007). For this reason, a portion of 30 mL of the small intestinal digesta was centrifuged at 5000 rpm during 20 min at room temperature (Centrifuge AVANTI™ J-25; Beckman Instruments Inc., Fullerton, CA, USA).

3.5. Bioactive compound analysis

3.5.1. Vitamin C

The extraction of vitamin C was performed according to the method validated by Odriozola-Serrano, Hernández-Jover, & Martín-Belloso (2007) with some modifications. Non-digested or digested beverages (5 mL) were mixed with 5 mL of a solution containing 45 g/L metaphosphoric acid and 7.2 g/L of dithiothreitol. After that, samples were filtered in 0.20 μm membrane. The filtrate was added into ultrafiltration devices and centrifuged at 6000 rpm during 30 min at 4 ºC (Centrifuge AVANTI™ J-25; Beckman Instruments Inc.) in order to purify samples.

The HPLC system consisted of a 600 Controller, a 486 absorbance detector and a reverse-phase C18 Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm × 250 cm) (Waters Corporation, Milford, MA, USA). A 0.01% sulfuric acid solution adjusted to pH 2.6 was used as mobile phase and the flow rate was fixed to 1 mL/min at room

temperature. Detection was performed at 245 nm. The identification of vitamin C was performed by comparing the retention time and the UV-visible absorption spectrum of samples with the standard (ascorbic acid). Quantification was carried out by integrating the peak areas and using a calibration curve (R^2=0.9989, concentration in the range of 10 to 1000 mg/L) Results were expressed as mg of ascorbic acid /100 mL of sample.

3.5.2. Individual phenolic compounds

Phenolic profile was determined by HPLC, using the methodology reported by Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso (2008), slightly modified. A portion of 5 mL of non-digested or digested beverages was mixed with 5 mL of 62% aqueous methanol and 1 mL of 6 M HCl. The mixture was refluxed at 90 °C for 2 h, cooled and diluted to 25 mL with methanol. Finally, the extracts were sonicated 3 min, filtered (0.20 µm membrane) and frozen (-45 ºC) until HPLC analysis.

The HPLC system was equipped with a 600 Controller and a 2996 diode array detector (Waters Corporation, Milford, MA, USA) which was set to scan from 200 to 600 nm. Separations were performed on a reverse-phase C18 Spherisorb ODS2 (5 µm) stainless steel column (4.6 mm x 250 mm) operating at room temperature with a flow rate of 1 mL/min. A gradient elution was employed with a mixture of two solvents: (A) 2.5% of acetic acid in water and (B) 2.5% of acetic acid in methanol (Odriozola-Serrano et al., 2008). Each phenolic compound was identified by comparing its retention time and spectrum with the standards. Quantification of individual phenols was carried out integrating the peak areas and using calibration curves (R^2 in the range of 0.9978 to 0.9999, concentration from 5 to 500 mg/L). Results were expressed as mg of phenolic compound/100 mL of sample. Total phenolic compounds were calculated as the sum of individual phenolic compounds identified by HPLC (TPC by HPLC).

3.5.3. Total phenolic content

Total phenolic content was determined through a modified version of the methodology reported by Singleton, Orthofer, & Lamuela-Raventós (1998), using the Folin-Ciocalteu (F-C) reagent. A portion of 0.5 mL of non-digested or digested beverages was mixed with 0.5 mL of F-C reagent and 10 mL of Na_2CO_3 (20 %). The mixture was made up to 25 mL with distilled water and kept in the dark during 1 h at room temperature. Afterwards, the samples were filtered using a 0.2 µm membrane and

the absorbance was measured at 725 nm (Cecil Instruments Ltd., Cambridge, UK). Concentrations of total phenolic compounds determined by Folin-Ciocalteu (TPC by F-C) methodology were determined by comparing the samples absorbance with a calibration curve built with gallic acid (R^2=0.9990, concentration from 50 to 2000 mg/L). Results were expressed as mg of gallic acid/100 mL of sample.

3.5.4. Isoflavones

Isoflavones were analyzed according to the method of Morales-de la Peña et al. (2010), with some modifications. An aliquot of 4 mL of non-digested or digested beverages was mixed with 4 mL of 80% ethanol acidified to 1 M with HCl. Then, this mixture was incubated at 80 °C for 1 h. After incubation, samples were cooled, shaken vigorously for 2 min and centrifuged at 9,000 rpm during 10 min at 4 °C (Centrifuge AVANTI™ J-25; Beckman Instruments Inc.). Supernatant was decanted into 10 mL volumetric flask and the residue was re-extracted with 2 mL of 80% ethanol and centrifuged under the same conditions previously described. Both supernatants were combined into a volumetric flask, which was filled up to 10 mL with 80% ethanol. The samples were filtered through 0.2 μm membrane and kept at -45 °C until HPLC analysis.

HPLC system was equipped with a 600 Controller and a diode array detector (Waters Corporation, Milford, MA, USA) which was set to scan from 200 to 350 nm. Separations were performed using a C18 SunFire (3.5 μm) stainless steel column (3 mm x 150 mm) and a C18 SunFire (5 μm) guard column, operating at 37 ºC with a flow rate of 0.3 mL/min. A gradient elution was employed with a mixture of two solvents: (A) water/methanol (80:20 v/v) and (B) water/methanol/acetonitrile (40:40:20 v/v/v) (Morales-de la Peña et al., 2010). Isoflavones were identified by comparing their spectrum and retention time with the standards. Quantification of isoflavones was carried out by integrating the peak areas and using calibration curves (R^2 in the range of 0.9921 to 0.9994, concentration from 0.5 to 200 mg/L). Results were expressed as mg of isoflavone/100 mL of sample. Total isoflavone concentration was calculated as the sum of individual isoflavones determined by HPLC.

3.5.6. Carotenoids

Carotenoids were extracted following the methodology previously reported by Morales-de la Peña et al. (2011), slightly modified. Non-digested or digested beverages (6 mL) were mixed with 0.01 g of magnesium hydroxide carbonate, 0.01 g butylhydroxytoluene (BHT) and 15 mL ethanol/hexane solution (4:3 v/v) in an amber round-bottom flask under N_2 atmosphere and continuous agitation during 45 min. Afterwards, the mixture was filtered using a low ash filter paper 70 mm (Albert-Hahnemuehle, S.L.U., Barcelona, Spain) and the residue was washed and again filtered once with 10 mL of ethanol/hexane solution (4:3 v/v), twice with 5 mL of ethanol and once with 5 mL of hexane. All the filtrates were combined and washed with 10 mL of distilled water and 10 mL of 10% NaCl solution in an amber decanting funnel, discarding the aqueous phase each time. The organic phase was roto-evaporated at 40 ºC until dryness. Then, the residue was saponified with 5 mL of methanolic KOH 0.5 M + 0.1% of BHT (v/w) and 5 mL of diethyl ether, under N_2 atmosphere during 30 min. Later, 5 mL of diethyl ether were added and the solution was washed with 10 mL of distilled water and 10 mL of 10% NaCl solution. The organic phase was mixed with 5 mL of ethanol and roto-evaporated at 45 ºC until dryness. The residue was dissolved with 4 mL of diethyl ether and placed in an amber glass vial. Finally, the solvent was evaporated under N_2 atmosphere and stored (-45 ºC) until analysis.

The HPLC system was equipped with a 600 Controller and a 2996 diode array detector (Waters Corporation, Milford, MA, USA) which was set to scan from 200 to 600 nm. Carotenoids were separated using a reverse-phase C18 Spherisorb ODS2 (5 µm) stainless steel column (4.6 mm x 250 mm) operating at 30 ºC with a flow rate of 1 mL/min. A gradient elution was carried out to separate these compounds (Morales-de la Peña et al., 2011). The mobile phase consisted of four eluents: (1) methanol/ammonium acetate 0.1 M, (2) milli-Q water, (3) methyl tert-butyl ether and (4) methanol. Individual carotenoids were identified by comparing their retention time and spectrum with the standards and/or those reported in the literature. Carotenoid quantification was carried out by integrating the peak areas and using calibration curves (R^2 in the range of 0.9961 to 0.9995, concentration from 0.1 to 50 mg/L). Results were expressed as µg of carotenoid/100 mL of sample. Total carotenoid concentration was calculated as the sum of individual carotenoids determined by HPLC.

3.6. Hydrophilic and lipophilic antioxidant activity

A portion of 5 mL of sample and 10 mL of methanol were mixed and centrifuged at 6,000 rpm for 20 min at 4 ºC (Centrifuge AVANTI™ J-25; Beckman Instruments Inc.). The supernatant was considered as the hydrophilic fraction, whereas the residue was mixed with 10 mL of tetrahydrofuran and centrifuged in the same conditions described above. The second supernatant was considered as the lipophilic fraction.

The antioxidant activity was evaluated using the colorimetric method reported by Brand-Williams, Cuvelier, & Berset (1995), which is based on the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) assay. Aliquots of 0.2 mL of hydrophilic or lipophilic extracts were mixed with 3.8 mL of $DPPH^{\bullet}$ methanolic solution (0.025 g/L). The homogenate was shaken vigorously and kept in the dark for 30 min. Later, the absorbance was measured at 515 nm against a blank of methanol. Results were expressed as percentage of $DPPH^{\bullet}$ inhibition (Eq. 1), which can be related to the decrease in absorbance with respect to the control (methanolic solution of $DPPH^{\bullet}$ radical without extract).

$$DPPHinhibition(\%) = 100x\left(\frac{A_0 - A_1}{A_0}\right) \quad \text{Eq. 1}$$

where A_0 is the absorbance of control (methanolic solution of $DPPH^{\bullet}$ radical without extract) and A_1 is the absorbance of sample extract (non-digested or digested beverages).

3.7. Bioaccessibility calculations

Bioaccessibility was determined through Eq. 2. Results were expressed as percentage.

$$Bioaccessibility(\%) = 100x\left(\frac{BC_{digested}}{BC_{non-digested}}\right) \quad \text{Eq. 2}$$

where $BC_{digested}$ corresponded to the bioactive compound concentration in the digested beverage (dialyzed fraction for vitamin C, phenolic compounds and isoflavones; micellar fraction for carotenoids). $BC_{non\text{-}digested}$ was the concentration of these compounds in the non-digested beverage.

3.8. Statistical analysis

The *in vitro* gastrointestinal digestion of each beverage was conducted in duplicated. Bioactive compounds contained in gastric digesta, small intestinal digesta, dialyzed fraction and micellar fraction were extracted and analyzed three times in each digestion for experiments addressing the stability of these constituents during the *in vitro* gastrointestinal digestion. Bioactive compounds were extracted and analyzed two times from two independent experiments studying the influence of the food matrix and processing.

Analysis of variance (ANOVA) followed by the least significant difference test (LSD) were applied to the results obtained to verify whether there were significant differences ($p < 0.05$) in the concentration and bioaccessibility of bioactive compounds from beverages in relation to the factors studied in this doctoral thesis (*in vitro* gastrointestinal digestion, food matrix and processing). All statistical analyses were performed with the program Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD, USA). Results were reported as the mean ± standard deviation.

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4. PUBLICATIONS

CHAPTER I

Soymilk phenolic compounds, isoflavones and antioxidant activity as affected by *in vitro* gastrointestinal digestion

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Food Chemistry (2013) 136: 206–212

Abstract

The aim of this research was to evaluate changes in the phenolic compounds, isoflavones and antioxidant activity of soymilk following *in vitro* gastrointestinal digestion (including dialysis). Gastric digestion significantly influenced the release of bioactive substances from the soymilk matrix, increasing the con-centration of total phenolic components (35% as the sum of individuals and 14% by Folin–Ciocalteu [F–C] method), total isoflavone content (22%) and total antioxidant activity (76%). The concentration of all those compounds was reduced significantly in the duodenal fraction in comparison to gastric digestion and their lowest concentration was observed in the dialysed fraction, where phenolic acids were not detected. The bioaccessibility of soymilk phenolic compounds was 15% as the sum of individuals and 20% by F–C assay; isoflavones 36% and constituents with antioxidant activity 27%. Results suggest that most of these compounds were sufficiently available to be absorbed and could contribute health benefits.

Keywords: Soymilk; I*n vitro* gastrointestinal digestion; Bioaccessibility; Phenolic compounds; Isoflavones; Antioxidant activity

1. Introduction

Soymilk is an aqueous extract of whole soybeans, containing high amounts of protein, iron and niacin, but low concentration of fat, carbohydrates and calcium compared to cow and human milks (Jinapong, Suphantharika and Jamnong, 2008). Additionally, soymilk is cholesterol and lactose free. For these reasons, soymilk is considered an excellent alternative to cows' milk for people with milk protein allergy, lactose intolerance or galactosemia (Xu and Chang, 2009).

There is growing evidence that consumption of soy-derived food has potential health benefits related to cardiovascular diseases, menopausal symptoms, osteoporosis, breast and prostate cancers because they are rich sources of bioactive phenolic compounds (Devi et al., 2009). The major phenolic constituents contained in soy-derivatives are flavonoids, specifically isoflavones in their glucoside (genistin, daidzin and glycitin) or aglycone (genistein, daidzein, and glycitein) forms (Sanz and Luyten, 2006), while their principal phenolic acids are syringic, chlorogenic, gallic, vanillic and ferulic (Tyug, Prasad and Ismail, 2010). The concentrations and profile of these components depend on the type of soy-derivative and the processing conditions (Grün et al., 2001).

Bioactive compounds must be released from the food matrix and modified in the gastrointestinal tract before becoming bio-available. Bioavailability is defined as the fraction of ingested nutrient that is absorbed and utilised (Wood, 2005). The overall bioavailability process includes gastrointestinal digestion, absorption and metabolism. Thus, the first step in assessing the bioavailability of food constituents is gastrointestinal digestion, where a bioaccessible fraction (portion of bioactive compound available for absorption) is obtained. However, it is difficult to study the *in vivo* changes of these components during their passage through the digestive tract. Consequently, *in vitro* methodologies have been developed as an alternative approach to *in vivo* studies and they are considered simple, cheap and reproducible tools to asses the digestive stability of different food constituents (Failla, M.L. and Chitchumroonchokchai, C., 2005). *In vitro* gastrointestinal digestion followed by the determination of bioactive compound concentrations, which are able to cross a semipermeable membrane with a specific pore size, have been well correlated to animal and human studies (Luten et al., 1996; Van Campen and Glahn, 1999).

Bioaccessibility is affected by different factors such as the food matrix and the interaction with other food components (Parada and Aguilera, 2007). Little information is available in the literature about the bioaccessibility of bioactive compounds in soy-derivatives. To the best of our knowledge, this is the first study that has focused on evaluating the changes in phenolic compounds, isoflavones and antioxidant activity during the *in vitro* gastrointestinal digestion of soymilk.

2. Materials and methods

2.1. Materials

A commercial soymilk (Yosoy, Girona, Spain) was purchased at a local supermarket. It contained 3.6 g of protein, 0.7 g of carbohydrates, 1.8 g of fat and 1 g of fibre in 100 mL (data supplied by the manufacturer). Soymilk pH was measured in a pH-meter (Crison Instruments S.A., Alella, Barcelona, Spain), and was found to be 7.34 ± 0.02. The soluble solids content was also determined (Comecta S.A., Abrera, Barcelona, Spain) resulting 5.83 ± 0.14 ºBrix.

Pepsin (from porcine gastric mucosa), pancreatin (from porcine pancreas), bile (from bovine bile), 4-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, sinapic acid, hesperidin, naringenin, rutin, quercetin, (+)-catechin, daidzin, genistin, glycitin, daidzein, genistein, glycitein, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and the cellulose dialysis membrane (molecular weight cutoff of 12,000 Da) were acquired from Sigma–Aldrich (St. Louis, MO, USA). Gallic acid and F–C reagent were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Filters (0.2 μm) were purchased from Millipore Iberica S.A. (Madrid, Spain).

2.2. In vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion was carried out in two sequential phases: gastric and intestinal digestion (including dialysis), as previously described by Vallejo, Gil-Izquierdo, Pérez-Vicente and García-Viguera (2004), with some modifications.

2.2.1. Gastric digestion. A portion of soymilk (50 mL) and 32,700 pepsin units were mixed in a beaker. Then the pH was adjusted immediately to 2 by addition of HCl (12

M) and the mixture was incubated at 37 ºC and 90 rpm during 2 h in an incubator chamber with orbital agitation (Ovan, Badalona, Spain).

2.2.2. Intestinal digestion with dialysis membrane. Dialysis membrane segments (12 cm of length) were filled with 25 mL of water–$NaHCO_3$ (0.5 N) mixture. The amount of $NaHCO_3$ (0.5 N) contained in the dialysis membrane was that required to titrate the gastric digest to pH 7.5. To simulate intestinal digestion, the gastric digest (20 mL) was placed into a polyethylene tube and the dialysis membrane (containing the water-NaHCO3 mixture) was completely immersed in that digest until reaching pH 5.0. Later, 5 mL of pancreatin (4 g/L) - bile (25 g/L) mixture was added to the tube, and the incubation continued for 2 h at 37 ºC and 90 rpm. The dialysis membrane was removed and rinsed with distilled water and the dialysate was analysed. Therefore, two fractions were obtained after intestinal digestion: duodenal and dialysed fractions. The duodenal fraction corresponded to the portion of bioactive compounds that remained outside the dialysis membrane and were considered as unabsorbed compounds. The bioactive compounds available for absorption were in the dialysed fraction (inside the dialysis membrane).

Aliquots were taken at the end of each digestive phase and immediately placed in a cold water bath for 10 min. Afterwards, aliquots were frozen (-45 ºC) until the analyses of phenolic com-pounds, isoflavones and antioxidant activity.

2.3. Bioactive compounds analysis

2.3.1. Phenolic compounds

2.3.1.1. Phenolic compounds determined by HPLC. The phenolic profile of samples was evaluated using the methodology reported by Odriozola-Serrano, Soliva-Fortuny and Martín-Belloso (2008), slightly modified. Briefly, 5 mL of non-digested or digested soymilk were mixed with 5 mL of 62% aqueous methanol (containing 2 g/L tert-butylhydroquinone) and 1 mL of 6 M HCl. The mixture was refluxed at 90 ºC for 2 h, cooled and made up to 25 mL with methanol. Later, the extracts were sonicated for 3 min, passed through a 0.20lm filter and stored at -45 ºC until HPLC analysis.

The HPLC system was equipped with a 600 Controller and a diode array detector (Waters Corporation, Milford, MA, USA) which was set to scan from 200 to 600 nm.

Separations were performed on a reverse-phase C18 Spherisorb ODS2 (5 μm) stainless steel column (4.6*x*250 mm) operating at room temperature with a flow rate of 1 mL/min. A gradient elution was employed with two sol-vent mixtures of 2.5% acetic acid in water (solvent A) and 2.5% acetic acid in methanol (solvent B), following the methodology of Odriozola-Serrano et al. (2008). HPLC chromatograms of phenolic compounds in non-digested soymilk are shown in Fig. 1. Individual phenols were identified by comparing their retention time and spectrum to those of the standards: gallic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, sinapic acid, hesperidin, naringenin, rutin, quercetin and (+)-catechin. Quantification of individual phenols was carried out by integration of the peak areas and using calibration curves. Results were expressed as milligrams of each phenolic compound/100 mL of soymilk. Total phenolic compounds (TPC) were calculated as the sum of individuals.

2.3.1.2. Total phenolic content determined by F–C methodology. Total phenolic content was analysed using the colorimetric method described by Morales-de la Peña, Salvia-Trujillo, Rojas-Graü and Martín-Belloso (2010a). An aliquot of 0.5 mL of non-digested or digested soymilk was mixed with 0.5 mL of F–C reagent and 10 mL of Na_2CO_3 (20%). The mixture was made up to 25 mL with distilled water and kept in the dark at room temperature for 1 h. Then, mixtures were filtered (0.2 μm membrane) and the absorbance was measured at 725 nm (Cecil Instruments Ltd., Cambridge, UK). Concentrations were determined by comparing the absorbance of the samples with a calibration curve constructed using gallic acid. Results were expressed as mg of gallic acid/100 mL of soymilk.

2.3.2. Isoflavones

Isoflavones were analysed using the method of Morales-De La Peña, Salvia-Trujillo, Rojas-Graü and Martín-Belloso (2010b), with some modifications. For the extraction and hydrolysis of isoflavones, 4 mL of non-digested or digested soymilk were mixed with 4 mL of 80% ethanol acidified to 1 M with HCl. Then, this mixture was incubated at 80 ºC for 1 h. After incubation, samples were cooled, shaken vigorously for 2 min and centrifuged at 9,000 rpm for 10 min at 4 ºC. Supernatant was decanted into a 10 mL volumetric flask and the residue was re-extracted with 2 mL of 80% ethanol and centrifuged under the conditions previously described. Both supernatants were

combined into the volumetric flask, which was filled to 10 mL with 80% ethanol. The samples were filtered through a 0.2 μm membrane and kept at -45 ºC until HPLC analysis.

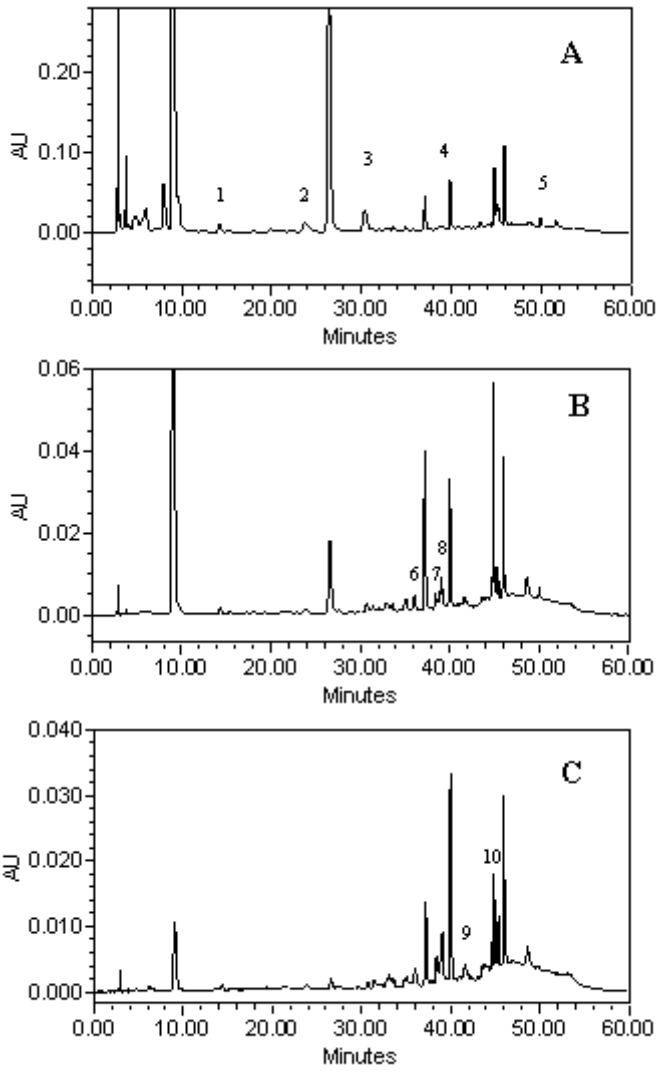

Figure 1. HPLC chromatograms of phenolic compounds in non-digested soymilk. Detection: 280 nm (A), 320 nm (B) and 360 nm (C). Peaks: 1 gallic acid; 2 4-hydroxybenzoic acid; 3 (+)-catechin; 4 hesperidin; 5 naringenin; 6 p-coumaric acid; 7 ferulic acid; 8 sinapic acid; 9 rutin and 10 quercetin.

The HPLC system was equipped with a 600 Controller and a diode array detector (Waters Corporation, Milford, MA, USA) set to scan from 200 to 350 nm. Separations were performed using a C18 SunFire (3.5 μm) stainless steel column (3 mm x 150 mm) and a C18 SunFire (5 μm) guard column, operating at 37 ºC with a flow rate of 0.3 mL/min. A gradient elution was employed with a mixture of two solvents: (A) water/methanol (80:20 v/v) and (B) water/methanol/acetonitrile (40:40:20 v/v/v), as

described by Morales-De La Peña et al. (2010b). Fig. 2 shows the HPLC chromatograms of isoflavones in non-digested soymilk. Isoflavones were identified by comparison of their spectrum and retention time with the standards of daidzein, genistein, glycitein, daidzin, genistin and glycitin. Quantification of isoflavones was carried out by integration of the peak areas. Data were compared with calibration curves of each isoflavone and results were expressed as mg of isoflavone/100 mL of soymilk. Total isoflavone concentration was calculated by the sum of individuals.

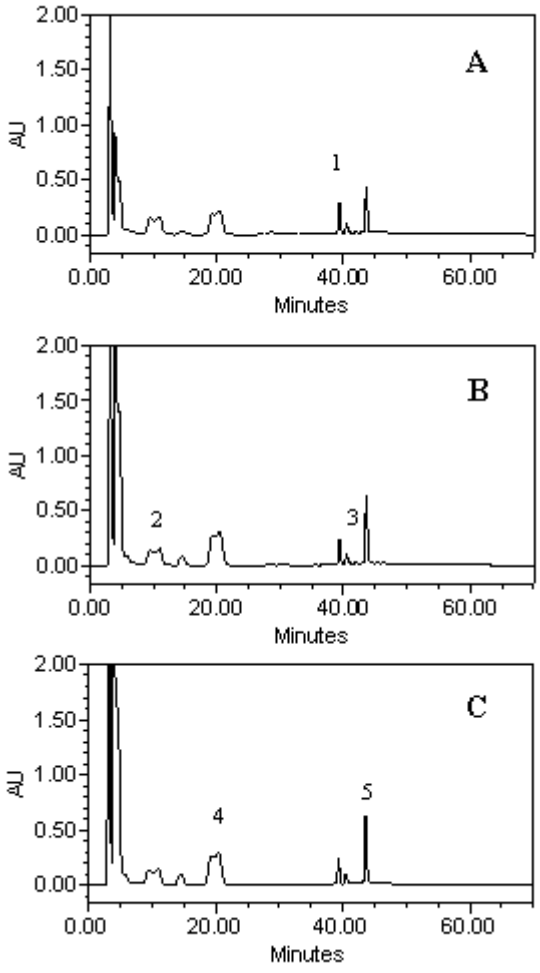

Figure 2. HPLC chromatograms of isoflavones in non-digested soymilk. Detection: 248 nm (A), 260 nm (B) and 262 nm (C). Peaks: 1 daidzein; 2 daidzin; 3 glycitein, 4 genistin, 5 genistein.

2.3.3. Hydrophilic and lipophilic antioxidant activity

A portion of 5 mL of non-digested or digested soymilk was mixed with 10 mL of methanol and centrifuged at 6,000 rpm for 20 min at 4 ºC. The supernatant was

considered as the hydrophilic fraction and the residue was mixed with 10 mL of tetrahydrofuran and centrifuged using the conditions described above. The second supernatant was considered as the lipophilic fraction. Antioxidant activity was evaluated using the methodology reported by Morales-de la Peña et al. (2010a), slightly modified. Aliquots of 0.2 mL of hydrophilic or lipophilic extracts were mixed with 3.8 mL of methanolic solution of DPPH (0.025 g/L). The homogenate was shaken vigorously and kept in the dark for 30 min. Absorbance of non-digested and digested soymilk were measured at 515 nm against a methanol blank. Results were calculated using Eq.1 and expressed as percentage of inhibition of DPPH radical.

$$\%DPPHinhibition = \left(\frac{C_{abs} - SM_{abs}}{C_{abs}} \right) x100 \qquad \text{(Eq. 1)}$$

where C_{abs} is the absorbance of control (methanolic solution of DPPH radical without extract) and SM_{abs} is the absorbance of non-digested or digested soymilk extract.

2.4. Bioaccessibility calculations

Bioaccessibility was considered as the concentration of bioactive compounds released from the food matrix by *in vitro* gastrointestinal digestion and which is available for absorption. Bioaccessibility was calculated using Eq.2.

$$Bioaccessibility(\%) = \left(\frac{BC_{dialyzed}}{BC_{non-digested}} \right) x100 \qquad \text{(Eq. 2)}$$

where $BC_{dialysed}$ and $BC_{non\text{-}digested}$ corresponded to the bioactive compound concentration (mg/100 mL) in dialysed fraction and non-digested soymilk, respectively.

2.5. Statistical analysis

The *in vitro* gastrointestinal digestion was assayed in duplicate. Three analyses were carried out on each studied parameter and results were reported as mean ± standard deviation. Analysis of variance (ANOVA) of the results was carried out in order to determine significant differences ($p< 0.05$) between the concentration of bio-active compounds in non-digested soymilk and that obtained in the different phases of the *in vitro* gastrointestinal digestion (Stat-graphics Plus v.5.1, Rockville, Md, USA).

3. Results and discussion

3.1. Phenolic compounds

The effect of *in vitro* gastrointestinal digestion on soymilk phenolic constituents is presented in Table 1. Two main phenolic groups were identified in soymilk by HPLC: phenolic acids (hydroxybenzoic and hydroxycinnamic derivatives) and flavonoids (flavanones, flavonols, and flavanols).

Table 1. Concentration of phenolic compounds during *in vitro* digestion of soymilk.[a]

Phenolic compound	Concentration (mg/100 mL)				Bioaccessibility (%)
	Non-digested	Gastric digestion	Intestinal digestion		
			Duodenal fraction	Dialyzed fraction	
Phenolic acids					
Hydroxybenzoic acids					
Gallic acid	1.214 ± 0.011a	1.833 ± 0.014b	1.748 ± 0.019c	nd	0
4-Hydroxybenzoic	0.372 ± 0.020a	0.687 ± 0.022b	0.741 ± 0.015c	nd	0
Hydroxycinnamic acids					
p-Coumaric	0.234 ± 0.013a	0.350 ± 0.018b	0.253 ± 0.014c	nd	0
Ferulic	0.20 ± 0.01a	0.369 ± 0.015b	0.194 ± 0.014a	nd	0
Sinapic	0.351 ± 0.008a	0.795 ± 0.020b	0.195 ± 0.015c	nd	0
Total phenolic acids	2.38 ± 0.04a	4.03 ± 0.04b	3.13 ± 0.05c	nd	0
Flavonoids					
Flavanones					
Hesperidin	26.1 ± 0.6a	33.7 ± 1.6b	15.8 ± 0.6c	3.84 ± 0.16d	14
Naringenin	2.98 ± 0.13a	2.98 ± 0.09a	2.6 ± 0.1b	0.61 ± 0.03c	21
Flavonols					
Rutin	1.060 ± 0.015a	1.30 ± 0.04b	0.92 ± 0.08c	nd	0
Quercetin	1.431 ± 0.012a	2.72 ± 0.07b	1.95 ± 0.03c	0.246 ± 0.014d	17
Flavan-3-ols					
(+)-Catechin	2.69 ± 0.09a	4.95 ± 0.08b	2.60 ± 0.15a	0.758 ± 0.018c	28
Total flavonoids	34.3 ± 0.7a	45.6 ± 1.7b	23.9 ± 0.6c	5.46 ± 0.17d	16
Total phenolic compounds					
Sum of individuals	36.7 ± 0.7a	49.6 ± 1.7b	27.0 ± 0.6c	5.46 ± 0.17d	15
Folin-Ciocalteu method	61.4 ± 1.2a	70.3 ± 1.8b	69.8 ± 1.7b	12.0 ± 0.3c	20

[a] Values are expressed as mean ± standard deviation. Different letters in the same row indicate significant differences ($p<0.05$) between the digestive phases. nd= not detected.

In non-digested soymilk, the concentration of TPC determined as the sum of individuals and by F–C were 36.7 and 61.4 mg/100 mL, respectively. The F–C method is simple and useful in assessing the overall phenolic content in food. However, other

non-phenolic substances from food, such as ascorbic acid, sugars, aromatic amines, organic acids and proteins, are also reduced by the F–C reagent (Prior, Wu and Schaich, 2005), thus leading to overestimation of the phenolic content. This could explain the results observed in this research, where the TPC determined by F–C showed higher concentration than the sum of individuals. Furthermore, soymilk may contain other phenolic compounds which were not quantified by HPLC. A wide range of TPC in soymilk has previously been reported, varying from 104 to 676 mg/100 g (quantified as the sum of individuals) and between 96 and 320 mg gallic acid equivalent/100 g (determined by F–C), depending on the soybean variety and the processing conditions to produce soymilk (Tyug et al. 2010; Xu and Chang, 2009).

As can be seen in Table 1, gastric digestion increased significantly ($p< 0.05$) the concentration of all phenolic compounds (total phenolic acids increased by 70% and total flavonoids by 33%), with the exception of naringenin, which remained unchanged with respect to its content in non-digested soymilk. These results suggest that gastric digestion improves the release of phenolic compounds from the soymilk matrix. This fact could be mainly attributed to the acidic pH and enzymatic activity during this digestive phase, which can induce the hydrolysis of some phenolic compounds bound to other food constituents. Similarly, Baublis, Decker and Clydesdale, 2000 and Liyana-Pathirana and Shahidi, 2005 suggested that gastric conditions increase the extractability of phenolic compounds from the food matrix. Saura-Calixto, Serrano and Goñi (2007) reported that phenols linked to high molecular weight compounds, such as proteins and carbohydrates, may be released by digestive enzyme action, leading to a significant increase in their concentrations after gastric digestion. Tagliazucchi, Verzelloni, Bertolini and Conte (2010), observed an increase of approximately 22% and 77% in the content of TPC (by F–C) and total flavonoids (measured by a colorimetric assay), respectively, after two hours of gastric digestion of grapes. The same authors observed that pure phenolic compounds, such as gallic acid, caffeic acid, catechin and quercetin, remained stable after gastric digestion, highlighting the importance of the food matrix and the interaction with dietary constituents in the release of phenolic compounds. The concentration of phenolic acids and flavonoids in the duodenal fraction significantly diminished between 5% and 76%, during gastric digestion. However, 4-hydroxybenzoic acid increased significantly by 8% when the duodenal fraction was compared to gastric digestion. Losses in the concentration of chokeberry juice flavonols (between 25% and

30%) and TPC of fruit beverages (47%), both determined by HPLC analysis after intestinal conditions, were reported (Bermúdez-Soto, Tomás-Barberán and García-Conesa, 2007; Cilla, Lagarda, Barberá and Romero, 2010). On the other hand, the dialysed fraction showed the lowest phenolic concentration. Total flavonoids decreased by 77% and 88% in the dialysed fraction, with respect to the duodenal fraction and gastric digestion, respectively. Phenolic acids were not detected in the dialysed fraction. The interactions between phenolic compounds and other constituents may favour the formation of complexes with low solubility or large molecular weight, which can not cross the dialysis membrane, causing a reduction in phenolic concentration. Scalbert and Williamson (2000) also related the molecular weight of phenolic compounds to their capability of absorption in the gut. Saura-Calixto, Serrano and Goñi (2007) and Argyri, Komaitis and Kapsokefalou (2006) observed that solubility and availability of phenolic compounds are influenced by pH and interaction with dietary constituents, such as iron, fibre or proteins. In addition, Argyri, Proestos, Komaitis and Kapsokefalou (2005) reported that many phenolic compounds, such as hydroxybenzoic acid, rutin and catechin, were observed in non-digested red wine, but that these compounds were not detected in the dialysed fraction at the end of an *in vitro* digestion.

Total phenolic compounds determined as the sum of individuals and by F–C showed a significant correlation (r^2= 0.8663, p= 0.0001) along the *in vitro* gastrointestinal digestion of soymilk. Therefore, in spite of the great differences in the phenolic concentration obtained by both methods, the behaviour of these compounds followed a similar pattern under these digestive conditions.

Bioaccessibilities of phenolic compounds are displayed in Table 1. Total flavonoids showed a bioaccessibility of 16%, whereas total phenolic acids were not bioaccessible under the conditions assayed in this research. The bioaccessibility of TPC determined as the sum of individuals and by F–C was 15% and 20%, respectively. These results suggest that many changes in phenolic compounds, such as chemical structure, solubility and interaction with other constituents, may occur during the gastrointestinal digestion of soymilk, influencing their bioaccessibilities. Likewise, Gil-Izquierdo, Gil, Ferreres and Tomás-Barberán (2001) observed flavanone (hesperidin and narirutin) bioaccessibilities between 11% and 36% in orange juice. Vallejo et al. (2004) obtained a total flavonoid bioaccessibility of 6% (quantified as the sum of individuals) in broccoli inflorescences. Akillioglu and Karakaya (2010) reported that the bioaccessibility of

TPC obtained by F–C was between 19% and 39%, depending on the bean variety. Human intervention studies also showed a low percentage (between 5% and 10%) of phenolic compounds available for absorption in the intestine (Clifford, 2004), demonstrating that the bioaccessible fraction of phenols is small, but significant enough to exert their functional and nutritional effects.

3.2. Isoflavones

The influence of *in vitro* gastrointestinal digestion on soymilk isoflavone profiles is shown in Table 2, where the concentration of individual isoflavones and their distribution into glucoside and aglycone forms are displayed. Total isoflavones content was calculated as the sum of individuals, resulting 48.9, 59.89, 47.8 and 17.4 mg/100 mL in non-digested soymilk, gastric digestion, duodenal fraction and dialysed fraction, respectively. The isoflavone con-centration of non-digested soymilk was in the range reported by Bhagwat, Haytowitz and Holden (2008), who obtained between 0.7 and 196.05 mg/100 g in different soymilk types. As it can be seen inTable 2, glucosides were the predominant form of isoflavones in non-digested soymilk (76%), whereas aglycones were detected in a small percentage (24%). Similar results were reported by Grün et al. (2001) who obtained a higher content of glucoside than aglycone forms in fermented soymilk.

After gastric digestion, the concentration of total isoflavones increased by a significant 22%, relative to their content in non-digested soymilk. These results suggest that acidic conditions of gastric digestion could improve the release of isoflavones from the food matrix. However, this hypothesis has been discussed by different authors, with controversial results about the effect of pH and/or the enzymatic activity on the increase of isoflavones due to gastric digestion. For instance, Peñalvo, Nurmi and Adlercreutz (2004) observed that mild acid hydrolysis separated the acetyl and malonyl groups of the isoflavone conjugates, releasing the 7-O-glucosides and the free aglycones from food. Walsh, Zhang, Vodovotz, Schwartz and Failla (2003) did not find differences in the concentration of soy-bread isoflavones during *in vitro* oral and gastric digestion, whereas, Piskula (2000) reported a decrease in the content of isoflavones during gastric digestion due to a lack of solubility under acidic conditions. Sanz and Luyten (2006) observed that gastric pH (not enzyme action) significantly influenced the concentration and partition of isoflavones. Hendrich (2002) reported that the stomach does not contain

enzymes able to hydrolyse isoflavone glucosides and that the hydrolysis by non-enzymatic activity is difficult. Based on those findings, isoflavone stability under gastric digestion could mainly depend on the pH effect and the food matrix.

Table 2. Concentration of isoflavones during *in vitro* digestion of soymilk.[a]

Isoflavones	Concentration (mg/100 mL)				Bioaccessibility (%)
	Non-digested	Gastric digestion	Intestinal digestion		
			Duodenal fraction	Dialyzed fraction	
Glucosides					
Daidzin	15.7 ± 2.0a	20.4 ± 0.6b	16.9 ± 1.9a	4.19 ± 0.21c	27
Genistin	21.3 ± 0.5a	24.0 ± 0.3b	19.6 ± 0.3c	11.2 ± 0.5d	53
Glycitin	nd	nd	nd	nd	nd
Total glucosides	37.0 ± 2.3a	44.4 ± 0.8b	36.5 ± 1.8a	15.4 ± 0.7d	42
Aglycones					
Daidzein	3.40 ± 0.12a	4.88 ± 0.19b	3.37 ± 0.06a	0.58 ± 0.02c	17
Genistein	7.13 ± 0.05a	8.97 ± 0.14b	6.4 ± 0.1c	1.23 ± 0.05d	17
Glycitein	1.34 ± 0.03a	1.67 ± 0.04b	1.483 ± 0.019c	0.202 ± 0.006d	15
Total aglycones	11.87 ± 0.14a	15.52 ± 0.21b	11.30 ± 0.09c	2.01 ± 0.06d	17
Total isoflavones	48.9 ± 2.3a	59.89 ± 0.8b	47.8 ± 1.8a	17.4 ± 0.8c	36

[a] Values are expressed as mean ± standard deviation. Different letters in the same row indicate significant differences ($p<0.05$). nd= not detected.

In the duodenal fraction, a significant decrease ($p< 0.05$) in the concentration of each isoflavone was observed compared to that after gastric digestion. However, when non-digested soymilk and the duodenal fraction were compared, there were no significant differences ($p> 0.05$) in the content of total glucosides, while aglycones diminished just 5%. In the same way, Walsh et al. (2003) observed that daidzein, genistein, their respective β-glucosides and glycitein remained stable during simulated small intestinal digestion of soy-bread, in comparison to the non-digested sample. Sanz and Luyten (2006) reported that small intestine digestion enhances the solubility of isoflavones in enriched custards. The lowest isoflavone concentration was observed in the dialysed fraction. The glucoside forms daidzin and genistin decreased by 75% and 43%, respectively, when the dialysed fraction was compared to the duodenal fraction, whereas the aglycones diminished between 81% and 86%. To the best of our

knowledge, this is the first study addressing the dialysability of isoflavones in soymilk, and so it is not possible to compare these results with the literature.

Bioaccessibility data of soymilk isoflavones are displayed in Table 2. Glucosides and aglycones showed a bioaccessibility of 42% and 17%, respectively, suggesting that glucosides contained in soymilk could be more available for absorption than aglycones. However, there is controversy about whether glucoside forms of isoflavones are more bioavailable compared to aglycones. Previous researches reported that glucosides were more bioaccessible than aglycones in the aqueous fraction of soy-bread after *in vitro* digestion (Walsh et al., 2003), as well as in human plasma (Setchell et al., 2002). However, Zubik and Meydani (2003) did not find differences in the human absorption of aglycones and glucosides, while Izumi et al. (2000) reported greater bioavailability of aglycones. Nevertheless, other factors may affect the *in vivo* bioavailability of isoflavones, such as β-glucosidase activity (along the brush border surface in the small intestine) and the resident microbiota (in the large intestine), which could hydrolyse conjugated isoflavones, releasing the aglycones for absorption, metabolism or further reconjugation (Larkin, Price and Astheimer, 2008).

3.3. Hydrophilic and lipophilic antioxidant activity

Soymilk is a rich source of hydrophilic and lipophilic antioxidants. Some phenolic compounds and water-soluble vitamins (as vitamin B1, B2 and B3) contribute to its hydrophilic antioxidant activity, whereas the fatty acids and lipid-soluble vitamins (as A and E) are related to its lipophilic antioxidant capacity. As far as we know, the hydro and lipophilic antioxidant activity of soymilk has not been documented and so both fractions were evaluated in this research.

The antioxidant activity of non-digested soymilk showed 17% DPPH inhibition (Fig. 3), corresponding to 10% and 7% for hydrophilic and lipophilic fractions, respectively. Devi et al. (2009) reported higher total DPPH inhibition (42%) in soymilk than that observed in this research, while Rekha and Vijayalakshmi (2008) observed only 7% inhibition. Differences in these results may be due to the soybean varieties or the process conditions to obtain soymilk, which are expected to affect their antioxidant activities (Lee et al., 2002).

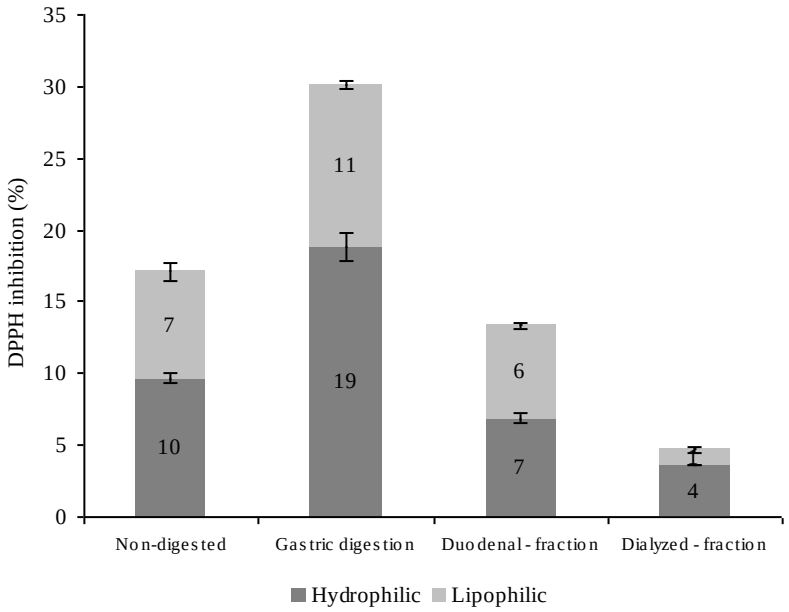

Figure 3. Changes in hydrophilic and lipophilic antioxidant activity during *in vitro* digestion of soymilk.

The DPPH inhibition increased to 30% after gastric digestion (76% more than non-digested soymilk), with the hydrophilic antioxidant activity (19%) being higher than the lipophilic antioxidant activity (11%). The hydrophilic antioxidant activity showed a significant correlation with total phenolic acids (r^2= 0.9291, p= 0.0074) during gastric digestion, as well as with total flavonoids (r^2= 0.9595, p= 0.0024). Therefore, the increment in the hydrophilic antioxidant activity of soymilk could be related to the rise in phenolic compounds after gastric digestion, showing that acidic conditions might improve the release of antioxidants from the food matrix. Similarly, Baublis et al. (2000) reported that the acidic conditions of gastric digestion altered the activity, composition and concentration of water-soluble, low-molecular-weight antioxidants of wheat-based ready-to-eat breakfast cereals, producing a significant increase in their antioxidant activity. Noguer et al. (2008) observed 100–1000 times greater antioxidant capacity after gastric digestion of red wine due to the formation of compounds during digestion that were not necessarily present in the food itself. Liyana-Pathirana and Shahidi (2005) reported between 160% and 580% higher DPPH inhibition following the gastric digestion of soft and hard wheat compared to the non-digested equivalents.

A significant loss ($p< 0.05$) of total antioxidant activity was observed during intestinal digestion. In the duodenal fraction, the hydrophilic and lipophilic antioxidant activity decreased by 63% and 43%, respectively, regarding gastric digestion. These data showed that hydrophilic compounds of soymilk were more susceptible to the

alkaline medium and to interaction with dietary or digestive constituents than lipophilic compounds. The lowest antioxidant activity was observed in the dialysed fraction, being 4% of DPPH inhibition for hydrophilic compounds and 1% for lipophilic compounds. The decrease in the antioxidant activity under intestinal conditions might be attributed to the fact that some substances with antioxidant activity, such as phenolic compounds, may be transformed into different structural forms with other chemical properties due to their sensitivity to alkaline pH (Bermúdez-Soto et al., 2007). Additionally, these compounds are able to bond with other constituents, resulting in the formation of complexes which could not pass through the dialysis membrane. In the same way, Bouayed, Hoffmann and Bohn (2011) reported a decrease in the total antioxidant activity of dialysable compounds, compared to those observed in fresh apples (57% and 46% for FRAP and ABTS test, respectively). Akillioglu and Karakaya (2010), observed a reduction of 1.6–2.1-fold in the DPPH inhibition of pinto beans after simulated gastrointestinal digestion.

The bioaccessibility of hydrophilic and lipophilic constituents was 38% and 14%, respectively. Probably, hydrophilic compounds showed greater bioaccessibility than lipophilic compounds due to the fact that soymilk is an aqueous extract of soybeans with a low fat concentration.

4. Conclusion

Gastric digestion improved the release from the food matrix of phenolic compounds, isoflavones, as well as bioactive compounds with antioxidant activity. In contrast, the concentration of these compounds decreases significantly during intestinal digestion, mainly in the dialysed fraction. Although the concentration of bioactive compounds contained in soymilk after *in vitro* gastrointestinal digestion was significantly lower than in the non-digested product, most soymilk bioactive substances were bioaccessible. Glucoside forms of isoflavones were the most bioaccessible soymilk compounds (42%), followed by the isoflavones in their aglycone form (17%) and the flavonoids (16%). However, phenolic acids were not bioaccessible under the assay conditions. Hydrophilic constituents displayed higher bioaccessibility (38%) than those lipophilics (14%). The results obtained in this research suggest that soymilk might be considered as a rich source of available bioactive compounds. Further research is needed to investigate the bioavailability and biological effect of soymilk bioactive compounds in humans.

Acknowledgement

The present research has been financed by the Ministerio de Ciencia y Tecnología (Spain), reference AGL2006-12758-C02-02/ALI. María Janeth Rodríguez-Roque thanks to the Comissionat per a Universitats i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya and European Social Fund for the predoctoral grant, and to the Secretaría de Educación Pública de México for their support. Prof. Olga Martín-Belloso thanks to the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia Award 2008.

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CHAPTER II

Changes in vitamin C, phenolic and carotenoid profiles throughout an *in vitro* gastrointestinal digestion of a blended fruit juice

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Journal of Agricultural and Food Chemistry (2013) 61: 1859–1867

Abstract

The aim of this research was to evaluate the influence of an *in vitro* gastrointestinal digestion on the stability and bioaccessibility of vitamin C, phenolic compounds and carotenoids, as well as the antioxidant activity in a blended fruit juice (BFJ) containing orange, pineapple and kiwi. Vitamin C and most of the analyzed phenolic compounds were quite stable under gastric conditions (recovery over 75%), while carotenoids diminished significantly (until a 64%). The concentration of all the evaluated compounds decreased during small intestinal digestion. The bioaccessibility of hydrophilic constituents was higher than that of those lipophilic. Flavonoids, vitamin C and phenolic acids showed a bioaccessibility of 20.1%, 15.0% and 12.7%, respectively. However, carotenes and xanthophylls were around 7.6% and 17.4% available for absorption. In spite of the decrease in the concentration of these bioactive compounds after being subjected to an *in vitro* gastrointestinal digestion, results suggest that BFJ is an important source of bioaccessible constituents.

Keywords: Blended fruit juice; Bioactive compounds; *In vitro* gastrointestinal digestion; Bioaccessibility; Vitamin C; Phenolic compounds; Carotenoids; Antioxidant activity

1. Introduction

Fruit and vegetable consumption plays an important role in improving human health. The World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) recommend eating at least 400 g of fruits and vegetables per day (the“5 a day”program) for the prevention of chronic diseases such as heart disease, cancer, diabetes, and obesity (FAO/WHO, 2003). However, fruit and vegetable intake per day in Spain, among other countries, is below the dietary recommendations at this time (Zulueta, Esteve, Frasquet, & Frígola, 2007).

Consumption of fruit juices has become a dietary concern worldwide. Fruit juices retain the physicochemical and organoleptical characteristics of fruits from which they are produced; therefore, their intake also should contribute to maintain health. Health benefits of fruit juices are attributed to a large number of compounds with biological activity present in these foods. These biological functions include radical scavenging activity, protecting proteins, lipids, and DNA from oxidative damage (Liu, 2003). The major bioactive antioxidant compounds of fruit and vegetables juices are vitamin C and phenolic compounds (Perales, Barberá, Lagarda, & Farré, 2008), as well as carotenoids. The intake of vitamin C reduces the risk of several cardiovascular and neurodegenerative diseases, among others (Harrison & May, 2009). The main biological functions of phenolic compounds are preventing some cancer types and cardiovascular and inflammatory diseases, and carotenoids avoid age-related macular degeneration (Scalbert & Williamson, 2000; Daly, Jiwan, O'Brien, & Aherne, 2010). For these reasons, the potential market of fruit juices is currently growing, and new fruit-derived products have been designed. Among the new products, blended fruit juices (BFJ) stand out to enhance the sensorial and nutritional characteristics of these products. Mixing different fruit juices provides increased concentrations of selected bioactive compounds, adds new nutrients, or improves flavor and appearance. Besides, it has been reported that absorption of bioactive compounds in fruit juices exceeds that after consumption of intact fruits (Perales, Barberá, Lagarda, & Farré, 2008). Therefore, the bioavailability of these substances could also be enhanced through BFJ.

Many bioactive compounds must be released from the food matrix to exert their biological effects. In this sense, *in vitro* methodologies have been developed as a simple and fast approach to in vivo trials because the latter are expensive long-term studies

with high variability between subjects (Failla & Chitchumroonchokchai, 2005). In general, *in vitro* gastrointestinal digestion is useful in assessing the bioaccessibility of compounds with biological activity from food. This method followed by a dialysis has been mainly applied to study the bioaccessibility of food micronutrients (iron, calcium, zinc, copper, and manganese, among others) (Briones-Labarca, Muñoz, & Maureira, 2011; Cámara, Amaro, Barberá, & Clemente, 2005; Hemalatha, Platel, & Srinivasan, 2007; Velasco-Ryenold et al., 2008). The whole premise of dialyzability methods is that dialyzable compounds will be available for absorption (Cámara, Amaro, Barberá, & Clemente, 2005). In fact, iron dialyzability data have been reported to agree reasonably well with human absorption (Luten et al., 1996). This could be one of the reasons why the application of gastric digestion with dialysis has been extended to other food constituents, including vitamin C, phenolic compounds, and isoflavones (Bouayed, Hoffmann, & Bohn, 2011; Bouayed, Deußer, Hoffmann, & Bohn, 2012; Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013; Vallejo, Gil-Izquierdo, Pérez-Vicente, García-Viguera, 2004).

It is known that not all of the quantity of food bioactive phytochemicals is able to be absorbed by the gastrointestinal tract. Information related to the concentration of substances with biological activity in individual fruit juices is available in the literature. However, studies in relation to the bioaccessibility of different bioactive compounds contained in BFJ are scarce (Cilla et al., 2011, 2012). Therefore, the aim of this research was to assess the changes in the concentration of vitamin C, phenolic compounds, and carotenoids, as well as the antioxidant activity, during an in vitro gastrointestinal digestion of a BFJ and to determine their bioaccessibility.

2. Materials and methods

2.1. Materials

A BFJ was manufactured by mixing three different fruits juices (orange, kiwi, and pineapple). Fruits were purchased at commercial maturity in a local supermarket. Each fruit was washed and peeled and the juice extracted. Afterward, every juice was filtered with a cheesecloth using a vacuum pump. Finally, the juices were combined in the following proportions: orange, 50%; kiwi, 36%; and pineapple, 14%. Fruit juice formulation was selected according to previous studies, in which the blend of these fruit

juices showed high vitamin C and phenolic concentrations, as well as antioxidant activity (Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2010). The pH of BFJ was 3.40±0.04 (Crison Instruments SA, Alella, Barcelona, Spain). The soluble solid content was measured in a Comecta refractometer (Abrera, Barcelona, Spain), being 10.75±0.01°Brix.

Pepsin from porcine stomach, pancreatin from porcine pancreas, bovine bile,DL-1,4-dithiothreitol (DTT), metaphosphoric acid, phenol standards (caffeic, chlorogenic,p-coumaric, ferulic, and sinapic acids; hesperidin, naringenin, rutin, quercetin, and (+)-catechin), carotenoid standards (α-carotene, β-carotene, zeaxanthin, lutein, α-cryptoxanthin, and β-cryptoxanthin), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and cellulose dialysis membrane (molecular weight cutoff of 12000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid, gallic acid, and Folin−Ciocalteu (F−C) reagent were acquired from Scharlau Chemie S.A. (Barcelona, Spain). Ultrafiltration devices (Amicon Ultra 3 K) and 0.2μm membranes were bought from Millipore Iberica S.A. (Madrid, Spain).

2.2. In Vitro Gastrointestinal Digestion

The in vitro gastrointestinal digestion was carried out following the methodology described by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013), which consisted of two sequential phases: gastric and small intestinal digestion (including dialysis). In addition, a micellar fraction was prepared from digesta following the completion of the small intestinal phase.

2.2.1. Gastric Digestion. BFJ (200 mL) and pepsin (0.2 g) were mixed in a beaker. Afterward, the pH was immediately adjusted to 2 by addition of 12 M HCl, and the mixture was incubated at 37 °C and 90 rpm during 2 h (incubation chamber with orbital agitation Ovan, Badalona, Spain).

2.2.2. Small Intestinal Digestion with Dialysis Membrane. Segments of dialysis membrane were cut at 12 cm of length and filled with 25 mL of water/$NaHCO_3$ (0.5 N) mixture. The required amount of $NaHCO_3$ (0.5 N) to titrate the gastric digesta to pH 7.5 was that contained in the dialysis membrane. Every 20 mL of gastric digesta was placed into a polyethylene tube, and a dialysis membrane was completely immersed until a pH

of 5.0 was reached. Later, 5 mL of pancreatin (4 g/L) and bile (25 g/L) mixture was added to each tube and incubated during 2 h at 37 °C and 90 rpm (incubation chamber with orbital agitation Ovan). Finally, the dialysis membrane was removed, rinsed with distilled water, and weighed. Bioactive compounds of small intestinal digesta corresponded to those obtained outside the dialysis membrane, and bioactive compounds that could be available for absorption were inside the dialysis membrane (bioaccessible fraction).

2.2.3. Micellar Fraction. Some lipophilic constituents, such as carotenoids, are usually micellarized before being absorbed, packed into chylomicrons, and secreted to the lymphatic system. To quantify the amount of carotenoids transferred to the aqueous-micellar fraction, a portion of small intestinal digesta (30 mL) was centrifuged at 5000 rpm during 20 min at room temperature (Granado-Lorencio et al., 2007a).

To monitor the changes in bioactive compound concentration during the in vitro gastrointestinal digestion of BFJ, aliquots were taken at the end of each digestive phase and immediately placed in a cold water bath during 10 min and frozen (−45°C) until analysis.

2.3. Bioactive Compound Analysis

2.3.1. Vitamin C

Vitamin C extraction was performed through the method validated by Odriozola-Serrano, Hernández-Jover, & Martín-Belloso (2007), with some modifications. A sample of 5 mL of nondigested or digested BFJ was mixed with 5 mL of a solution containing 45 g/L metaphosphoric acid and 7.2 g/L DTT. Then, samples were filtered in 0.20 μm membrane. The filtrate was added into the ultrafiltration devices and centrifuged at 6000 rpm during 30 min at 4 °C to purify samples. The recovery of vitamin C after ultrafiltration was between 70 and 80%.

An aliquot of 20μL was injected into the HPLC system consisting of a 600 controller, a 486 absorbance detector, and a reverse-phase C18 Spherisorb ODS2 (5μm) stainless steel column (4.6 mm×250cm) (Waters Corp., Milford, MA, USA). A 0.01% sulfuric acid solution adjusted to pH 2.6 was used as mobile phase, and the flow rate was fixed to 1 mL/min at room temperature. Detection was performed at 245 nm.

Vitamin C identification was carried out by comparing the retention time and UV−visible absorption spectrum of samples with the standard (ascorbic acid). Results were expressed as milligrams of ascorbic acid per 100 mL of sample.

2.3.2. Phenolic Compounds

(a) Phenolic Compounds Determined by HPLC. The phenolic profile was analyzed using the methodology reported by Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso (2008). Briefly, a portion of 5 mL of nondigested or digested BFJ was mixed with 5 mL of 62% aqueous methanol and 1 mL of 6 M HCl. The mixture was refluxed at 90 °C for 2 h, cooled, and diluted to 25 mL with methanol. Finally, the extracts were sonicated for 3 min, filtered (0.20 μm membrane), and frozen (−45°C) until HPLC analysis.

The HPLC system was equipped with a 600 controller and a 2996 diode array detector (Waters Corp.), which was set to scan from 200 to 600 nm. Separations were performed on a reverse-phase C18 Spherisorb ODS2 (5μm) stainless steel column (4.6 mm×250 mm) operating at room temperature with a flow rate of 1 mL/min. A gradient elution was employed with a mixture of two solvents: (A) 2.5% of acetic acid in water and (B) 2.5% of acetic acid in methanol as described by Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso (2008). Individual phenols were identified by comparison of their retention times and spectra to those of the standards. Quantification of individual phenols was carried out by integrating the peak areas and using calibration curves. Results were expressed as milligrams of each phenolic compound per 100 mL of sample. Total phenolic compounds (TPC) were calculated as the sum of individuals.

(b) Total Phenolic Content Determined by Folin−Ciocalteu (F−C) Methodology. Total phenolic content was evaluated following the methodology reported by Singleton, Orthofer, & Lamuela-Raventós (1998), with some modifications. A portion of 0.5 mL of nondigested or digested BFJ was mixed with 0.5 mL of F−C reagent and 10 mL of Na2CO3 (20%). The mixture was made up to 25 mL with distilled water and kept in the dark during 1 h at room temperature. Later, the samples were filtered using a 0.2 μm membrane, and the absorbance was measured at 725 nm (Cecil Instruments Ltd., Cambridge, UK). Concentrations were determined by comparing the sample's

absorbance with a calibration curve built with gallic acid. Results were expressed as milligrams of gallic acid per 100 mL of sample.

2.3.3.Carotenoids

Carotenoids were extracted and quantified by HPLC following the procedure reported by Morales-de La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2011), with some modifications. Nondigested or digested BFJ (6 mL) was mixed with 0.01 g of magnesium hydroxide carbonate, 0.01 g of butylhydroxytoluene (BHT), and 15 mL of ethanol/hexane solution (4:3 v/v) in an amber round-bottomflask under N2 atmosphere and continuous agitation during 45 min. Afterward, the mixture was filtered using a low-ash filter paper 70 mm (Albert-Hahnemuehle, S.L.U., Barcelona, Spain), and the residue was washed and again filtered once with 10 mL of ethanol/hexane solution (4:3 v/v), twice with 5 mL of ethanol, and once with 5 mL of hexane. All of the filtrates were combined and washed with 10 mL of distilled water and 10 mL of 10% NaCl solution in an amber decanting funnel, discarding the aqueous phase each time. The organic phase was rotoevaporated at 40 °C until dryness. Then, the residue was saponified with 5 mL of methanolic KOH 0.5 M + 0.1% of BHT (v/w) and 5 mL of diethyl ether, under N_2 atmosphere during 30 min. Later, 5 mL of diethyl ether was added, and the solution was washed with 10 mL of distilled water and 10 mL of 10% NaCl solution. The organic phase was mixed with 5 mL of ethanol and rotoevaporated at 45 °C until dryness. The residue was dissolved with 4 mL of diethyl ether and placed in an amber glass vial. Finally, the solvent was evaporated under N_2 atmosphere and stored at (−45 °C) until analysis.

Carotenoids were determined in the same HPLC equipment used to assess phenolic compounds. The diode array detector 2996 (Waters Corp.) was set to scan from 200 to 600 nm. Carotenoids were separated using a reverse-phase C18 Spherisorb ODS2 (5 μm) stainless steel column (4.6 mm×250 mm) operating at 30 °C with a flow rate of 1 mL/min. A gradient elution was carried out to separate these compounds (Morales-de La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2011). Four eluents were employed as mobile phase: (1) methanol/ammonium acetate 0.1 M, (2) Milli-Q water, (3) methyl tert-butyl ether, and (4) methanol. Individual carotenoids were identified according to the retention time and spectrum of standards, as well as by comparison of those reported in the literature. Carotenoid quantification was carried out by integrating

the peak areas and using calibration curves. Results were expressed as micrograms of carotenoid compound per 100 mL of sample.

2.4. Hydrophilic and Lipophilic Antioxidant Activity

Extraction of hydrophilic and lipophilic fractions of nondigested or digested BFJ was performed using the colorimetric method described by Brand-Williams, Cuvelier, & Berset (1995), as reported by Rodrıíguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013). Briefly, 5 mL of sample and 10 mL of methanol were mixed and centrifuged at 6000 rpm for 20 min at 4 °C. The supernatant was considered as the hydrophilic fraction, whereas the residue was mixed with 10 mL of tetrahydrofuran and centrifuged in the same conditions described above. The second supernatant was considered as the lipophilic fraction. The antioxidant activity was studied by evaluating the free radical scavenging effect of extracts on DPPH radical. Aliquots of 0.2 mL of hydrophilic or lipophilic extracts were mixed with 3.8 mL of DPPH methanolic solution (0.025 g/L). The homogenate was shaken vigorously and kept in the dark for 30 min. Afterward, the absorbance was measured at 515 nm against a blank of methanol. Results were expressed as percentage of DPPH inhibition, which can be related to the decrease in absorbance with respect to the control (methanolic solution of DPPH radical without extract).

2.5. Bioaccessibility Calculations

Bioaccessibility was expressed as percentage and determined using equation 1:

$$Bioaccessibility\,(\%) = \left(\frac{BC_{dial/micellar}}{BC_{non-digested}} \right) x100 \qquad \text{(Equation 1)}$$

where $BC_{dial/micellar}$ corresponds to the bioactive compound concentration in the dialyzed (for vitamin C and phenolic compounds) or micellar fractions (for carotenoids) and $BC_{non\text{-}digested}$ is the concentration in nondigested BFJ.

2.6. Statistical analysis

The *in vitro* gastrointestinal digestion of BFJ was carried out twice. Each studied parameter was analyzed three times in every *in vitro* gastrointestinal digestion (n = 6).

Results were reported as the mean ±standard deviation. Analysis of variance (ANOVA) of the results was performed to determine significant differences ($p < 0.05$) between the concentration of bioactive compounds in nondigested BFJ and that obtained in each digestive phase (Statgraphics Plus v.5.1, Rockville, MD, USA).

3. Results and discussion

3.1. Vitamin C

Vitamin C concentration of nondigested BFJ was 30.1 mg ascorbic acid/100 mL. The recommended nutrient intake (RNI) for vitamin C ranges between 40 and 45 mg per day (Cuervo et al., 2009). Therefore, the daily requirement of vitamin C is achieved by drinking 150 mL of the BFJ containing orange, pineapple, and kiwi.

The vitamin C concentration was reduced 17% in the gastric digesta, with regard to the content in nondigested BFJ (Figure 1). The acidic conditions of the gastric environment could protect the vitamin C against its chemical or enzymatic oxidation. This hypothesis is supported by Ball (2006), who reported that ascorbic acid was slowly attacked by oxygen when this molecule was fully protonated at low pH. Moreover, other authors demonstrated that gastric digestion had little effect on vitamin C stability, recovering 93% of this bioactive compound in broccoli inflorescences and 71% in pomegranate juice (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004).

On the other hand, the vitamin C concentration decreased 39% in the small intestinal digesta with regard to gastric digesta of BFJ. These results showed that vitamin C was unstable under intestinal conditions. The alkaline pH and some factors inherent to in vitro gastrointestinal digestion, such as temperature, oxygen, light, and the enzyme activity, could enhance the vitamin C oxidation or complex formation with other constituents. In this sense, Jeney-Nagymate & Fodor (2008) observed that the concentration of ascorbic acid decayed when the pH was >4. In addition, Ball (2006) reported that vitamin C oxidation in the gastrointestinal tract occurs due to its pro-oxidant behavior, maintaining the reduced state of other nutrients such as iron. This author also related the vitamin C degradation to the formation of metal−oxygen−ascorbate complexes (Ball, 2006).

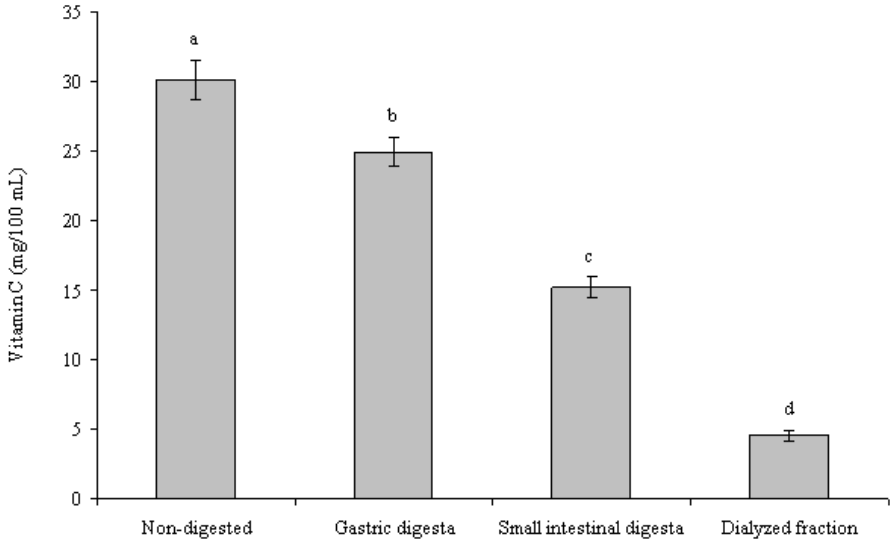

Figure 1. Vitamin C concentration during *in vitro* gastrointestinal digestion of a blended fruit juice. Different lowercase letters indicate significant differences ($p<0.05$).

The vitamin C contained in the BFJ displayed a bioaccessibility of 15.0%. As far as we know, few papers have studied the in vitro bioaccessibility of vitamin C from food. Cilla et al. (2012) reported a similar bioaccessibility (12.58%) of this compound in a beverage made with soy milk and fruit juices (orange, pineapple, kiwi, and mango). However, they obtained higher vitamin C bioaccessibility (up to 70.19%) when the juice was blended with whole or skimmed milk. A high vitamin C bioaccessibility (around 44 and 83.7%) has been also reported by other authors in blended fruit juices made with grape, sweet orange, apricot, and peach after 135 days of storage (Cilla et al. 2011).

Differences in these results and those found in the present research could be attributed to the food matrix composition, as well as to the fact that Cilla et al. (2011, 2012) did not use a dialysis membrane. In contrast, the vitamin C bioaccessibility was around 2.5% and about 3.2% in pomegranate juice and broccoli inflorescences, respectively (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004). The vitamin C obtained in this research was between 4- and 6-fold more highly bioaccessible than that found in pomegranate juice and broccoli inflorescence when a dialysis membrane was also used. Probably, synergistic interactions between the vitamin C and other constituents were stronger in a BFJ than in food prepared with a single fruit or vegetable. Although the vitamin C concentration is significantly reduced during small intestinal digestion,

human intervention studies demonstrated that the intake of orange juice and other vitamin C sources, such as potatoes and vegetables, increased the plasmatic concentration of this compound (Sánchez-Moreno et al. 2003; Kondo et al., 2012; Van Het Hof, Tijburg, Pietrzik, & Weststrate, 1999).

3.2. Phenolic Profile

Changes in phenolic profile due to in vitro gastrointestinal digestion of BFJ are shown in Table 1, where two main groups (phenolic acids and flavonoids) were identified by HPLC. Total phenolic compounds (TPC) were calculated either as the sum of individuals or by F−C method.

The concentrations of phenolic acids and flavonoids in nondigested BFJ were 6.49 and 29.3 mg/100 mL, respectively. TPC determined by F−C were 2.0-fold higher than TPC calculated by HPLC. It is well-known that F−C reagent could be reduced by other nonphenolic substances, such as sugars, amines, organic acids, proteins, and ascorbic acid, leading to overestimation of the phenolic content in samples (Prior, Wu, & Schaich, 2005).

Phenolic compounds showed different stabilities under gastric conditions. Chlorogenic and *p*-coumaric acids, as well as naringenin and rutin, increased their concentration, whereas ferulic and sinapic acids, hesperidin, quercetin, and (+)-catechin diminished. Caffeic acid remained unchanged in the gastric digesta. These results suggest that the phenolic stability might depend on some factors such as their physicochemical properties and the interaction with dietary or gastric constituents. Additionally, the low pH and the enzyme action of gastric digestion could hydrolyze some phenolic substances bound to proteins and carbohydrates from the food matrix, increasing the concentration of these bioactive compounds (Saura-Calixto, Serrano, & Goñi, 2007). Other studies also support these findings. Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013) and Tagliazucchi, Verzelloni, Bertolini, & Conte (2010) showed that gastric digestion improved the phenolic release in soy milk and grapes, respectively. Bermúdez-Soto, Tomás-Barberán, & García-Conesa (2007) reported that the concentration of caffeic acid derivatives of chokeberry juice did not change in the gastric digesta, whereas theflavan-3-ols content was reduced a 15%. On the other hand, Laurent, Besançon, & Caporiccio (2007) and Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán (2001) reported that gastric digestion had no effect on the

stability of flavonoids in grape seeds and orange juice, respectively. Differences between these results and those obtained in the present research could be explained by the fact that phenolic constituents may display antagonistic or synergistic interactions among themselves or with other substances, depending on the food matrix (Rice-Evans, Miller, & Paganga, 1997).

Table 1. Concentration of Phenolic Compounds During *in vitro* Gastrointestinal Digestion of a Blended Fruit Juice.[a]

Phenolic compounds	Concentration (mg/100 mL)				Bioaccessibility
	Non-digested	Gastric digesta	Small intestinal digesta	Dialyzed fraction	(%)
Phenolic acids					
Hydroxycinnamic acids					
Caffeic acid	0.332 ± 0.018*a*	0.337 ± 0.015*a*	0.267 ± 0.007*b*	nd	0.00*A*
Chlorogenic acid	3.00 ± 0.10*a*	3.279 ± 0.016*b*	2.97 ± 0.08*a*	0.329 ± 0.009*c*	11.0 ± 0.5*B*
p-Coumaric	1.06 ± 0.04*a*	1.204 ± 0.014*b*	1.007 ± 0.018*c*	0.176 ± 0.007*d*	16.7 ± 0.7*C*
Ferulic	0.81 ± 0.05*a*	0.452 ± 0.006*b*	0.293 ± 0.013*c*	0.211 ± 0.009*d*	25.97 ± 1.6*D*
Sinapic	1.30 ± 0.03*a*	0.470 ± 0.015*b*	0.411 ± 0.016*c*	0.229 ± 0.013*d*	17.7 ± 1.0*E*
Total phenolic acids	6.49 ± 0.15*a*	5.74 ± 0.04*b*	4.95 ± 0.06*c*	0.83 ± 0.08*d*	12.7 ± 1.3*F*
Flavonoids					
Flavanones					
Hesperidin	12.1 ± 0.4*a*	11.2 ± 0.3*b*	8.46 ± 0.06*c*	2.22 ± 0.10*d*	18.4 ± 0.7*E,G*
Naringenin	7.9 ± 0.3*a*	16.0 ± 1.0*b*	7.7 ± 0.6*a*	1.48 ± 0.03*c*	18.7 ± 0.7*G*
Flavonols					
Rutin	1.34 ± 0.06*a*	3.51 ± 0.08*b*	1.71 ± 0.08*c*	0.297 ± 0.010*d*	22.2 ± 0.8*H*
Quercetin	0.916 ± 0.008*a*	0.68 ± 0.03*b*	0.288 ± 0.014*c*	0.264 ± 0.012*d*	28.9 ± 1.5*I*
Flavan-3-ols					
(+)-Catechin	7.04 ± 0.05*a*	6.13 ± 0.07*b*	3.95 ± 0.04*c*	1.631 ± 0.018*d*	23.16 ± 0.13*J*
Total flavonoids	29.3 ± 0.6*a*	37.5 ± 0.8*b*	22.1 ± 0.7*c*	5.90 ± 0.09*d*	20.1 ± 0.4*K*
Total phenolic compounds					
Sum of individuals	35.8 ± 0.6*a*	43.3 ± 0.8*b*	27.1 ± 0.8*c*	6.73 ± 0.13*d*	18.8 ± 0.4*G*
Folin-Ciocalteu method	73.4 ± 1.3*a*	78.9 ± 1.3*b*	64.1 ± 1.1*c*	8.4 ± 0.5*d*	11.5 ± 0.6*B*

[a] Values are expressed as mean ± standard deviation. Lowercase letters in the same row show significant differences ($p<0.05$) among digestive phases. Capital letters within the column indicate significant differences among the bioaccessibility of phenolic compounds. nd= not detected.

During small intestinal digestion, the concentration of BFJ phenolic constituents significantly diminished. Flavonoid concentration was more reduced (41%) than that of phenolic acids (14%) in the small intestinal digesta with respect to gastric digesta. The phenolic instability under alkaline pH suggests that these compounds undergo several chemical reactions, mainly oxidation and polymerization, affording the formation of

other phenolic derivatives, such as chalcones, which are not available for absorption because of their high molecular weight and low solubility. In fact, it has been reported that alkaline conditions transform 50−60% of flavanones into chalcones (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001). Furthermore, some dietary constituents such as fiber, proteins, and iron reduce the solubility and availability of phenolic compounds (Saura-Calixto, Serrano, & Goñi, 2007; Argyri, Komaitis, & Kapsokefalou, 2006).

As it can be seen in Table 1, the bioaccessibility of TPC determined as the sum of individuals was 18.8 and 11.5% by F−C assay. Phenolic acids and flavonoids were 12.7 and 20.1% bioaccessible, respectively. Ferulic acid was the most bioaccessible phenolic acid (26.0%) and quercetin the most bioaccessible flavonoid (28.9%). In contrast, caffeic acid did not show bioaccessibility under the conditions assayed in this research. These results demonstrate that phenolic bioaccessi-bility is widely influenced by pH changes and interactions with other constituents during the gastrointestinal digestion of food. Similar results were displayed by Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán (2001), who observed aflavanone bioaccessibility between 11 and 36% in orange juice. On the other hand, Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera (2004) showed that ferulic and sinapic acids derivatives had a low bioaccessibility (8.2 and 1.8%, respectively). In other studies, the bioaccessibility of phenolic compounds was >35%. For instance, Cilla, González-Sarrías, Tomás-Barberán, Espín, & Barberá (2009) reported that hydroxycinnamic acids, flavanones, flavones, andflavan-3-ols from a fruit beverage made with grape, orange, and apricot were between 36 and 63% bioaccessible. Cilla et al. (2011) obseved a bioaccessibility of 90% in TPC determined by F−C in a fruit beverage (containing grape, sweet orange, apricot, and peach) after 135 days of storage. However, Cilla et al. (2011) & Cilla, González-Sarrías, Tomás-Barberán, Espín, & Barberá (2009) obtained the bioaccessible fraction by centrifugation instead of dialysis. This fact, as well the food matrix composition, could explain differences between the results obtained by these authors and that obtained in this research.

3.3. Carotenoid Profile

The in vitro gastrointestinal digestion influence on BFJ carotenoid profile is shown in Table 2. The concentrations of carotenes and xanthophylls in nondigested BFJ were 140 and 400μg/100 mL, respectively.

Table 2. Concentration of Carotenoids During *in vitro* Gastrointestinal Digestion of a Blended Fruit Juice.[a]

Carotenoids	Concentration (µg/100 mL)				Bioaccessibility (%)
	Non-digested	Gastric digesta	Small intestinal digesta	Micellar fraction	
Carotenes					
α-carotene	9.2 ± 0.3*a*	5.8 ± 0.4*b*	3.4 ± 0.3*c*	0.85 ± 0.06*d*	9.3 ± 0.8*A*
ß-carotene	131 ± 4*a*	65.8 ± 0.7*b*	30.2 ± 1.4*c*	13.7 ± 1.6*d*	10.42 ± 1.04*A,B*
Total carotenes	140 ± 4*a*	71.6 ± 0.8*b*	33.6 ± 1.6*c*	14.5 ± 1.6*d*	10.3 ± 1.0*A,B*
Xanthophylls					
Cis-violaxanthin+neoxanthin	81.8 ± 2.2*a*	29.8 ± 2.4*b*	9.17 ± 0.24*c*	6.20 ± 0.20*d*	7.6 ± 0.3*C*
Cis-antheraxanthin	141 ± 11*a*	60 ± 3*b*	19.7 ± 1.5*c*	10.6 ± 1.6*d*	7.5 ± 0.9*C*
Antheraxanthin	30.9 ± 1.6*a*	17.9 ± 1.1*b*	8.1 ± 0.3*c*	4.3 ± 0.5*d*	13.9 ± 1.6*D*
Lutein	109 ± 3*a*	97.3 ± 1.5*b*	63 ± 3*c*	18.9 ± 0.9*d*	17.4 ± 1.0*E*
α-cryptoxanthin	12.2 ± 1.0*a*	6.45 ± 0.22*b*	4.45 ± 0.22*c*	1.75 ± 0.07*d*	14.4 ± 1.4*D*
ß-cryptoxanthin	25.2 ± 1.5*a*	14.7 ± 1.2*b*	8.06 ± 0.24*c*	3.73 ± 0.23*d*	14.8 ± 1.4*D*
Total xanthophylls	400 ± 16*a*	226.6 ± 7*b*	113 ± 3*c*	45.5 ± 2.1*d*	11.4 ± 0.4*B*
Total carotenoids	540 ± 15*a*	298.2 ± 7*b*	146 ± 4*c*	60 ± 3*d*	11.1 ± 0.4*B*

[a] Values are expressed as mean ± standard deviation. Lowercase letters in the same row show significant differences ($p<0.05$) among digestive phases. Capital letters within the column indicate significant differences among the bioaccessibility of carotenoids.

Carotenes diminished 49% and xanthophylls 43% in the gastric digesta. Lutein was the carotenoid with the highest stability after gastric digestion (90%). However, the recoveries of α-andβ-carotenes, α-and β-cryptoxanthines, cis-violaxanthin + neoxanthin, cis-antheraxanthin, and antheraxanthin ranged from 36 to 63%. Most of the BFJ carotenoids were unstable under gastric conditions perhaps due to oxidation reactions. Carotenoids are known to be unstable in acidic media because they are susceptible to oxidation owing to the numerous double bonds of their chemical structure (Hedrén, Diaz, & Svanberg, 2002). Moreover, other factors, such as temperature and pH changes, can produce their oxidation (Rao & Rao, 2007). In line with the results obtained in this research, Rich et al. (2003) reported that trans-β-carotene concentration of raw spinach was reduced to 50% at low pH (2.5). Granado-Lorencio et al. (2007b) also showed that lutein was stable after gastric digestion of orange and kiwi fruits, recovering 80 and 100%, respectively.

The concentration of carotenoids decreased between 31 and 69% when the small intestinal digesta of BFJ was compared to gastric digesta. In this research, carotenoids were not detected in the dialyzed fraction. Likely, their concentration was below the limit of detection due to the fact that the digestion of BFJ was carried out without dietary fat, which enhances the absorption of carotenoids (Unlu, Bohn, Clinton, & Schwartz, 2005). Similarly, Faulks and Southon (2005) showed that when carotenoids were ingested with a meal, these compounds were almost completely absorbed. However, when carotenoids were consumed apart from a meal, their absorption was exceedingly smaller. In addition, intestinal epithelium contain other constituents (that were not present in the dialysis membrane) as membrane receptors (type B residual receptors, cluster of differentiation 36, and Niemann-PickC1-like 1 protein), enzymes (such as carboxyl-ester lipase), and proteins that facilitate the absorption of liposoluble compounds (Fernández-García et al., 2012). Carotenoids are highly hydrophobic compounds, which once released from the food matrix are dispersed in the gastrointestinal tract and solubilized in mixed micelles. Therefore, the formation of mixed micelles is one of the critical factors in carotenoid bioavailability (Nagao, 2011). With these findings taken into account, in the present study, the small intestinal digesta was centrifuged (5000 rpm/20 min at room temperature) to obtain the micellar fraction, which was considered to be the bioaccessible carotenoids (Granado-Lorencio et al., 2007a).

As can be seen in Table 2, the bioaccessibility of carotenoids ranged from 7.6% (cis-violaxanthin + neoxanthin and cis-anteraxanthin) to 17.4% (lutein). Similar results were reported by Granado-Lorencio et al., (2007a) who observed 25% lutein bioaccessibility in orange fruit. Dhuique-Mayer et al. (2007) showed that β-cryptoxanthin was between 16 and 40% bioaccessible in different citrus juices (orange, mandarin, and lemon). Daly, Jiwan, O'Brien, & Aherne (2010) observed thatβ-carotene was up to 19% bioaccessible in different herb types, whereas β-cryptoxanthin and lutein + zeaxanthin were up to 27% bioaccessible. Hedrén, Diaz, & Svanberg (2002) showed that 3% of β-carotene from raw carrot pieces was bioaccessible. Hedrén, Diaz, & Svanberg (2002) also observed an increase of β-carotene bioaccessibility when the raw carrot pieces were made pure, cooked pulp or added oil, highlighting the importance of the particle size and oil presence on carotene bioaccessibility. Moreover, Cilla et al. (2012) reported that the food matrix has a significant influence on the bioaccessibility of carotenoids, such

as β-carotene, lutein, and β-cryptoxanthin, which displayed higher bioaccessibility when a blended juice was combined with whole milk (up to 148%) than with skimmed or soy milk (up to 63 and 38%, respectively).

In general, xanthophylls contained in the BFJ were more bioaccessible than carotenes. Changes in the molecular structure of carotenoids and a competitive inhibition between themselves could occur, affecting their incorporation into micelles, intestinal uptake, or lymphatic transport (Maiani et al., 2009). Granado-Lorencio et al. (2007a), Nagao (2011), and Chitchumroonchokchai, Schwartz, & Failla (2004) observed the same trend in in vitro carotenoid bioaccessibility: the xanthophylls zeaxanthin and β-cryptoxanthin were more available for absorption than carotenes. In addition, the results obtained in this research are in accordance with those reported in animal and human intervention studies, where the bioaccessibility and absorption rate of carotenoids is low because they interact with macromolecules within the food matrix, such as fiber, which decreases carotenoid absorption by entrapping them and increasing their fecal excretion (Hoffmann, Linseisen, Riedl, & Wolfram, 1999).

3.4. Antioxidant Activity

Total antioxidant activity (TAA) of BFJ was determined as the sum of hydrophilic and lipophilic antioxidant activity (HAA and LAA, respectively). The antioxidant activity in fruit juices depends on the composition and concentration of its antioxidants, such as vitamins, phenols, and carotenoids (Liu, 2003). Nondigested BFJ displayed 98% DPPH inhibition, of which 81% corresponded to the hydrophilic fraction and 17% to the lipophilic (Figure 2). Ryan and Prescott (2010) observed that the antioxidant activities of different fruit juices, including orange and pineapple juices, had a variation between 31 and 85% DPPH inhibition.

The HAA decreased 9% and LAA 38% in the gastric digesta, demonstrating that hydrophilic constituents were less affected by gastric digestion than those lipophilics. Phenolic compounds are among the most important BFJ bioactive compounds with hydrophilic antioxidant activity. These compounds exhibited a high stability under gastric conditions, which could explain the results obtained in this research. Furthermore, a significant correlation between the HAA and the TPC determined by HPLC was observed (r^2= 0.8198, p= 0.0458).

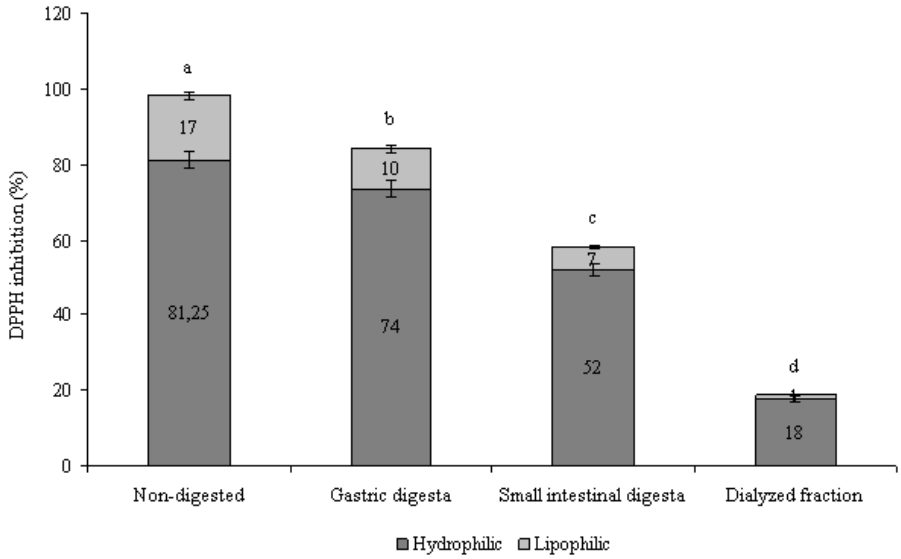

Figure 2. Changes in hydrophilic and lipophilic antioxidant activity during *in vitro* gastrointestinal digestion of a blended fruit juice. Different lowercase letters indicate significant differences (p<0.05) in the total antioxidant activity.

The antioxidant activity of both hydrophilic and lipophilic compounds diminished significantly (p< 0.05) during the small intestinal digestion of BFJ, for which HAA and LAA were reduced 30 and 37%, respectively, compared to that obtained in the gastric digesta. The greatest losses of antioxidant activity were observed in the dialyzed fraction, where the DPPH inhibition for HAA was 18 and 1% for LAA. These results suggest that most BFJ bioactive compounds with antioxidant activity are unstable under intestinal conditions. The alkaline pH, as well as the digestive enzymes action, could transform these bioactive compounds in other substances with different chemical and physical properties, such as the conversion of flavanones into chalcones or complex formation among the bioactive compounds and other dietary constituents. Moreover, HAA displayed a good correlation with the vitamin C (r^2=0.9819, p= 0.0001) and the phenolic compounds determined as the sum of individuals (r^2= 0.9502, p= 0.0002) during the in vitro gastrointestinal digestion. These data related the decrease in the HAA to the loss of both vitamin C and phenolic compounds during digestion of BFJ. A significant correlation was also found between LAA and total carotenoids (r^2=0.9770, p= 0.0001). This could explain the similar behaviour between the LAA and the carotenoids during the in vitro gastrointestinal digestion of BFJ, although the contribution of other lipophilic compounds, such as fatty acids and some liposoluble vitamins, is not discarded.

The bioaccessibilities of hydrophilic and lipophilic com-pounds contained in BFJ were 21.6 and 7.89%, respectively. A similar pattern was reported by Rodríguez-Roque et al. (2013), who observed greater bioaccessibility of hydrophilic than of lipophilic soy milk constituents. Changes in the food environment, such as heat, pH, additives, and modification of particle size, may alter the functional and structural properties of vitamins (Ball, 2006), phenolic compounds, and carotenoids. As a result, the bioaccessibility of these compounds could also be influenced by these factors.

Results obtained in this research reveal the amount of bioactive compounds from a blended juice containing orange, pineapple, and kiwi that could be released from the food matrix and could be available for absorption in vivo. Therefore, in vitro methodologies, such as gastrointestinal digestion, allow rapid progress in understanding physicochemical changes, inter-actions, and bioaccessibility of bioactive compounds.

As can be seen in previous sections, the quantity of ingested compound differs widely from the bioaccessible fraction using this in vitro methodology. If these results could be corroborated by in vivo studies, it would mean that small quantities of bioaccessible compounds could influence cellular activities that modify the risk of several diseases and could be potentially beneficial in improving health.

On the other hand, the assessment of bioaccessibility and bioavailability of compounds with biological activity contained in food might provide more specific information concerning dietary requirements of these constituents to achieve health benefits beyond recommended dietary patterns reported at this time. Consequently, in vivo experimental designs are necessary to understand the biological effects and mechanisms of action of bioactive constituents of food.

In conclusion, most BFJ bioactive compounds were quite stable in the gastric digesta. Only small changes in the concentration of phenolic compounds and vitamin C were observed after acidic conditions. However, the concentration of all the analyzed compounds, as well as the antioxidant activity, diminished significantly during small intestinal digestion. The bioaccessibility of hydrophilic bioactive compounds was higher (vitamin C, 15%; phenolic acids, 13%; and flavonoids, 20%) than that of lipophilics (carotenes, 10%; and xanthophylls, 11%). Results suggest that, despite the significant decrease in the concentration of these bioactive compounds after being subjected to in vitro gastrointestinal digestion, the bioaccessi-bility of BFJ constituents could be high enough to be absorbed and utilized. The results obtained in this research

should be compared with additional in vivo studies to correlate the bioaccessibility of BFJ bioactive compounds between *in vivo* and *in vitro* methodologies.

Funding

This research has beenfinanced by the Ministerio de Ciencia y Tecnología (Spain), reference AGL2006-12758-C02-02/ALI. M.J.R.-R. thanks the Comissionat per a Universitats i Recerca, del Departament d'Innovació , Universitats i Empresa de la Generalitat de Catalunya (AGAUR), and European Social Fund for the predoctoral grant, as well as the Secretaría de Educación Pública de Mé xico (SEP) for their support. O.M.-B. thanks the Institució Catalana de Recerca i Estudis Avanç ats (ICREA) for the Academia Award 2008.

Abbreviations list

BFJ Blended fruit juice
BHT Butylhydroxytoluene
DPPH 1,1-diphenyl-2-picrylhydrazyl
DTT DL-1,4-dithiotreitol
FAO Food Agriculture Organization of the United Nations
F-C Folin-Ciocalteu
HAA Hydrophilic antioxidant activity
HPLC High-performance liquid chromatography
LAA Lipophilic antioxidant activity
RDA Recommended dietary allowances
TAA Total antioxidant activity
TPC Total phenolic compounds
WHO The World Health Organization

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CHAPTER III

In vitro bioaccessibility of health-related compounds from a blended fruit juice-soymilk beverage: Influence of the food matrix

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Journal of Functional Foods (2014) 7: 161-169

Abstract

This research evaluated the effect of an *in vitro* gastrointestinal digestion on the concentration of vitamin C, phenolic compounds, isoflavones, carotenoids, as well as antioxidant activity of a beverage containing a blended fruit juice and soymilk (BFJ–SMB). Additionally, the influence of the food matrix on the bioaccessibility of these compounds was studied. BFJ–SMB hydrophilic constituents displayed higher bioaccessibility (12–26.5%) than those lipophilics (6.5–13.8%). The most bioaccessible compounds were quercetin and genistein, while cis-violaxanthin+neoxanthin were the lowest. Several compounds were less bioaccessible in BFJ–SMB (6.5–14%) with respect to those of BFJ or SM. Conversely, phenolic acids and aglycone isoflavones displayed their highest bioaccessibility in BFJ–SMB. Results showed that both the *in vitro* gastrointestinal digestion and the food matrix exerted a significant influence on the bioaccessibility of these compounds. BFJ–SMB can be considered as a beverage with an important nutritional quality and highly bioaccessible substances.

Keywords: Blended fruit juice–soymilk beverage; Bioaccessibility; Bioactive compounds; Antioxidant activity; Food matrix

1. Introduction

Consumption of five portions of fruits and vegetables per day contributes to prevent several cardiovascular and neurodegenerative diseases, as well as some cancer types (FAO/WHO, 2003). However, the actual intake of fruit and vegetables in adult population is below this recommendation. In this sense, fruit and vegetable juices provide a convenient way for increasing the consumption of several nutrients, such as phenolic compounds, vitamins and carotenoids (Ryan and Prescott, 2010; Wootton-Beard, Moran, and Ryan, 2011).

On the other hand, the popularity of soy-based beverages, including soymilk, is growing due to they contain great amounts of phenols, isoflavones, amino acids and minerals (Jinapong, Suphantharika, & Jamnong, 2008; Tyug, Prasad, & Ismail, 2010). In addition, soymilk is a good alternative for people that cannot consume dairy products due to they have lactose intolerance, milk protein allergy or galactosemia(Xu & Chang, 2009). The disadvantage of soy-derived products is their characteristic beany flavour that is not accepted by a great part of population (Oliveira et al., 2010). As alternative, mixed beverages based on blended fruit juices (BFJ) and soy-milk (SM) not only mask the soymilk beany flavour, but also combine the sensorial and nutritional properties of both products.

Previous reports have documented that mixing fruit juices with soymilk improve the nutritional properties of these beverages (Morales-De La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2010a, b; 2011; Potter, Dougherty, Halteman, & Camire, 2007; Rau de Almedia Callou, Sadigov, Lajolo, & Genovese, 2010). Nevertheless, it is important to take into consideration that only a small fraction from the amount of ingested compound is absorbed and utilized by the human body. Several bioactive compounds must be first released from the food matrix and/or modified by the gastrointestinal tract to exert their biological functions. In this sense, *in vitro* gastrointestinal digestion is a quick and useful methodology for assessing the bioaccessibility of dietary compounds, in comparison to *in vivo* trials which are expensive and long term studies (Failla, & Chitchumroonchokchai, 2005).

Information about the bioaccessibility of some bioactive compounds in fruit juices is available in the literature (Cilla, González-Sarrías, Tomás-Barberán, Espín, and Barberá, 2009; Cilla et al., 2011; Gil-Izquierdo, Gil, Ferreres, and Tomás-Barberán,

2001; Pérez-Vicente, Gil-Izquierdo, and García-Viguera, 2002; Granado-Lorencio, Herrero-Barbudo, Blanco-Navarro, Pérez-Sacristán and Olmedilla-Alonso, 2009; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, and Martín-Belloso, 2013a). However, although it is well known that the food matrix is one of the most important factors that affect the bioaccessibility of bioactive substances, there is really scarce information about the bioaccessibility of these compounds in complexes matrices (Cilla et al., 2012).

Due to the increasing interest in functional beverages and the few studies that have evaluated the bioaccessibility of bioactive compounds contained in mixed beverages, the aims of this research were: first, to determine the effect of an *in vitro* gastrointestinal digestion on the con-centration of vitamin C, phenolic compounds, carotenoids, as well as antioxidant activity of a beverage made with a blend of fruit juices (orange, pineapple and kiwi) and soy-milk (BFJ–SMB); second, to compare the bioaccessibilities of BFJ–SMB bioactive substances with those previously obtained in our laboratory using the same *in vitro* gastrointestinal methodology in both soymilk (SM) and a blended-fruit juice (BFJ), separately, in order to evaluate the influence of the food matrix.

2. Materials and methods

2.1. Reagents

Pepsin from porcine stomach, pancreatin from porcine pancreas, bovine bile, phenol standards (caffeic, chlorogenic, *p*-coumaric, ferulic, sinapic and 4-hydroxybenzoic acids; hesperidin, naringenin, rutin, quercetin and [+]-catechin), isoflavone standards (daidzin, genistin, glycitin, daidzein, genistein and glycitein), carotenoid standards (α-carotene, β-carotene, zeaxanthin, lutein,α-cryptoxanthin and β-crypto-xanthin), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and cellulose dialysis membrane (molecular weight cutoff of 12,000 Da) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ascorbic acid, gallic acid and Folin–Ciocalteu (F–C) reagent were acquired from Scharlau Chemie S.A. (Barcelona, Spain).

2.2. Beverage preparation

Orange, kiwi and pineapple fruits were purchased at commercial maturity in a local supermarket. Each fruit was washed, peeled and the juice extracted. Later, every juice was filtered through a cheesecloth using a vacuum pump. Fruit juices were combined in

the following proportions to obtain a blended fruit juice (BFJ): orange 50%, kiwi 36% and pineapple 14%.

The blended fruit juice–soymilk beverage (BFJ–SMB) was prepared by mixing 50.0% of BFJ, 42.5% of soymilk (SM) (Yosoy, Girona, Spain) and 7.5% of sugar. Finally, the pH of the beverage was adjusted to 3.7 with citric acid (Crison Instruments SA, Alella, Barcelona, Spain). The solid soluble content was measured in a refractometer (Comecta S.A., Abrera, Barce-lona, Spain), resulting in 15.0 ± 0.12 ºBrix.

The formulation of this beverage was selected based on a previous study where the combination of these fruit juices displayed high concentrations of vitamin C, carotenoids and phenolic compounds, as well as antioxidant activity (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013a)

2.3. *In vitro* gastrointestinal digestion

An *in vitro* gastrointestinal digestion was performed following the methodology previously carried out in our laboratory (Rodríguez-Roque et al., 2013a), which consisted of gastric digestion (pH 2, containing pepsin) and small intestinal digestion (pH 7, containing a pancreatin–bile mixture). Small intestinal digestion includes dialyzed (bioactive compounds of inside the dialysis membrane) and micellar fractions (supernatant of small intestinal digesta centrifuged at 5000 rpm for 20 min), containing the bioaccessible hydrophilic and lipophilic compounds, respectively.

Aliquots were taken at the end of each digestive phase and immediately placed in a cold water bath during 10 min and frozen (-45 ºC) until analysis to monitor the changes in bioactive compounds concentration along the *in vitro* gastrointestinal digestion of BFJ–SMB.

2.4. Bioactive compound analysis

2.4.1. Vitamin C

Extraction, separation, identification and quantification of vitamin C were performed according to the method validated by Odriozola-Serrano, Hernández-Jover, & Martín-Belloso (2007), with some modifications (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013a). The identification of vitamin C was performed by

HPLC, comparing the retention time and UV–visible absorption spectrum of samples with the standard (ascorbic acid).
Results were expressed as mg of ascorbic acid/100 mL of sample.

2.4.2. Phenolic compounds

Phenolic compounds were determined by both HPLC and Folin–Ciocalteu methodologies, as follows:

2.4.2.1. Phenolic compounds determined by HPLC. Phenolic compounds were extracted, separated, identified, and quantified using the methodology of Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b). Quantification of individual phenols was carried out integrating the peak areas and using calibration curves. Results were expressed as mg of phenolic compound/100 mL of sample. Total phenolic compounds (TPC) were calculated as the sum of individuals.

2.4.2.2. Total phenolic content determined by Folin–Ciocalteu (F–C) methodology. Total phenolic content was performed through the methodology reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b), which is based on the colorimetric method previously described by Singleton, Orthofer, and Lamuela-Raventós (1998). Concentrations were determined by comparing the absorbance of samples with a calibration curve built with gallic acid. Results were expressed as mg of gallic acid/100 mL of sample.

2.4.3. Isoflavones

Extraction, separation, identification and quantification of isoflavones were performed according to the method of Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b), which is based on the methodology reported by Morales-de La Peña et al. (2010b). Isoflavones were identified by HPLC, comparing their spectrum and retention time with the standards. Quantification of isoflavones was carried out by integration of the peak areas. Results were expressed as mg of isoflavone/100 mL of sample. Total isoflavone concentration was calculated as the sum of individuals.

2.4.4. Carotenoids

Carotenoids were extracted, separated, identified and quantified following the methodology of Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a). Individual carotenoids were identified by HPLC, comparing their retention time and spectrum with the standards or with those reported in the literature. Quantification of carotenoids was carried out by integration of the peak areas and using calibration curves. Results were expressed as μg of carotenoid/100 mL of sample. Total carotenoids were calculated as the sum of individuals.

2.5. Hydrophilic and lipophilic antioxidant activity

Extraction of hydrophilic and lipophilic fractions of non-digested or digested BFJ–SMB was carried out as reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b). The antioxidant activity was evaluated through the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Results were expressed as percentage of DPPH inhibition, which can be related to the decrease in the absorbance with respect to the control (methanolic solution of DPPH radical without extract).

2.6. Bioaccessibility calculations

Bioaccessibility was determined using Eq. 1 and expressed as percentage.

$$Bioaccessibility(\%) = \left(\frac{BC_{dialyzed/micellar}}{BC_{non-digested}} \right) x100 \qquad \text{(Eq. 1)}$$

where $BC_{dialyzed/micellar}$ corresponded to the bioactive compound concentration in the dialyzed (for vitamin C, phenolic compounds and isoflavones) or micellar (for carotenoids) fractions and $BC_{non\text{-}digested}$ was the concentration in non-digested BFJ-SMB.

2.7. Statistical analysis

The *in vitro* gastrointestinal digestion was carried out twice. Each studied parameter was evaluated three times in every *in vitro* digestion (n= 6). Results were reported as the mean ± standard deviation. Analysis of variance (ANOVA) was performed to determine significant differences ($P< 0.05$) between the concentration of bioactive compounds in non-digested and digested samples, as well as among the bioaccessibilities. All the

statistical analyses were carried out with the program Statgraphics Plus v.5.1 (Rockville, MD, USA).

3. Results and discussion

3.1. Influence of in vitro gastrointestinal digestion on the concentration and bioaccessibility of bioactive compounds from BFJ–SMB

3.1.1. Vitamin C

The concentration of vitamin C along the *in vitro* gastrointestinal digestion of BFJ–SMB is showed in Fig. 1. Non-digested beverage showed a concentration of this bioactive compound of 23.1 mg/100 mL.

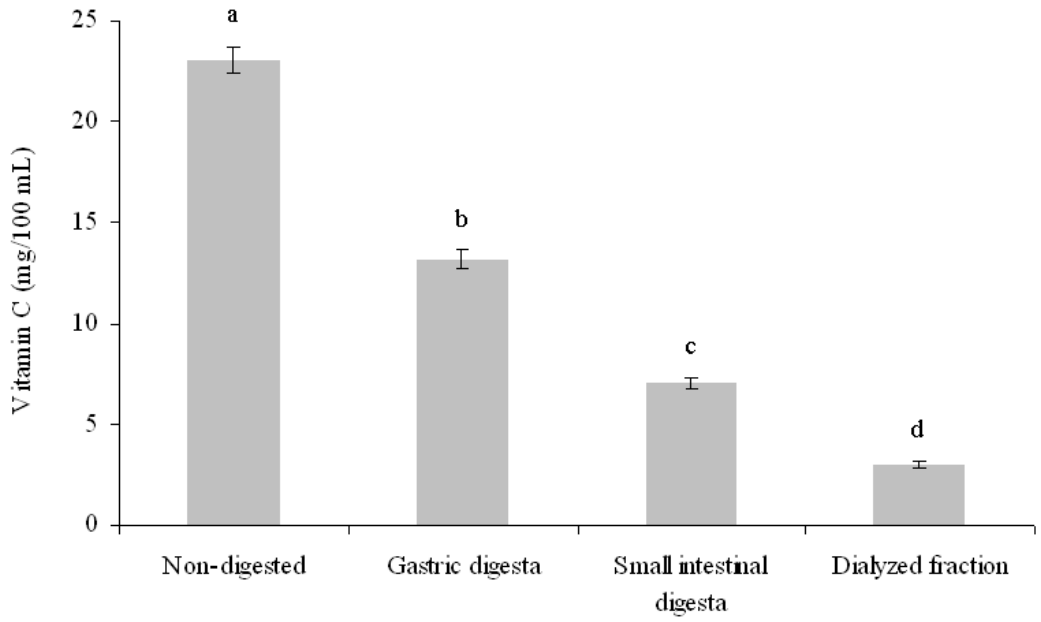

Figure 1. Changes in the concentration of vitamina C along the *in vitro* gastrointestinal digestion of a blended fruit juice-soymilk beverage (BFJ-SMB). Different lower case letters show significant differences ($P < 0.05$) among digestive phases.

Vitamin C was significantly affected by the gastric digestion ($P < 0.05$), where losses of 43% were observed in comparison to non-digested product. A similar decrease of this compound (46%) was observed when the small intestinal digesta and the gastric digesta were compared. In the dialyzed fraction, the concentration of vitamin C diminished a 57% with the regard to that found in the small intestinal digesta. Results suggest that

vitamin C is affected by the changes of pH during *in vitro* gastrointestinal digestion which could modify the physicochemical properties of this compound (i.e. chemical structure and solubility). Additionally, it has been reported that vitamin C is easily oxidized at pH > 4 and degraded by interactions with metal ions (Ball, 2006; Jeney-Nagymate, E. and Fodor, P., 2008).

The vitamin C contained in BFJ–SMB displayed a bioaccessibility of 13%. Similarly, Cilla et al. (2012) reported a vitamin C bioaccessibility of 12.8% in a beverage containing a mixture of fruit juices and soymilk.

3.1.2. Phenolic profile

TPC determined as the sum of individuals were 36.6 mg/100 mL in non-digested BFJ–SMB and 74.0 mg/100 mL by F–C assay (Table 1).

Table 1. Changes in the concentration of phenolic compounds during the *in vitro* gastrointestinal digestion of a blended fruit juice-soymilk beverage (BFJ-SMB).[a]

Phenolic compounds	Concentration (mg/100 mL)				Bioaccessibility (%)
	Non-digested	Gastric digesta	Small intestinal digesta	Dialyzed fraction	
Phenolic acids					
Caffeic acid	0.453 ± 0.017a	0.314 ± 0.016b	0.219 ± 0.012c	0.00 ± 0.00d	0.00 ± 0.00A
Chlorogenic acid	2.10 ± 0.12a	2.16 ± 0.13a	1.50 ± 0.06b	0.251 ± 0.012c	12.0 ± 0.9B
Gallic acid	nd	nd	nd	nd	nd
p-Coumaric acid	0.91 ± 0.04a	0.75 ± 0.04b	0.345 ± 0.020c	0.141 ± 0.008d	15.6 ± 0.7C
Ferulic acid	1.34 ± 0.06a	0.80 ± 0.04b	0.501 ± 0.023c	0.255 ± 0.012d	19.0 ± 1.5D
Sinapic acid	1.74 ± 0.07a	1.04 ± 0.06b	0.726 ± 0.024c	0.241 ± 0.010d	13.9 ± 0.9E
4-Hydroxybenzoic acid	nd	nd	nd	nd	nd
Total phenolic acids	6.54 ± 0.17a	5.06 ± 0.17b	3.30 ± 0.08c	0.89 ± 0.03d	13.6 ± 0.6E
Flavonoids					
Hesperidin	12.5 ± 0.5a	15.2 ± 0.8b	9.8 ± 0.5c	1.98 ± 0.10d	15.9 ± 1.1CF
Naringenin	8.2 ± 0.3a	8.3 ± 0.4a	4.60 ± 0.20b	1.51 ± 0.07c	18.4 ± 1.1D
Rutin	1.67 ± 0.07a	1.67 ± 0.05a	0.97 ± 0.04b	0.283 ± 0.017c	17.0 ± 1.0FG
Quercetin	1.26 ± 0.06a	1.81 ± 0.08b	0.96 ± 0.05c	0.332 ± 0.016d	26.5 ± 1.7H
(+)-Catechin	6.4 ± 0.3a	4.7 ± 0.3b	4.22 ± 0.16c	1.54 ± 0.05d	24.0 ± 1.7I
Total flavonoids	30.1 ± 0.6a	31.7 ± 1.0a	20.5 ± 0.7b	5.65 ± 0.18c	18.8 ± 0.7D
Total phenolic compounds					
Sum of individuals	36.6 ± 0.7a	36.8 ± 1.0a	23.8 ± 0.7b	6.54 ± 0.19c	17.9 ± 0.7DG
Folin-Ciocalteu method	74.0 ± 2.3a	89 ± 5b	63.3 ± 1.6c	13.4 ± 0.7d	18.1 ± 0.8DG

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same row indicate significant differences ($P < 0.05$) among digestive phases.

The concentration of phenolic acids decreased from 18% (*p*-coumaric acid) to 40% (ferulic and sinapic acids) during gastric digestion, with the exception of chlorogenic acid, which remained unchanged ($P > 0.05$). In contrast, the concentration of flavonoids varied under acidic conditions: hesperidin and quercetin increased 22% and 44%, respectively; naringenin and rutin did not change their concentration, and (+)-catechin diminished 26%. Likely, the acidic pH and the digestive enzyme action liberate some of these compounds from the food matrix, increasing their concentration. In line to these results, Saura-Calixto, Serrano, & Goñi (2007) reported that gastric enzymes hydrolyze the phenolic compounds bound to proteins and carbohydrates from different foods. Liyana-Pathirana and Shahidi (2005) and Tagliazucchi, Verzelloni, Bertolini, and Conte (2010) also observed an improvement in the release of phenolic substances after gastric digestion. On the other hand, the decrease in the concentration of these compounds could be explained by interactions with other food constituents, causing changes in their molecular weight, solubility and chemical structure, among others (Scalbert& Williamson, 2000).

It was observed that (+)-catechin showed the highest stability (89% of recovery) when the small intestinal digesta was compared to gastric digesta; whereas *p*-coumaric acid showed the lowest stability (46%). In the dialyzed fraction, the decrease in the concentration of phenolic compounds ranged from 74% to 88%, except for caffeic acid (loss of 100%). The instability of these compounds under alkaline conditions could be attributed to the fact that these com-pounds undergo several changes, such as oxidation, polymerization and transformation. In fact, the reduction in the concentration of phenolic substances under alkaline conditions was related to the complexes formation between these compounds and metal ions, proteins and/or fiber (Argyri, Komaitis, & Kapsokefalou, 2006; Saura-Calixto, Serrano, & Goñi, 2007). In addition, Gil-Izquierdo et al. (2001) observed that between 50% and 60% of flavanones from orange juice were transformed into chalcones after *in vitro* intestinal digestion.

The bioaccessibility of TPC determined either as the sum of individuals or by F–C methodology was around 18%. Flavonoids were 15.9–26% bioaccessible, while phenolic acids from 0% to 19% (Table 1). Quercetin was the most bioaccessible phenolic compound of BFJ–SMB (26.5%) and caffeic acid the lowest (0%). In line to these results, Gil-Izquierdo et al. (2001) reported a flavanone bioaccessibily between

11% and 36% in orange juice. Vallejo, Gil-Izquierdo, A., Pérez-Vicente, A., & García-Viguera (2004) obtained bioaccessibilities of ferulic and sinapic acids of 8.2% and 1.8%, respectively. Cilla et al. (2011) and Cilla et al. (2009) reported higher concentration of phenolic compounds (up to 90%) in different beverages, but they did not use a dialysis membrane, which could explain differences in the results.

3.1.3. Isoflavone profile

Two glucoside (daidzin and genistin) and two aglycone forms of isoflavones (daidzein and genistein) were detected throughout the *in vitro* gastrointestinal digestion of BFJ–SMB (Table 2). The concentrations of glucosides and aglycones were 15.5 and 4.92 mg/100 mL, respectively, in non-digested BFJ–SMB.

Table 2. Changes in the concentration of isoflavones during the *in vitro* gastrointestinal digestion of a blended fruit juice-soymilk beverage (BFJ-SMB).[a]

Isoflavones	Concentration (mg/100 mL)				Bioaccessibility (%)
	Non-digested	Gastric digesta	Small intestinal digesta	Dialyzed fraction	
Glucosides					
Daidzin	6.25 ± 0.21a	2.78 ± 0.14b	1.70 ± 0.11c	0.93 ± 0.04d	14.8 ± 1.0A
Genistin	9.26 ± 0.17a	4.81 ± 0.18b	4.07 ± 0.08c	2.41 ± 0.08d	26.0 ± 1.1B
Glycitin	nd	nd	nd	nd	nd
Total glucosides	15.5 ± 0.3a	7.6 ± 0.3b	5.77 ± 0.11c	3.33 ± 0.09d	21.5 ± 0.8C
Aglycones					
Daidzein	1.91 ± 0.08a	1.52 ± 0.04b	1.23 ± 0.05c	0.312 ± 0.015d	16.4 ± 1.3D
Genistein	3.01 ± 0.12a	2.81 ± 0.18b	1.95 ± 0.06c	0.80 ± 0.03d	26.5 ± 0.9B
Glycitein	nd	nd	nd	nd	nd
Total aglycones	4.92 ± 0.14a	4.33 ± 0.21b	3.19 ± 0.08c	1.11 ± 0.03d	22.6 ± 1.0C
Total isoflavones	20.4 ± 0.4a	11.9 ± 0.4b	8.96 ± 0.14c	4.44 ± 0.09d	21.7 ± 0.6C

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same row indicate significant differences ($P< 0.05$) among digestive phases. Different capital letters in the same column show significant differences among the bioaccessibility of isoflavones. nd = not detected.

Daidzein and genistein were less affected by gastric conditions (recovery > 79%) than their glucoside form (recovery from 44% to 52%). The isoflavone concentration diminished significantly when the small intestinal digesta was compared with that of non-digested BFJ–SMB (losses between 35% and 73%). Similarly, the concentration of glucosides and aglycones diminished 41–75% in the dialyzed fraction with regard to the

small intestinal digesta. The decrease in the concentration of isoflavones during gastric digestion could be attributed to the lack of solubility of these compounds under acidic conditions (Piskula, 2000). In addition, it was found that the pH of gastric digestion cause changes in the concentration of isoflavones and the combination of intestinal enzymes plus bile played an active role in the recovery and partition of these compounds (Sanz & Luiten, 2006).

As shown in Table 2, total glucoside and aglycone forms of isoflavones showed the same bioaccessibility. Around 26% of genistin and genistein were bioaccessible, followed by daidzein (16.4%) and daidzin (14.8%). Information regarding the bioaccessibility of isoflavones in beverages is really limited but the bioaccessibilities of glucoside and aglycone forms of isoflavones in soymilk were 42% and 17%, respectively (Rodríguez-Roque et al., 2013b).

3.1.4. Carotenoid profile

The effect of *in vitro* gastrointestinal digestion on BFJ–SMB carotenoid profile is presented in Table 3. Total carotenoid concentration was determined as the sum of individuals (carotenes and xanthophylls), resulting in 223 μg/100 mL in non-digested BFJ–SMB.

All the carotenoids analyzed in this study diminished their concentration after *in vitro* gastrointestinal digestion of BFJ–SMB. β-cryptoxanthin and α-carotene were the most stable carotenoids during gastric digestion, with recoveries of 79% and 81%, respectively. However, *cis*-violaxanthin + neoxanthin showed the lowest stability under acidic conditions (recovery of 33%). Significant losses in the content of carotenoids were observed when the small intestinal digesta was compared to gastric digesta (in the range of 11–58%). The smallest carotenoid concentration was observed in the micellar fraction, where 30% of total carotenes and 21% of total xanthophylls were obtained with respect to the small intestinal digesta. The decrease in the concentration of carotenoids could be related to the fact that carotenoids are molecules highly oxidized due to the double bounds of their chemical structure (Hedrén, Diaz, & Svanberg, 2002). Other important factor to take into consideration is the change of pH during the *in vitro* gastrointestinal digestion, which could affect the carotenoid stability (Rao & Rao, 2007).

On the other hand, the bioaccessibilities of α-carotene and β-carotene were 7.5% and 9.1%, respectively. Antheraxanthin and lutein were the most bioaccessible xanthophylls (13.8%). A similar carotenoid bioaccessibility was found in a blend of fruit juices (11%) (Rodríguez-Roque et al., 2013a). In contrast, Cilla et al. (2012) reported that the bioaccessibility of carotenoids was around 39.84% in a blend of fruit juices and soymilk. Differences in these results could be due to food matrix effect and methodological procedures.

Table 3. Changes in the concentration of carotenoids during the *in vitro* gastrointestinal digestion of a blended fruit juice-soymilk beverage (BFJ-SMB).[a]

Carotenoids	Concentration (µg/100 mL)				Bioaccessibility
	Non-digested	Gastric digesta	Small intestinal digesta	Micellar fraction	(%)
Carotenes					
α-carotene	4.20 ± 0.23a	3.30 ± 0.21b	2.91 ± 0.09c	0.317 ± 0.018d	7.5 ± 0.6A
ß-carotene	68 ± 4a	45 ± 4b	18.8 ± 0.9c	6.2 ± 0.3d	9.1 ± 0.6BC
Total carotenes	72 ± 4a	49 ± 4b	21.7 ± 0.9c	6.5 ± 0.3d	9.0 ± 0.6B
Xanthophylls					
Cis-violaxanthin+neoxanthin	28.8 ± 1.0a	9.6 ± 0.5b	7.7 ± 0.4c	1.86 ± 0.08d	6.5 ± 0.4D
Cis-antheraxanthin	54.8 ± 2.3a	27.1 ± 1.8b	24.2 ± 1.5c	3.95 ± 0.23d	7.20 ± 0.22A
Antheraxanthin	14.4 ± 0.8a	8.6 ± 0.4b	7.6 ± 0.5c	1.98 ± 0.06d	13.8 ± 0.9EF
Lutein	38.7 ± 2.4a	26.0 ± 1.7b	22.7 ± 1.0c	5.3 ± 0.3d	13.8 ± 0.4F
α-cryptoxanthin	4.6 ± 0.3a	3.20 ± 0.19b	2.55 ± 0.17c	0.54 ± 0.04d	11.8 ± 0.7G
ß-cryptoxanthin	9.7 ± 0.4a	7.9 ± 0.4b	6.0 ± 0.4c	1.28 ± 0.05d	13.2 ± 0.5E
Total xanthophylls	151 ± 5a	82 ± 3b	70.8 ± 2.3c	14.9 ± 0.5d	9.90 ± 0.16H
Total carotenoids	223 ± 7a	131 ± 4b	93 ± 3c	21.4 ± 0.7d	9.6 ± 0.2CH

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same row indicate significant differences ($P < 0.05$) among digestive phases. Different capital letters in the same column show significant differences among the bioaccessibility of carotenoids. nd=not detected.

3.1.5. Hydrophilic and lipophilic antioxidant activity

The total antioxidant activity (TAA) was determined as the sum of both hydrophilic (HAA) and lipophilic antioxidant activities (LAA). As can be seen in Fig. 2, TAA of non-digested BFJ-SMB showed a 78.2% of DPPH inhibition (66.3% of HAA and 11.9% of LAA).

The antioxidant activity of both hydrophilic and lipophilic constituents was reduced 17% under gastric conditions. However, the stability of lipophilic constituents was greater (71%) than that of hydrophilics (58%) in the small intestinal digesta, with

respect to gastric digesta. Finally, the hydrophilic constituents displayed more antioxidant activity (34%) than those lipophilics (22%) when the dialyzed and micellar fractions, respectively, were compared with the small intestinal digesta.

Hydrophilic compounds with antioxidant activity displayed higher bioaccessibility (16.6%) than that of lipophilics (12.7%). Few reports have evaluated the bioaccessibility of both hydrophilic and lipophilic bioactive compounds of foods but it was observed the same trend in SM and BFJ (Rodríguez-Roque et al., 2013a, b).

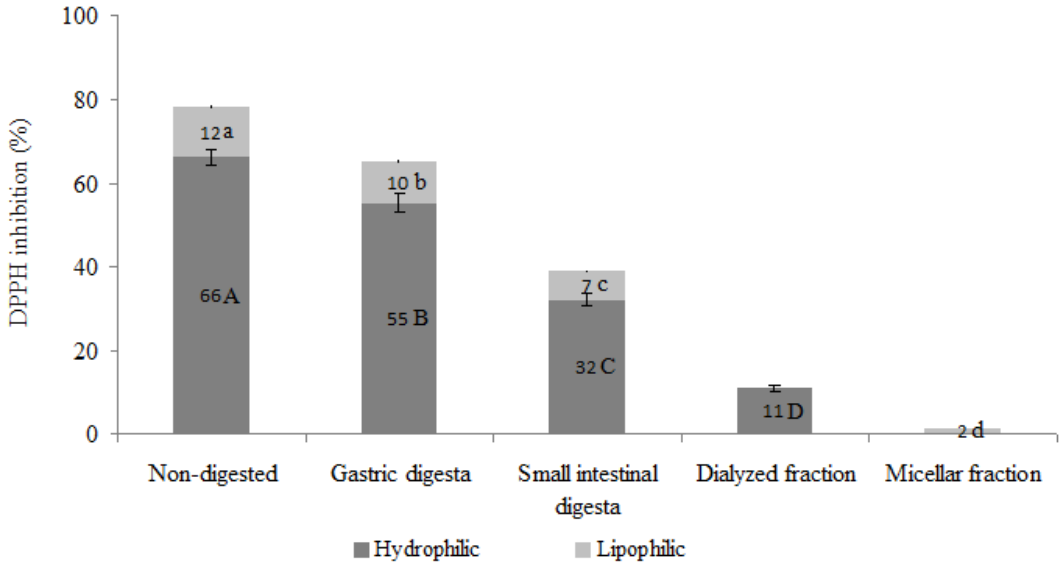

Figure 2. Changes in hydrophilic and lipophilic antioxidant activity during the *in vitro* gastrointestinal digestion of a blended fruit juice-soymilk beverage (BFJ-SMB). Different lower case letters mean significant differences ($P< 0.05$) in the lipophilic antioxidant activity along the digestive process. Different capital letters indicate significant differences ($P< 0.05$) in the hydrophilic antioxidant activity along the digestive process.

3.2. Influence of the food matrix on the bioaccessibility of bioactive compounds from BFJ–SMB

The bioaccessibility of the bioactive compounds from BFJ–SMB was compared with that previously obtained from both BFJ and SM in order to evaluate the impact of the food matrix. BFJ and SM were digested in our laboratory, using the same in vitro gastrointestinal digestion and analytical procedures (Rodríguez-Roque et al., 2013a, b).

As shown in Table 4, the food matrix had a significant influence ($P< 0.05$) on the bioaccessibility of the bioactive compounds contained in these beverages. However, it is important to note that the bioaccessibilities of most of these compounds were only

reduced 6.5–14% in BFJ–SMB with respect to BFJ or SM. For instance, the bioaccessibility of vita-min C was 14% higher in BFJ than in BFJ–SMB. In SM this compound was not detected. It is well known that apart from the health-promoting compounds, food also contains a number of substances which can diminish the bioaccessibility of nutrients. In this context, vitamin C is oxidized by the presence of heavy metal ions (such as cupper, iron, and zinc), sulfites and other vitamins (such as B_1, B_2, and B_{12}) (Ball, 2006; Berry, 2009), most of these compounds are contained in soy-milk, explaining why the bioaccessibility of vitamin C diminished in BFJ–SMB. In addition, vitamin C may acts as antioxidant, protecting other dietary substances, such as carotenoids, from oxidation (Berry, 2009).

Phenolic acids were not bioaccessible in SM but interestingly, the addition of SM to BFJ (BFJ–SMB) improved 6% the bioaccessibility of these compounds as compared with BFJ. The highest bioaccessibility of flavonoids was found in BFJ (20.1%), followed by BFJ–SMB (18.8%) and finally, SM (15.9%). The most affected compounds by the food matrix were the glucoside isoflavones, which showed a decrease of 51% in BFJ-SMB with respect to that of SM. Conversely, the aglycone forms of isoflavones were more bioaccessible in BFJ–SMB (22.6%) than in SM (17.0%). The decrease in the bioaccessibility of phenolic compounds has been attributed to the formation of complexes among these substances and fiber, proteins and metal ions. Some phenolic compounds may bind to proteins resulting in insoluble aggregates that precipitate and make difficult the digestibility of food. SM is a rich source of proteins and iron (Jinapong et al., 2008), therefore, interactions among phenolic compounds and these constituents could increase when BFJ and SM are mixed.

Both carotenes and xanthophylls showed a slight bioaccessibility reduction of 13% in BFJ–SMB as compared with BFJ. The decrease in the bioaccessibility of carotenoids from BFJ–SMB could be explained by interactions with other food constituents (i.e. other carotenoids and fiber). It was reported that β-carotene compete with lutein and lycopene for absorption when consumed at the same time (Higdon, 2007). Dietary fiber reduces the micellisation of carotenoids because biliary fluids become soluble in the gel formed during gastric digestion (Fernández-García et al., 2012).

Table 4. Bioaccessibility of bioactive compounds in soymilk (SM), a blended fruit juice (BFJ) and a blended fruit juice-soymilk beverage (BFJ-SMB).[a]

Bioactive compounds	Bioaccessibility (%)		
	SM[b]	BFJ[c]	BFJ-SMB
Vitamin C	nd	15.04 ± 1.8a	13.0 ± 0.6b
Phenolic compounds			
Phenolic acids	0a	12.8 ± 1.3b	13.6 ± 0.6b
Flavonoids	15.9 ± 0.7a	20.1 ± 0.4b	18.8 ± 0.7c
TPC as the sum of individuals	14.9 ± 0.6a	18.8 ± 0.4b	17.9 ± 0.7c
TPC by Folin-Ciocalteu method	19.6 ± 0.9a	11.5 ± 0.6b	18.1 ± 0.8c
Isoflavones			
Glucosides	42 ± 4a	nd	21.5 ± 0.8b
Aglycones	17.0 ± 0.4a	nd	22.6 ± 1.0b
Total isoflavones	35.7 ± 2.8a	nd	21.7 ± 0.6b
Carotenoids			
Carotenes	nd	10 ± 1a	9.0 ± 0.6b
Xanthophylls	nd	11.4 ± 0.4a	9.90 ± 0.16b
Total carotenoids	nd	11.1 ± 0.4a	9.6 ± 0.2b
Antioxidant activity			
Hydrophilic antioxidant activity (dialyzed fraction)	38 ± 4a	21.6 ± 0.5b	16.6 ± 1.1c
Lipophilic antioxidant activity (micellar fraction)	8.6 ± 0.5a*	11.6 ± 0.6b*	12.7 ± 0.9b

[a] Values are expressed as the mean ± standard deviation. Different letters in the same row indicate significant differences ($P < 0.05$) among beverages. nd=not detected.
[b] Data from Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b)
[c] Data from Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a)
*Unpublished data.

In overall, the bioactive compounds with hydrophilic antioxidant activity were more bioaccessible in SM, followed by BFJ and BFJ–SMB. There were not found significant differences between the bioaccessibility of lipophilic compounds with antioxidant activity from BFJ and that of BFJ–SMB. These data suggest that the antioxidant activity of food is influenced by synergistic and/or antagonistic interactions between antioxidants and other compounds, which depends on the food matrix in which they are contained.

Although it is clear that interactions among bioactive com-pounds and other food constituents could be favoured in complexes matrices, the main advantages of mixing SM and BFJ are the improvement in the sensorial and nutritional properties of the beverage. SM could provide bioactive compounds, such as isoflavones, that are not found in fruit juices; while BFJ contributes with carotenoids that are not contained in

SM. In addition, results obtained in this research showed that the bioaccessibilities of bioactive compounds from BFJ–SMB were similar to those of individual products (BFJ or SM). In our knowledge, there are scarce studies evaluating the bioaccessibility of several bioactive compounds from the same food matrix. Therefore, information gained through this study can contribute to elucidate the influence of the food matrix on the bioaccessibility of nutrients from complexes matrices and may help in the design of food products with enhanced nutritional and functional quality.

4. Conclusion

The concentration and bioaccessibility of BFJ–SMB bioactive compounds (vitamin C, phenolic compounds, isoflavones, carotenoids and bioactive compounds with antioxidant activity) were significantly influenced by the in vitro gastrointestinal digestion. Hydrophilic constituents displayed higher bioaccessibilities (in the range of 12–26.5%) than those lipophilics (between 7.5% and 13.8%). The bioaccessibility of most of the analyzed compounds was slightly lower in BFJ–SMB than in SM or BFJ (reduction of 6.5–14%), with the exception of the glucoside form of isoflavones which diminished 48.5%. Nevertheless, BFJ–SMB combines the healthy com-pounds of both SM (i.e. isoflavones) and BFJ (i.e. carotenoids), providing a higher variety of bioactive substances than individual products.

Results obtained in this study proved that *in vitro* gastrointestinal methodologies are useful tools for predictive purposes that allow obtaining information concerning to the stability, solubility, interactions, and bioaccessibility of bioactive compounds in heterogeneous food matrices. All these are important issues to take into consideration when developing new formulation of functional beverages. Further *in vivo* studies are required to confirm these results.

Acknowledgement

The present research has been financed by the Ministerio de Ciencia e Innovación (Spain), reference AGL2006-12758-C02-02/ALI. María Janeth Rodríguez-Roque thanks to the Comissionat per a Universitats i Recerca, del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR) and European Social Fund for the predoctoral grant, as well as to the Secretaría de Educación Pública de

México (SEP) for their support. Prof. Olga Martín-Belloso thanks to the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia Award 2008.

Abbreviations list

BFJ, blended fruit juice

BFJ-SMB, blended fruit juice-soymilk beverage

DPPH, 1,1-diphenyl-2-picrylhydrazyl

F−C, Folin−Ciocalteu

HAA, hydrophilic antioxidant activity

HPLC, high-performance liquid chromatography

LAA, lipophilic antioxidant activity

SM, soymilk

TAA, total antioxidant activity

TPC, total phenolic compounds

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CHAPTER IV

In vitro bioaccessibility of health–related compounds as affected by the formulation of fruit juice- and milk-based beverages

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Food Research International (Accepted)

Abstract

The purpose of this research was to evaluate the influence of the beverage formulation on the *in vitro* digestibility and bioaccessibility of phenolic compounds, vitamin C, and carotenoids, as well as antioxidant activity from milk, a blended fruit juice (BFJ) and a combination of both of them (BFJ-MB). The release of many phenolic substances was improved during gastric digestion of milk and BFJ-MB (around 5 and 75%), but not in BFJ. Vitamin C and carotenoids diminished significantly ($P < 0.05$) in the gastric and intestinal digesta of each beverage. Phenolic acids, flavonoids, vitamin C and hydrophilic constituents with antioxidant activity were more bioaccessible in BFJ (up to 3.4 times) than in milk and BFJ-MB. On the contrary, the bioaccessibility of carotenes, xanthophylls and those compounds with lipophilic antioxidant activity was improved when milk was added to BFJ (up to 1.9 times). Results suggest that the addition of milk improved the bioaccessibility of lipophilic constituents but not that of hydrophilics. Nevertheless, BFJ-MB combines the nutritional ingredients of milk and BFJ. As a result, BFJ-MB could supply a higher diversity of bioaccessible compounds in comparison to that of milk and BFJ alone, promoting health and protecting against several diseases.

Keywords: Fruit juice- and milk-based beverages; Beverage formulation; Bioaccessibility; Bioactive compounds; Antioxidant activity

1. Introduction

Nowadays, there is a clear trend toward consumption of food that beyond nutritional value, improve health and well-being, reducing the risk of disease. These products are usually known as functional food (Howlet, 2008) and their potential market is currently growing. In fact, new functional food and beverages have been designed in order to satisfy the demand of consumers, standing out the fruit juice- and milk-based beverages.

Fruit juices are considered as the main dietary sources of bioactive substances, such as vitamins, phenolic compounds and carotenoids, which reduce the risk of cardiovascular and neurodegenerative diseases, as well as some cancer types (Aboul-Enein, Berczynski, & Kruk, 2013; Gülçin, 2012). On the other hand, milk contains proteins (essential amino acids), fat (unsaturated fatty acids), vitamins (mainly, A and E), carotenoids (mainly β-carotene), and minerals (Claeys et al., 2013; Antone, Sterna & Zagorska, 2012). Therefore, both fruit juices and milk possess a high nutritional value and represent a good option to obtain beverages with functional properties.

Nevertheless, the knowledge of the quantity of nutrients contained in the food itself it is not enough to attribute functional properties. The most important feature is the proportion of these compounds that is available to exert their biological function. Bioaccessibility corresponds to the fraction of bioactive substance that is released from the food matrix after digestion and solubilised into the gut lumen for uptake in the intestinal mucosa (Ferruzzi et al., 2010). Bioavailability is defined as the fraction of nutrient secreted into circulation that is available for tissue uptake and metabolism. In this context, *in vitro* gastrointestinal digestion is usually utilized to assess the bioaccessibility of food constituents and represents an easy and fast approach to *in vivo* trials (Failla & Chitchumroonchokchai, 2005).

There are some studies assessing the *in vitro* bioaccessibility of bioactive compounds in simple beverages. For instance, Gil-Izquierdo, Gil, Ferreres & Tomás-Barberán, (2001) analyzed the bioaccessibility of phenolic compounds in orange juice. Pérez-Vicente, Gil-Izquierdo & García-Viguera (2002) studied the bioaccessibility of pomegranate juice phenolic compounds, anthocyanins and vitamin C. Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b) evaluated the changes in the concentration of vitamin C, phenolic compounds and carotenoids throughout an *in vitro* gastrointestinal digestion of a blend of orange, pineapple and kiwi juices.

However, information on the bioaccessibility of bioactive compounds in heterogeneous food matrices is still very limited. In this sense, Cilla et al. (2012) investigated the bioaccessibility of tocopherols, carotenoids, and ascorbic acid from milk- and soy-based fruit beverages. Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2014) evaluated the bioaccessibility of vitamin C, phenolic compounds, isoflavones and carotenoids from a blended fruit juice-soymilk beverage. Granado-Lorencio et al. (2009) analyzed the bioaccessibility of carotenoids and α-tocopherol from fruit juices in the presence of absorption modifiers, such as milk. The extent to which these compounds could be available for absorption depends on their stability, interactions with other compounds and the type of food matrix where these substances form part (Rein et al. 2013). Therefore, the aim of this study was to evaluate the influence of the beverage formulation on the *in vitro* digestibility and bioaccessibility of phenolic compounds, vitamin C, carotenoids, as well as antioxidant activity from milk, a blended fruit juice (BFJ) containing orange, kiwi, pineapple, and mango juices, and a blended fruit juice-milk beverage (BFJ-MB).

2. Material and methods

2.1. Reagents

Pepsin from porcine stomach, pancreatin from porcine pancreas, bovine bile, phenol standards (caffeic, chlorogenic, p-coumaric, ferulic, sinapic and 4-hydroxybenzoic acids; hesperidin, naringenin, quercetin, rutin, and [+]-catechin), carotenoid standards (α-carotene, β-carotene, zeaxanthin, lutein, α-cryptoxanthin and β-cryptoxanthin), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and cellulose dialysis membrane (molecular weight cutoff of 12,000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid and Folin-Ciocalteu (F-C) reagent were acquired from Scharlau Chemie S.A. (Barcelona, Spain).

2.2. Beverages preparation

Whole cow's milk was purchased at a local supermarket (Lleida, Spain). According to manufacturer, it contained 3.6% of fat, 3.0% of protein and 4.5% carbohydrates. In addition, the pH (Crison Instruments S.A., Alella, Barcelona, Spain) and the soluble

solid content (Comecta S.A., Abrera, Barcelona, Spain) were assessed, resulting in 6.75 ± 0.06 and 11.83 ± 0.29 ºBrix, respectively.

Orange, kiwi, pineapple and mango fruits were purchased at commercial maturity in a local supermarket (Lleida, Spain). Fruits were washed, peeled and the juice extracted. Each fresh-squeezed juice was filtered through a cheese cloth using a vacuum pump. A blended fruit juice (BFJ) was obtained by mixing 40% of orange, 33% of kiwi, 13.5% of pineapple and 13.5% of mango juices. The pH of BFJ was 3.38 ± 0.04 and the soluble solid content 11.0 ± 0.10 ºBrix.

The blended fruit juice – milk beverage (BFJ-MB) was manufactured by mixing 75% of BFJ, 17.5% of milk and 7.5% of sugar. The beverage was filtered through a cheese cloth using a vacuum pump. The pH of BFJ-MB was adjusted to 3.3 ± 0.05 with citric acid if necessary, and the soluble solid content was assessed (17.5 ± 0.03 ºBrix). The formulation of the beverage was selected based on a previous study where the combination of these fruit juices with milk displayed a high concentration of vitamin C, as well as antioxidant activity and stability (Salvia-Trujillo, Morales-De La Peña, Rojas-Graü & Martín-Belloso, 2011)

2.3. In vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion was carried out following the methodology described by Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2013b). This procedure consisted of gastric digestion (pH 2, containing pepsin) and small intestinal digestion (pH 7, containing a pancreatin-bile mixture). Small intestinal digestion included dialyzed (bioactive compounds of inside the dialysis membrane) and micellar fractions (centrifugation at 5000 rpm for 20 min), which contained the bioaccessible hydrophilic and lipophilic compounds, respectively.

Aliquots were collected at the end of each digestive phase and immediately placed in a cold water bath during 10 minutes. All samples were frozen (-45 ºC) until analysis.

2.4. Bioactive compound analysis

2.4.1. Phenolic compounds analyzed by HPLC

Extraction, separation, identification and quantification of phenolic compounds were performed using the methodology reported by Rodríguez-Roque, Rojas-Graü, Elez-

Martínez & Martín-Belloso (2013a). Individual phenolic compounds were identified by comparison of their retention time and spectra with those of the standards (caffeic, chlorogenic, ferulic, p-coumaric, sinapic and 4-hydroxybenzoic acids; hesperidin, naringenin, quercetin, rutin, and [+]-catechin). Quantification was carried out by integration of the peak areas and using calibration curves. Results were expressed as mg of phenolic compound/100 mL of sample. Total phenolic compounds were calculated as the sum of individuals (TPC by HPLC).

2.4.2. Total phenolic content analyzed by Folin-Ciocalteu (TPC by F-C) methology

Total phenolic compounds were determined according to the methodology of Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2013a), using the colorimetric method previously described by Singleton, Orthofer & Lamuela-Raventós (1998). A calibration curve of gallic acid was used to quantify the concentration of total phenolic compounds in each sample. Results were expressed as mg of gallic acid/100 mL of sample.

2.4.3. Vitamin C

Vitamin C was extracted, separated, identified and quantified (by HPLC) according to the methodology reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2013b). Vitamin C identification was carried out by comparison of its retention time and spectra with the standard (ascorbic acid). Results were expressed as mg of ascorbic acid /100 mL of sample.

2.4.4. Carotenoids

Carotenoids were extracted, separated, identified and quantified (by HPLC) following the methodology reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2013b). Each carotenoid was identified by comparison of its retention time and spectra with the standards (α-carotene, β-carotene, zeaxanthin, lutein, α-cryptoxanthin and β-cryptoxanthin). Quantification was carried out by integration of the peak areas and using calibration curves. Cis-violaxanthin+neoxanthin, cis-antheraxanthin and anteraxanthin were identify according to the retention time and spectra reported in the literature and they were quantified through the calibration curve of zeaxanthin. Results were expressed as µg of carotenoid/100 mL of sample.

2.5. Hydrophilic and lipophilic antioxidant activity

Extraction of hydrophilic and lipophilic fractions of non-digested or digested samples, as well as antioxidant activity were performed according to the procedure of Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2013a). The determination of the antioxidant activity of extracts was based on a colorimetric method (DPPH radical) reported by Brand-Williams, Cuvelier & Berset (1995). Results were expressed as percentage of DPPH inhibition.

2.6. Bioaccessibility calculations

Bioaccessibility was determined as the ratio between the concentration of bioactive compound in the dialyzed (for phenolic compounds and vitamin C, as well as hydrophilic compounds with antioxidant activity) or micellar (for carotenoids and lipophilic compounds with antioxidant activity) fractions and the non-digested beverage. Results were expressed as percentage.

2.7. Statistical analysis

The *in vitro* gastrointestinal digestion was conducted in duplicated. Each bioactive compound was analyzed three times (n = 6). Results were reported as the mean ± standard deviation. Analysis of variance (ANOVA) of the results followed by least significant difference (LSD) test were carried out to determine significant differences ($P < 0.05$) between the concentration of bioactive compounds in non-digested and digested samples, as well as among the bioaccessibilities. The statistical analyses were performed through the program Statgraphics Plus v.5.1 (Rockville, MD, USA).

3. Results and discussion

3.1. Digestibility

3.1.1. Phenolic compounds

The concentration of phenolic compounds from milk, a blended fruit juice (BFJ), and a blended fruit juice-milk beverage (BFJ-MB) is addressed in Table 1. Hesperidin and 4-hydroxybenzoic acid were the major phenolic substances contained in BFJ-MB and BFJ. Only two phenolic compounds (4-hydroxybenzoic acid and [+]-catechin) were

found in milk. The content of TPC by F-C method was between 1.5 and 6.2 times higher than that obtained as TPC by HPLC in all the studied samples. These results could be explained by the fact that Folin-Ciocalteu reagent could be reduced by other non-phenolic substances, such as carbohydrates, amines, ascorbic acid, among others (Prior, Wu & Schaich, 2005), leading to overestimate the content of phenolic substances in food.

In BFJ, the concentration of several phenolic compounds was reduced due to gastric digestion (caffeic, ferulic, p-coumaric and 4-hydroxybenzoic acids, as well as naringenin), whereas other phenols improved their release from 5 to 58% (chlorogenic acid, sinapic acid, rutin and [+]-catechin) or remained unchanged (hesperidin and quercetin). The recovery of 4-hydroxybenzoic acid from milk increased 38% but (+)-catechin decreased a 14.5% under acidic conditions. On the other hand, the release of most phenolic compounds contained in BFJ-MB was improved between 5% (chlorogenic acid) and 93% (naringenin) by gastric digestion. TPC were better recovered in the gastric digesta of BFJ-MB than in that of BFJ or milk. In heterogeneous matrices, such as BFJ-MB, some phenolic compounds could be bound to other food constituents (in this case proteins from milk and carbohydrates from fruits juices) that make difficult their full extraction. However, many of these phenolic compounds can be released from proteins and carbohydrates due to the action of digestive enzymes and the gastric pH, thus resulting in an increase in their concentration (Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso, 2013b, 2014; Saura-Calixto, Serrano & Goñi, 2007; Tagliazucchi, Verzelloni, Bertolini & Conte, 2010).

In general, the concentration of phenolic compounds of the three beverages was reduced under intestinal conditions. BFJ-MB displayed the greatest losses in the phenolic concentration (10.7 - 63.3%) during small intestinal digestion in comparison to gastric digesta, followed by BFJ (4.9 - 49.4%) and milk (32.3 - 37.2%). However, the lowest recovery of phenolic substances from all the studied samples was obtained in the dialyzed fraction, varying from 0 to 36.4% with respect to the small intestinal digesta. Caffeic acid from BFJ-MB, as well as ferulic and sinapic acids from both BFJ-MB and BFJ, were not detected in the dialyzed fraction. Results suggest that phenolic compounds could undergo several reactions under intestinal digestion, such as polymerization, epimerization and auto-oxidation, leading to the formation of phenolic derivatives with different physicochemical properties. In this sense, a great portion of

flavanones from orange juice (50 – 60%) were transformed into chalcones, which are not dialyzable because of their high molecular weigh and low solubility under intestinal conditions (Gil-Izquierdo, Gil, Ferreres & Tomás-Barberán, 2001). Similarly, some phenolic acids from soymilk and red wine were not dialyzable due to modifications in their chemical structure and solubility (Argyri, Proestos, Komaitis & Kapsokefalou, 2005; Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso, 2013a). Other authors reported that the molecular weight of phenolic compounds have a significant influence on their absorption in the gut (Scalbert & Williamson, 2000).

3.1.2. Vitamin C

As can be seen in Table 2, the concentration of vitamin C in non-digested BFJ-MB and BFJ was 26.4 and 33.1 mg of ascorbic acid/100 mL, respectively. Vitamin C was not detected in milk under the assayed conditions, likely because milk contains a low concentration of this compound (Berry, 2009).

Under gastric conditions, vitamin C was recovered 67.7% in BFJ and 63.3% in BFJ-MB. It has been suggested that the molecules of vitamin C are protonated at low pH, avoiding the interaction with oxygen (Ball, 2006) or other substances. In this sense, Pérez-Vicente, Gil-Izquierdo & García-Viguera (2002) and Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2013b) observed a similar trend, obtaining 71% of vitamin C recovery in pomegranate juice and 83% in a blend of fruit juices, respectively.

Greater losses of vitamin C were observed in the small intestinal digesta of both BFJ-MB and BFJ, being recovered between 47.3% (BFJ-MB) and 52.2% (BFJ), with respect to gastric digesta. However, when the dialyzed fraction was compared to the small intestinal digesta, the vitamin C losses reached up to 62.6%. Intestinal environment, such as pH > 4, dissolved oxygen, heat and the enzyme activity, particularly favour the oxidation of this compound (Ball, 2006; Jeney-Nagymate & Fodor, 2008). The vitamin C oxidation in the gastrointestinal tract could also be related to the fact that this compound keeps metal ions in reduced state (i.e. iron), or regenerate the active form of other dietary constituents (i.e. vitamin E) by donating an electron (Ball, 2006; Schlueter & Johnston, 2011).

Table 1. Concentration of phenolic compounds through the *in vitro* gastrointestinal digestion of fruit juice- and milk-based beverages.[a]

	Phenolic compounds (mg/ 100 mL)						
Digestive phase	**Caffeic acid**	**Chlorogenic acid**	**Ferulic acid**	**p-coumaric acid**	**Sinapic acid**	**4-hydroxybenzoic acid**	**Total phenolic acids**
Blended fruit juice - milk beverage (BFJ-MB)							
Non-digested	0.47 ± 0.03a	1.16 ± 0.07a	0.212 ± 0.014a	2.09 ± 0.14a	0.337 ± 0.020a	6.29 ± 0.18a	10.56 ± 0.24a
Gastric digesta	0.61 ± 0.03b	1.22 ± 0.07b	0.225 ± 0.011b	2.20 ± 0.14b	0.224 ± 0.011b	10.4 ± 0.7b	14.9 ± 0.6b
Small intestinal digesta	0.444 ± 0.021c	1.09 ± 0.03c	0.135 ± 0.008c	1.616 ± 0.023c	0.134 ± 0.008c	5.1 ± 0.3c	8.54 ± 0.25c
Dialyzed fraction	nd	0.154 ± 0.008d	nd	0.279 ± 0.016d	nd	0.778 ± 0.017d	1.21 ± 0.03d
Blended fruit juice (BFJ)							
Non-digested	0.59 ± 0.03d	1.70 ± 0.05e	0.211 ± 0.011a	1.38 ± 0.05e	0.535 ± 0.010d	8.29 ± 0.24e	12.71 ± 0.19e
Gastric digesta	0.562 ± 0.017e	1.80 ± 0.07f	0.186 ± 0.007d	1.26 ± 0.06f	0.63 ± 0.03e	7.00 ± 0.16f	11.44 ± 0.22f
Small intestinal digesta	0.562 ± 0.020e	1.37 ± 0.05g	0.152 ± 0.006e	0.638 ± 0.018g	0.349 ± 0.008a	5.74 ± 0.13g	8.81 ± 0.18c
Dialyzed fraction	0.097 ± 0.005f	0.270 ± 0.007h	nd	0.232 ± 0.006d	nd	1.21 ± 0.05h	1.81 ± 0.06g
Milk							
Non-digested	nd	nd	nd	nd	nd	2.91 ± 0.10i	2.91 ± 0.10h
Gastric digesta	nd	nd	nd	nd	nd	4.01 ± 0.19j	4.01 ± 0.19i
Small intestinal digesta	nd	nd	nd	nd	nd	2.52 ± 0.10k	2.52 ± 0.10j
Dialyzed fraction	nd	nd	nd	nd	nd	0.265 ± 0.011*l*	0.265 ± 0.011k

[a] Values are expressed as the mean ± standard deviation. Different letters in the same column show significant differences ($P < 0.05$) among digestive phases and beverages. TPC by HPLC, means total phenolic compounds determined as the sum of individual phenolic compounds quantified by HPLC. TPC by F-C, means total phenolic compounds determined by Folin-Ciocalteu method. nd=not detected.

..... Continuation Table 1. Concentration of phenolic compounds through the *in vitro* gastrointestinal digestion of fruit juice- and milk-based beverages.[a]

	Phenolic compounds (mg/ 100 mL)							
Digestive phase	**Hesperidin**	**Naringenin**	**Quercetin**	**Rutin**	**(+)-catechin**	**Total flavonoids**	**TPC by HPLC**	**TPC by F-C**
Blended fruit juice - milk beverage (BFJ-MB)								
Non-digested	9.7 ± 0.3a	1.28 ± 0.07a	1.18 ± 0.07a	1.95 ± 0.11a	2.80 ± 0.03a	16.93 ± 0.19a	27.5 ± 0.3a	61.4 ± 1.6a
Gastric digesta	15.8 ± 0.5b	2.48 ± 0.13b	1.21 ± 0.07ad	3.41 ± 0.18b	2.09 ± 0.13b	25.0 ± 0.6b	39.9 ± 0.8b	68.2 ± 1.5b
Small intestinal digesta	8.45 ± 0.12c	1.25 ± 0.03a	1.05 ± 0.06b	1.08 ± 0.04c	1.85 ± 0.05c	13.68 ± 0.15c	22.2 ± 0.4c	47.6 ± 1.2c
Dialyzed fraction	1.23 ± 0.03d	0.160 ± 0.008c	0.130 ± 0.006c	0.135 ± 0.006d	0.348 ± 0.011d	2.00 ± 0.03d	3.21 ± 0.04d	6.7 ± 0.3d
Blended fruit juice (BFJ)								
Non-digested	9.9 ± 0.3a	6.26 ± 0.10d	1.26 ± 0.05de	3.66 ± 0.07e	2.34 ± 0.16e	23.4 ± 0.5e	36.1 ± 0.7e	69.2 ± 1.5b
Gastric digesta	9.97 ± 0.17a	4.53 ± 0.13e	1.27 ± 0.04de	3.846 ± 0.017f	3.70 ± 0.06f	23.3 ± 0.3e	34.74 ± 0.23f	79.3 ± 1.4e
Small intestinal digesta	11.3 ± 0.4e	4.31 ± 0.17f	1.29 ± 0.05e	3.50 ± 0.15b	2.72 ± 0.13a	23.1 ± 0.4e	31.9 ± 0.3g	48.5 ± 0.9c
Dialyzed fraction	1.44 ± 0.03d	0.938 ± 0.021g	0.16 ± 0.01c	0.433 ± 0.011g	0.33 ± 0.03d	3.30 ± 0.04f	5.11 ± 0.09h	13.6 ± 0.6f
Milk								
Non-digested	nd	nd	nd	nd	4.20 ± 0.16g	4.20 ± 0.16g	7.11 ± 0.10i	22.7 ± 0.7g
Gastric digesta	nd	nd	nd	nd	3.59 ± 0.05h	3.59 ± 0.05f	7.60 ± 0.21j	33.7 ± 0.8h
Small intestinal digesta	nd	nd	nd	nd	2.43 ± 0.04e	2.43 ± 0.04h	4.95 ± 0.12h	30.8 ± 1.1i
Dialyzed fraction	nd	nd	nd	nd	0.539 ± 0.020i	0.539 ± 0.020i	0.803 ± 0.017k	3.28 ± 0.13j

[a] Values are expressed as the mean ± standard deviation. Different letters in the same column show significant differences ($P < 0.05$) among digestive phases and beverages. TPC by HPLC, means total phenolic compounds determined as the sum of individual phenolic compounds quantified by HPLC. TPC by F-C, means total phenolic compounds determined by Folin-Ciocalteu method. nd=not detected.

Table 2. Concentration of vitamin C through the *in vitro* gastrointestinal digestion of fruit juice- and milk-based beverages.[a]

	Vitamin C (mg/100 mL)		
Digestive phase	**BFJ-MB**	**BFJ**	**Milk**
Non-digested	26.4 ± 0.8Aa	33.1 ± 1.1Ab	nd
Gastric digesta	16.7 ± 0.3Ba	22.4 ± 0.7Bb	nd
Small intestinal digesta	7.9 ± 0.4Ca	11.7 ± 0.3Cb	nd
Dialyzed fraction	3.03 ± 0.15Da	4.92 ± 0.09Db	nd

[a] Values are expressed as the mean ± standard deviation. Different capital letters in the same column indicate significant differences ($P < 0.05$) among digestive phases of each sample. Different lower case letters in the same row show significant differences ($P < 0.05$) among beverages. BFJ-MB= blended fruit juice-milk beverage; BFJ= blended fruit juice; nd=not detected.

3.1.3. Carotenoids

The concentration of carotenoids before and along the *in vitro* digestion is showed in Table 3. Cis-anteraxanthin and β-carotene were the major carotenoids contained in both BFJ-MB and BFJ. β-carotene was the predominant carotenoid in milk, representing 83.5% of the total amount of these compounds.

The recovery of carotenoids was in the range of 26 to 93% after gastric digestion of all beverages. The most stable carotenoid of both BFJ-MB and BFJ was α-cryptoxanthin (recovery >92%), while cis-violaxanthin+neoxanthin was the lowest (recovery <30%). It has been reported that carotenoids are sensitive to acidic pH (Rao & Rao, 2007; Berry, 2009), explaining why their concentration decrease under gastric conditions. In addition, carotenoids are known to be highly oxidized molecules owing to double bounds of their chemical structure (Hedrén, Diaz & Svanberg, 2002). A similar recovery of carotenoids was observed in the gastric digesta of a blended fruit juice (between 36 and 90%) and in a beverage containing soymilk and a blend of fruit juice (between 33 and 81%) (Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso, 2013b, 2014).

Table 3. Concentration of carotenoids through the *in vitro* gastrointestinal digestion of fruit juice- and milk-based beverages.[a]

Digestive phase	Carotenoids (µg/ 100 mL)										
	α-carotene	**β-carotene**	**Total carotenes**	**Cis-violaxanthin +neoxanthin**	**Cis-anteraxanthin**	**Anteraxanthin**	**Lutein**	**α-cryptoxanthin**	**β-cryptoxanthin**	**Total xantophylls**	**Total carotenoids**
Blended fruit juice – milk beverage (BFJ-MB)											
Non-digested	6.2 ± 0.3a	113 ± 5a	120 ± 5a	58.8 ± 2.2a	138 ± 6a	20.7 ± 0.9a	60.4 ± 1.3a	6.49 ± 0.23a	16.9 ± 0.6a	301 ± 9a	421 ± 12a
Gastric digesta	5.7 ± 0.3b	81.1 ± 1.9b	86.8 ± 1.9b	15.1 ± 0.8b	56 ± 3b	12.2 ± 0.5b	39.4 ± 1.8b	6.01 ± 0.13b	12.2 ± 0.5b	141 ± 4b	228 ± 4b
Small intestinal digesta	3.51 ± 0.13c	58.1 ± 2.2c	61.6 ± 2.3c	9.3 ± 0.3c	31.1 ± 1.6c	8.3 ± 0.3c	36.5 ± 0.9c	5.58 ± 0.13c	5.6 ± 0.3c	96.4 ± 1.5c	158 ± 2c
Micellar fraction	1.44 ± 0.04d	30.3 ± 0.7d	31.8 ± 0.7d	7.2 ± 0.3d	18.2 ± 0.5d	3.55 ± 0.08d	14.5 ± 0.7d	1.32 ± 0.05d	3.04 ± 0.15d	47.8 ± 1.1d	79.6 ± 1.2d
Blended fruit juice (BFJ)											
Non-digested	8.4 ± 0.4e	160 ± 4e	168 ± 4e	84 ± 3e	203 ± 9e	28.4 ± 1.2e	121 ± 3e	9.7 ± 0.5e	26.8 ± 0.9e	472 ± 11e	640 ± 12e
Gastric digesta	6.5 ± 0.3a	125.5 ± 2.3f	132 ± 3f	24.0 ± 1.1f	62 ± 3f	10.1 ± 1.1f	104 ± 3f	9.0 ± 0.3f	22.7 ± 0.8f	232 ± 5f	364 ± 6f
Small intestinal digesta	2.77 ± 0.06f	81.8 ± 4b	85 ± 4b	7.3 ± 0.3d	31.6 ± 1.6c	5.88 ± 0.19g	60.6 ± 2.0a	5.05 ± 0.25g	10.9 ± 0.4g	121.4 ± 1.1g	206 ± 5g
Micellar fraction	1.06 ± 0.05g	22.8 ± 0.6g	23.8 ± 0.6g	6.5 ± 0.3d	25.7 ± 0.9g	3.18 ± 0.07d	23.7 ± 0.8g	1.85 ± 0.08h	3.57 ± 0.08h	64.5 ± 1.5h	88.3 ± 1.2h
Milk											
Non-digested	nd	25.9 ± 0.6h	25.9 ± 0.6g	nd	nd	nd	3.04 ± 0.10h	nd	2.04 ± 0.10i	5.08 ± 0.20i	31.0 ± 0.6i
Gastric digesta	nd	15.9 ± 0.4i	15.9 ± 0.4h	nd	nd	nd	2.05 ± 0.08hi	nd	1.33 ± 0.05j	3.38 ± 0.12i	19.3 ± 0.4j
Small intestinal digesta	nd	10.9 ± 0.4j	10.9 ± 0.4i	nd	nd	nd	1.18 ± 0.06i	nd	0.92 ± 0.03j	2.10 ± 0.07i	13.0 ± 0.4j
Micellar fraction	nd	5.7 ± 0.3k	5.7 ± 0.3j	nd	nd	nd	0.624 ± 0.023i	nd	0.342 ± 0.017k	0.97 ± 0.04i	6.6 ± 0.3k

[a] Values are expressed as the mean ± standard deviation. Different letters in the same column show significant differences ($P < 0.05$) among digestive phases and beverages. Total xantophylls and total carotenoids were determined as the sum of individual carotenoids quantified by HPLC. nd=not detected.

Significant changes in the carotenoid concentration were observed during intestinal digestion. Among the analyzed samples, BFJ-MB showed the highest stability of total carotenes (71%), followed by milk (68.5%) and BFJ (65%) when the small intestinal digesta was compared to gastric digesta. Total xantophylls displayed a similar trend, being more recovered in the small intestinal digesta of BFJ-MB (68%) than that of milk (62%) and BFJ (52%) with regard of gastric digesta. Before being available for absorption, carotenoids must be solubilised and incorporated into mixed micelles. Micelles are molecular aggregates that transport the lipophilic material to the intestinal epithelium (Fernández-García, Carvajal-Lérida, Jarén-Galán, Garrido-Fernández, Pérez-Gálvez & Hornero-Méndez, 2012). Therefore, the degree of carotenoids incorporation into micelles is an important factor to take into consideration when assessing the bioaccessibility of these compounds. The recovery of total carotenoids from BFJ-MB (50%) and milk (51%) was similar when the micellar fraction was compared to the small intestinal digesta. However, total carotenoids of BFJ were the lowest recovered (43%) in the micellar fraction with respect to the small intestinal digesta. These results could be explained by the fact that carotenoid micellization is affected by several factors, such as the physicochemical properties of carotenoids, food matrix, and the fat solubility of individual carotenoids (Van Het Hof, West, Weststrate & Hautvast, 2000).

3.1.4. Antioxidant activity

Both hydrophilic and lipophilic antioxidant activities (HAA and LAA, respectively) were determined in each sample (Table 4). Total antioxidant activity (TAA) was considered as the sum of HAA and LAA. Non-digested BFJ showed the highest TAA (98.5% of DPPH inhibition), followed by BFJ-MB (86.1%) and finally milk (7.6 %). These results are in agreement with previous reports, where the TAA of fruit-based beverages was in the range of 31 to 98% of DPPH inhibition (Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso, 2013b; Ryan & Prescott, 2010).

After gastric digestion, the HAA of both BFJ-MB and BFJ diminished 8.8% and 16.5%, respectively. In contrast, it was observed an increase of 96.6% in the HAA of milk. LAA was reduced 18% in BFJ-MB and 10.5% in BFJ, whereas in milk increased 30.4%. Vitamin C and phenolic compounds are among the main dietary food antioxidants (Gülçin, 2012; Schlueter & Johnston, 2011). Significant losses in the concentration of some phenolic compounds and vitamin C were observed in the gastric

digesta of BFJ-MB and BFJ. Therefore, the stability of these substances under acidic conditions also influence on their antioxidant activity. On the other hand, the increment in HAA of milk during gastric digestion could be attributed to the high recovery of phenolic substances obtained under acidic conditions. Moreover, the digestive enzyme action could release other milk constituents, such as bioactive peptides, thiamine, pyridoxine, among others (Claeys et al., 2013; Kalač, P., 2011), which could also contribute to the increase in the antioxidant activity.

Table 4. Hydrophilic and lipophilic antioxidant activity through the *in vitro* gastrointestinal digestion of fruit juice- and milk-based beverages.[a]

Digestive phase	DPPH inhibition (%)		
	BFJ-MB	**BFJ**	**Milk**
Hydrophilic antioxidant activity (HAA)			
Non-digested	69.5 ± 2.1Aa	83.3 ± 1.5Ab	2.04 ± 0.10Ac
Gastric digesta	63.4 ± 2.3Ba	69.6 ± 3Bb	4.01 ± 0.19Bc
Small intestinal digesta	42.6 ± 1.4Ca	45.4 ± 2.0Ca	3.58 ± 0.18Cb
Dialyzed fraction	10.5 ± 0.6Da	14.9 ± 0.4Db	0.107 ± 0.003Dc
Lipophilic antioxidant activity (LAA)			
Non-digested	16.6 ± 0.5Aa	15.2 ± 0.6Ab	5.6 ± 0.3Ac
Gastric digesta	13.6 ± 0.4Ba	13.6 ± 0.4Ba	7.3 ± 0.4Bb
Small intestinal digesta	9.6 ± 0.4Ca	10.0 ± 0.5Ca	4.69 ± 0.18Cb
Micellar fraction	6.5 ± 0.5Da	4.7 ± 0.4Db	3.14 ± 0.20Dc

[a] Values are expressed as the mean ± standard deviation. Different capital letters in the same column indicate significant differences ($P < 0.05$) among digestive phases of each sample. Different lower case letters in the same row show significant differences ($P < 0.05$) among beverages. BFJ-MB= blended fruit juice-milk beverage; BFJ= blended fruit juice.

Both HAA and LAA of all the beverages diminished between 11% and 36% in the small intestinal digesta, with respect to that observed in the gastric digesta. However, greater losses of HAA and LAA were observed when the dialyzed and micellar fractions, respectively, were compared to the small intestinal digesta (up to 97% for HAA and up to 53% for LAA). These findings could be attributed to the fact that some bioactive compounds with antioxidant activity undergo several reactions under intestinal digestion, such as oxidation, formation of other derivatives, complexes formation or antagonistic interactions among bioactive compounds or with other food derivatives (Gil-Izquierdo, Gil, Ferreres & Tomás-Barberán, 2001; Saura-Calixto,

Serrano & Goñi, 2007). All these changes could contribute to the decrease in the antioxidant activity of beverages.

3.2. Bioaccessibility

3.2.1. Phenolic compounds

As can be seen in Table 5, the bioaccessibility of phenolic constituents from the samples analyzed in this research was in the range of 0% to 16.8%.

In overall, the bioaccessibility of phenolic acids and flavonoids from BFJ was higher than that of BFJ-MB and milk. In the same way, total phenolic compounds analyzed by both methodologies (TPC by HPLC and TPC by F-C) showed their greatest bioaccessibility in BFJ. Results demonstrate that the complexity of the food matrix in which bioactive compounds are contained can affect their degree of digestibility and therefore, their bioaccessibility. In addition, gastrointestinal digestion could afford either the transformation of phenolic compounds into other phenolic derivatives (such as chalcones) or the interaction with other dietary constituents. Phenolic substances could interact with dietary proteins, iron or fiber, leading to the formation of complexes (Argyri, Proestos, Komaitis & Kapsokefalou, 2005; Saura-Calixto, Serrano & Goñi, 2007). Milk is commonly added to fruit-based beverages in order to improve their sensorial and nutritional properties (Zulueta, Esteve, Frasquet, & Frígola, 2007) but it contains high amounts of proteins. Although complexes can be formed reversibly or irreversibly, depending on factors such as the protein type, phenolic concentration, pH and temperature (Roura et al., 2008), they interfere in the bioaccessibility of phenolic constituents. Additionally, it has been observed that milk affects the flavonoid metabolism pathways by increasing sulfation in healthy subjects (Roura et al., 2008).

Other important dietary factor to take into consideration is the presence of carbohydrates. Some authors reported that carbohydrates enhance the bioaccessibility of phenolic compounds from cocoa (Schramm et al., 2003), chocolate products (Neilson et al., 2009) and green tea (Peters et al., 2010). However, this trend was not observed in the BFJ-MB, which contained additional sugar. Likely, differences in the food matrix composition, such as the type and amount of carbohydrates, could favour the interactions of these dietary constituents with phenolic compounds.

Table 5. Bioccessibility of hydrophilic bioactive compounds contained in fruit juice- and milk-based beverages.[a]

Bioactive compound	Bioaccessibility (%)		
	BFJ-MB	**BFJ**	**Milk**
Phenolic compounds			
Phenolic acids			
Caffeic acid	0.0 ± 0.0a	16.55 ± 0.13b	nd
Chlorogenic acid	13.28 ± 0.43a	15.91 ± 0.25b	nd
Ferulic acid	0.0 ± 0.0a	0.0 ± 0.0a	nd
p-Coumaric acid	13.4 ± 0.5a	16.8 ± 0.9b	nd
Sinapic acid	0.0 ± 0.0a	0.0 ± 0.0a	nd
4-hydroxybenzoic acid	12.4 ± 0.3a	14.6 ± 0.3b	9.1 ± 0.4c
Total phenolic acids	11.47 ± 0.14a	14.2 ± 0.3b	9.1 ± 0.4c
Flavonoids			
Hesperidin	12.6 ± 0.3a	14.6 ± 0.5b	nd
Naringenin	12.44 ± 0.20a	15.0 ± 0.3b	nd
Quercetin	11.0 ± 0.3a	12.94 ± 0.24b	nd
Rutin	7.0 ± 0.4a	11.8 ± 0.3b	nd
(+)-Catechin	12.4 ± 0.4a	13.9 ± 0.3b	12.8 ± 0.2c
Total flavonoids	11.82 ± 0.17a	14.10 ± 0.20b	12.84 ± 0.22c
Total phenolic compounds			
Sum of individuals	11.69 ± 0.13a	14.14 ± 0.11b	11.30 ± 0.17c
Folin-Ciocalteu method	10.92 ± 0.51a	19.68 ± 0.47b	14.48 ± 0.30c
Vitamin C	11.5 ± 0.7a	14.9 ± 0.5b	nd
Hydrophilic antioxidant activity	15.2 ± 0.8a	17.9 ± 0.5b	5.3 ± 0.3c

[a] Values are expressed as the mean ± standard deviation. Bioaccessibility of hydrophilic compounds was determined as the ratio of solubilised compound in the dialyzed fraction with respect to non-digested beverage. Different letters in the same row show significant differences ($P < 0.05$) among beverages. BFJ-MB= blended fruit juice – milk beverage; BFJ= blended fruit juice; nd=not detected.

3.2.2. Vitamin C

The bioaccessibility of vitamin C was 11.5% in BFJ-MB and 14.9% in BFJ, but it was detected neither before nor after the *in vitro* gastrointestinal digestion of milk (Table 5). Similar results were reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2013b), who observed 15% of vitamin C bioaccessibility in a blend of three fruit juices. As far as we know, few reports have been focused in assessing the vitamin C bioaccessibility in complexes matrices, such as fruit and milk based beverages. Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso (2014) reported that the bioaccessibility of vitamin C was 14% higher in a blended fruit

juice than in a beverage containing the same blend of fruit juices plus soymilk. Cilla et al. (2012) showed that this compound was around 13% bioaccessible in a beverage of fruit juice with soymilk, but they obtained between 68.57 and 70.19% of vitamin C bioaccessibility in a blend of fruit juices and milk. Variances in the results could be attributed to differences in methodological procedures (i.e. vitamin C extraction and *in vitro* gastrointestinal digestion), as well as differences in the beverage formulation (i.e. amount of milk).

On the other hand, the higher bioaccessibility of vitamin C in BFJ than in BFJ-MB suggest that this compound could interact with other food constituents, mainly those coming from milk, such as proteins, vitamins and minerals. This hypothesis is supported by the fact that Claeys et al. (2013) identified vitamin-binding proteins in milk, which could afford the formation of complexes among these compounds. Milk also contains other vitamins that are able to interact with vitamin C, such as B_{12}, B_2, and B_1, producing deleterious effects in food (Berry, 2009). In addition, important amounts of minerals (Ca, P, K, Cl, Mg, Na, among others) and in less quantity metal ions (Fe, Cu and Zn) have been identified in milk (Claeys et al., 2013). Berry (2009) established that trace amounts of metal ions are enough to catalyze the oxidation of vitamin C. All these interactions could explain the behaviour observed in the bioaccessibility of vitamin C in BFJ-MB.

3.2.3. Carotenoids

Carotenoid bioaccessibility of the analyzed beverages is shown in Table 6, being in the range of 7.8 to 26.8%. The most bioaccessible carotenoids of BFJ-MB, BFJ and milk were β-carotene, lutein and β-cryptoxanthin, respectively. In line to these results, Dhuique-Mayer & Amiot-Carlin (2009) and Granado-Lorencio et al. (2007) observed that the bioaccessibility of lutein, β-cryptoxanthin, zeaxanthin and trans- β-carotene was between 16 and 40% in citrus juices.

In general, individual carotenoids displayed higher bioaccessibility in BFJ-MB, followed by milk and BFJ when these compounds were individually analyzed. However, if total carotenoid content is taken into consideration, it was observed that the hierarchy of bioaccessibility was milk>BFJ-MB>BFJ. Results demonstrated that the bioaccessibility of carotenoids was improved in beverages containing milk. Likely, the milk fat content increased the solubilisation of carotenoids into mixed micelles and thus,

their bioaccessibility. In fact, Van Het Hof, West, Weststrate & Hautvast, (2000) reported that the formation of mixed micelles in the intestine is dependent on the type and amount of dietary fat. Fernández-García, Mínguez-Mosquera & Pérez-Galvez (2007) observed that the composition of the lipid matrix produce a differential incorporation of carotenoids into micelles. Fernández-García et al. (2012) reported that the fatty matter is a key factor that could increase the micellisation of carotenoids.

Table 6. Bioccessibility of lipophilic bioactive compounds contained in fruit juice- and milk-based beverages.[a]

Bioactive compound	Bioaccessibility (%)		
	BFJ-MB	**BFJ**	**Milk**
Carotenoids			
Carotenes			
α-carotene	23.1 ± 1.5a	12.6 ± 0.3b	nd
β-carotene	26.8 ± 1.5a	14.3 ± 0.5b	21.8 ± 1.4c
Total carotenes	26.6 ± 1.5a	14.2 ± 0.5b	21.8 ± 1.4c
Xantophylls			
Cis-vilaxanthin+neoxanthin	12.2 ± 0.6a	7.8 ± 0.4b	nd
Cis-anteraxanthin	13.2 ± 0.6a	12.7 ± 0.8a	nd
Anteraxanthin	17.2 ± 0.4a	11.2 ± 0.6b	nd
Lutein	24.1 ± 1.3a	19.7 ± 1.0b	20.5 ± 0.4a
α-cryptoxanthin	20.4 ± 1.0a	19.1 ± 0.5b	nd
β-cryptoxanthin	18.0 ± 0.6a	13.3 ± 0.7b	16.8 ± 0.3c
Total xantophylls	15.9 ± 0.3a	13.7 ± 0.5b	19.0 ± 0.3c
Total carotenoids	18.9 ± 0.5a	13.8 ± 0.3b	21.4 ± 1.2c
Lipophilic antioxidant activity	39 ± 3a	30.9 ± 2.1b	56.0 ± 2.5c

[a] Values are expressed as the mean ± standard deviation. Bioaccessibility of lipophilic compounds was determined as the ratio of solubilised compound in the micellar fraction with respect to non-digested beverage. Different letters in the same row show significant differences ($P < 0.05$) among beverages. BFJ-MB= blended fruit juice – milk beverage; BFJ= blended fruit juice; nd=not detected.

Parada & Aguilera (2007) found that absorption of carotenoids not only depends on their release from the food matrix but also on their incorporation into micelles, where dietary lipids play an important role. This trend was also showed by Granado-Lorencio et al. (2009) who observed an increase in the bioaccessibility of lutein (57%), zeaxanthin (116%) and β-cryptoxanthin (83%) when milk was added to blended fruit juices.

On the other hand, the low carotenoid bioaccessibility in BFJ (with less amount of fat as compared with milk and BFJ-MB) also support the hypothesis that certain quantity of fat in food favours the incorporation of carotenoids into micelles. Moreover, these results suggest that interactions carotenoid-carotenoid or carotenoid-fiber could be strongest in BFJ in comparison to BFJ-MB and milk. In this sense, Maiani et al. (2009), observed that the incorporation of carotenoids into mixed micelles is affected by a competitive inhibition between themselves. In addition, fruit juices contain certain amount of natural fiber, which might entrap carotenoids, leading to reduce their bioaccessibility and absorption, as well as increasing their faecal excretion (Hoffmann, Linseisen, Riedl & Wolfram, 1999).

3.2.3. Antioxidant activity

The bioaccessibility of hydrophilic compounds with antioxidant activity of the beverages analyzed in this study were in the range of 5.3 to 15.2 (Table 5), while that of lipophilics ranged from 30.9 to 56% (Table 6).

Milk addition reduced 15% the bioaccessibility of BFJ hydrophilic constituents with antioxidant activity. These results suggest that several bioactive compounds of BFJ, such as phenolic compounds and vitamin C, interact with proteins and minerals/metals from milk. It has been reported that phenolic substances possess a high affinity for proteins, including caseins (Luck et al., 1994). In fact, Jobstl et al. (2006) reported a non-covalent cross-link between epigallocatechin gallate and casein. Vitamin C also can interact with proteins and minerals from milk (Claeys et al., 2013), reducing its antioxidant power.

On the other hand, the bioaccessibility of lipophilic compounds was increased 27% in the blended fruit juice containing milk (BFJ-MB), with respect to the beverage without milk (BFJ). Likely, the solubilisation of liposoluble compounds, such as carotenoids, into mixed micelles was improved by the fat content of milk. This trend was corroborated by the findings obtained in the micellar and bioaccessible fractions of carotenoids as reported in Tables 3 and 6. Additionally, Cilla et al. (2012) found that the bioaccessibility of carotenoids, such as β-carotene, β-cryptoxanthin and lutein, was higher in blended fruit juices containing whole milk than in that of skimmed milk.

4. Conclusion

The digestibility and bioaccessibility of bioactive compounds from fruit juice- and milk-based beverages were significantly influenced by the beverage formulation. Under gastric conditions, the release of several phenolic substances was improved in milk and BFJ-MB but not in a simple matrix as BFJ. Vitamin C and carotenoids of the three beverages diminished significantly ($P < 0.05$) in both gastric and intestinal digesta. The highest bioaccessibility of phenolic compounds (phenolic acids and flavonoids), vitamin C and hydrophilic constituents with antioxidant activity was found in the beverage without milk content (BFJ). In contrast, the addition of milk to BFJ improved the bioaccessibility of lipophilic compounds (carotenes, xanthophylls, and those with lipophilic antioxidant activity). Although the bioaccessibility of the hydrophilic substances was reduced when milk was added to BFJ, BFJ-MB also contains important amounts of bioaccessible water-soluble compounds. As a result, BFJ-MB can be considered as an interesting food matrix that besides combining the healthy properties of BFJ and milk, improves the bioaccessibility of lipophilic compounds.

The beverage formulation should be taken into consideration when developing new foods and beverages in order to improve the bioaccessibility, bioavailability and functionality of their biologically active substances. All these are important challenges to manufacturers and the *in vitro* gastrointestinal digestion could help in the understanding of the essential first step (the bioaccessibility). The extent to which *in vitro* gastrointestinal digestion could predict with accuracy the *in vivo* bioaccessibility and bioavailability of the compounds analyzed in this research remains to be determined.

Acknowledgement

This research has been financed by the Ministerio de Ciencia e Innovación (Spain), reference AGL2006-12758-C02-02/ALI. María Janeth Rodríguez-Roque thanks to the Comissionat per a Universitats i Recerca, del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (AGAUR) and European Social Fund for the predoctoral grant, and to the Secretaría de Educación Pública de México (SEP) for their support. Prof. Olga Martín-Belloso thanks to the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia Award 2008.

Abbreviations list

BFJ, blended fruit juice

BFJ-MB, blended fruit juice-milk beverage

DPPH, 1,1-diphenyl-2-picrylhydrazyl

F−C, Folin−Ciocalteu

HAA, hydrophilic antioxidant activity

HPLC, high-performance liquid chromatography

LAA, lipophilic antioxidant activity

TPC, total phenolic compounds

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CHAPTER V

Impact of food matrix and processing on the *in vitro* bioaccessibility of vitamin C, phenolic compounds, and hydrophilic antioxidant activity from fruit juice-based beverages

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Abstract

The effect of food matrix (water, milk, and soymilk) and processing [high intensity pulsed electric fields (HIPEF) (35 kV/cm with 4 μs bipolar pulses at 200 Hz during 1800 μs); high-pressure processing (HPP) (400 MPa at 40 ºC for 5 min); and thermal treatment (TT) (90 ºC during 1 min)] on the *in vitro* bioaccessibility of vitamin C and phenolic compounds, as well as on the hydrophilic antioxidant activity (HAA) of fruit-juice based beverages was analyzed. HIPEF and HPP improved or did not change the bioaccessibility of vitamin C and certain phenolic compounds in comparison with untreated beverages. In contrast, TT diminished the bioaccessibility of most of these compounds. The greatest bioaccessibility of vitamin C was obtained in beverages made with soymilk (SB), whereas water-beverages (WB) favoured the bioaccessibility of phenolic compounds and HAA. Results showed that both food matrix and processing modulated the bioaccessibility of vitamin C and phenolic compounds of blended beverages. Furthermore, HPP and HIPEF allow obtaining beverages with improved nutritional and functional quality than TT.

Keywords: Fruit juice-based beverages; Bioaccessibility; Food matrix; Non-thermal and thermal technologies; Vitamin C; Phenolic compounds; Hydrophilic antioxidant activity

1. **Introduction**

Currently, the food industry is attracting the consumer attention through functional foods and beverages that besides being highly nutritious and healthy, they are easy to prepare and consume (Wootton-Beard & Ryan, 2011). Originality, convenience and quality are considered as important marketing tools in the food industry to increase sales (Marsellés-Fontanet, Elez-Martínez, & Martín-Belloso, 2012). In this context, fruit juice-based beverages are becoming more popular due to represent an easy and convenient way of consuming fruits, which are important sources of health-promoting compounds, such as vitamin C and phenolic compounds.

Vitamin C is an essential nutrient for the biosynthesis of collagen and certain hormones. Its intake has been related to reduce the risk of cancer and cardiovascular diseases (Li & Schellhorn, 2007). On the other hand, it has been reported that diets rich in phenolic compounds correlates with the decrease of neurodegenerative disease and some cancer types (Aboul et al. 2013). In addition, both bioactive compounds are good contributors to the antioxidant activity of food (Barba, Cortés, Esteve, & Frígola, 2012).

Blended fruit juices are often combined with milk and soymilk to improve the sensorial and nutritional characteristics of the finished product. Moreover, milk is a rich source of proteins, unsaturated fatty acids, vitamins, carotenoids, minerals, among others (Claeys et al., 2013; Antone, Sterna & Zagorska, 2012); while soymilk contains high amounts of phenolic compounds, isoflavones, proteins, iron and niacin (Jinapong, Suphantharika, & Jamnong, 2008; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013b).

Liquid foods have been traditionally preserved by thermal treatment (TT) to prevent microorganisms spoilage and contamination with pathogens that can cause severe toxinfections in humans who consume them. However, this treatment leads to the loss of healthy compounds and sensorial properties of food (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013). Non-thermal food preservation technologies, such as high-intensity pulsed electric fields (HIPEF) and high-pressure processing (HPP), have been developed as alternatives to heat treatments in order to satisfy consumer demand for nutritious, healthy and safety products with a fresh-like appearance (Barbosa-Cánovas, Tapia, & Cano, 2005). Previous studies had reported that both technologies (HIPEF and HPP) inactivate microorganisms and enzymes

without compromise the nutritional and sensorial quality of food (Kadam, Jadhav, Salve & Machewad, 2012; Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013; Sanchez-Moreno, De Ancos, Plaza, Elez-Martinez, & Cano, 2009).

Processing is expected to modify the natural matrix or the microstructure of food. These changes could exert a significant influence on the release, transformation and absorption of some nutrients during digestion (Parada & Aguilera, 2007). The fraction of bioactive compound released from the food matrix following digestion that is solubilised into the gut for intestinal uptake is usually known as bioaccessible fraction (Ferruzzi, 2010). Since the point of view of nutritional and functional value of beverages, the information about the concentration of bioactive compounds reaching the bioaccessible fraction is a more important data than only know the concentration of this compound in the corresponding beverage. Moreover, it is also important determining the influence of food matrix on the bioavailability of bioactive compounds, especially in beverages because they are a complex medium that allows interactions between these bioactive compounds, nutrients and/or other constituents of food (proteins, lipids, carbohydrates, minerals, etc) (Kilara, 2006). Analyzing the extent to which food matrix and processing may modify the interactions, the stability and the bioaccessibility of bioactive compounds is an essential first step to better understanding the biological activity of food constituents.

Although *in vivo* studies, like human intervention studies, provide more specific information about the bioavailability of bioactive compounds, *in vitro* digestion models are considered valuable and useful methodologies for estimating pre-absorptive events as stability and bioaccessibility of nutrients and bioactive compounds from food (Wood, 2005).

While the influence of HIPEF and HPP on the concentration of bioactive compounds of beverages has been previously evaluated (Barbosa-Cánovas, Tapia, & Cano, 2005; Kadam, Jadhav, Salve & Machewad, 2012; Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013; Sanchez-Moreno, De Ancos, Plaza, Elez-Martinez, & Cano, 2009), information about the impact of these technologies on the bioaccessibility of nutrients is really scarce (Cilla et al., 2012). For this reason, this research aimed to assess the effect of the food matrix (water, milk and soymilk) and processing (HIPEF, HPP and TT) on the *in vitro* bioaccessibility of vitamin C and phenolic compounds, as

well as on the hydrophilic antioxidant activity of beverages based on a blend of fruit juices (orange, pineapple, kiwi and mango).

2. **Material and methods**

2.1. Reagents

Pepsin from porcine stomach, pancreatin from porcine pancreas, bovine bile, DL-1,4-dithiothreitol (DTT), metaphosphoric acid, phenol standards (caffeic, chlorogenic, ferulic, sinapic, p-coumaric and 4-hydroxybenzoic acids; hesperidin, naringenin, rutin, quercetin and [+]-catechin), 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) and cellulose dialysis membrane (molecular weight cutoff of 12,000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid and Folin-Ciocalteu (F-C) reagent were acquired from Scharlau Chemie S.A. (Barcelona, Spain).

2.2. Fruit juice-based beverages

Fruits (orange, kiwi, pineapple and mango) were purchased at commercial maturity in a local supermarket (Lleida, Spain). Fruits were washed, peeled and the juice extracted. Each fresh-squeezed juice was filtered with a cheesecloth using a vacuum pump. A blended fruit juice was obtained by mixing 40% of orange, 33% of kiwi, 13.5% of pineapple and 13.5% of mango juices.

Whole milk (Hacendado, Cordoba, Spain) and soymilk (Yosoy, Girona, Spain) were also purchased at local supermarket. Milk composition consisted of 3.6% of fat, 3.0% of protein and 4.5% of carbohydrates; while 1.8% of fat, 3.6% of protein, 0.7% of carbohydrates and 1% of fibre were contained in soymilk (data provided by manufacturers).

Afterwards, three different fruit juice-based beverages were prepared by mixing 75% of the blended fruit juice (orange, kiwi, pineapple and mango juices); 17.5% of milk (MB, milk-fruit juice beverage), or soymilk (SB, soymilk-fruit juice beverage), or distilled water (WB, water-fruit juice beverage); and 7.5% of sugar. The pH of the beverages was adjusted to 3.30 ± 0.20 (Crison Instruments S.A., Alella, Barcelona, Spain) with citric acid. The soluble solid content was determined in a refractometer Comecta S.A., Abrera (Barcelona, Spain), resulting in 18.0 ± 0.2, 18.5 ± 0.2, 19.3 ± 0.3 ºBrix for WB, SB and MB, respectively. Beverages formulations were selected

according to previous studies, in which a high concentration of bioactive compounds (vitamin C and antioxidant activity) was reached (Salvia-Trujillo, Morales-De La Peña, Rojas-Graü & Martín-Belloso, 2011a).

2.3. Processing technologies

2.3.1. High Intensity Pulsed Electric Fields (HIPEF)

HIPEF treatment was performed in a continuous-flow bench scale system (OSU-4F, The Ohio State University, Colombus, OH, USA), using square-wave pulses. Eight collinear chambers serially connected were used as treatment system. Each chamber consisted of two stainless steel electrodes separated by a gap of 0.29 cm. The flow rate was adjusted to 60 mL/min and controlled by a variable speed pump (model 752210-25, Cole Palmer Instrument Company, Vermon Hills, IL, USA). HIPEF processing conditions for beverages were 35 kV/cm field strength in bipolar mode, 4 μs pulse width, 200 Hz pulse frequency and 1800 μs total treatment time. Temperature was always kept below 35 ºC using a cooling coil connected before and after each pair of chambers and submerged in an ice-water shaking bath. HIPEF conditions were selected based on previous studies carried out in our laboratory, where the nutritional and microbiological stability of similar beverages was accomplished (Morales-de La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2011; Salvia-Trujillo, Morales-de la Peña, Rojas-Graü, & Martín-Belloso, 2011b).

2.3.2. *High-Pressure Processing (HPP)*

Beverages were treated in a high hydrostatic pressure unit with a vessel of 2925 mL capacity, a maximum pressure of 900 MPa, and a maximum temperature of 100 ºC (High Pressure Iso-Lab System, Model FPG7100:9/2C, Stansted Fluid Power LTD., Essex, UK). Previously to HPP, all three beverages were vacuum packed in flexible Doypack® bags (Polyskin XL, Flexibles Hispania, S.L.) (300 mL). Afterwards, they were introduced in the pressure unit filled with pressure medium (water). Beverages were processed at 400 MPa with a holding time of 5 min. The rates of compression and decompression were both 3 MPa/s. Because of adiabatic compression, the maximum temperature in the vessel was 40 ºC at 400 MPa. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the

process. These conditions were selected based on previous studies where the nutritional and microbiological stability of HPP fruit juices and similar beverages were achieved (Sánchez-Moreno et al., 2005; Muñoz, De Ancos, Sánchez-Moreno, & Cano, 2007)

2.3.3. *Thermal Treatment (TT)*

A tubular stainless-steel heat exchanger coil immersed in a hot water shaking bath was used to treat beverages by heat (University of Lleida, Spain). The flow rate of beverages was maintained through a gear pump. Beverages were thermally treated at 90 ºC for 60 s. After heating, the beverages were immediately cooled down to 5 ± 1 ºC in an ice-water bath.

2.4. In vitro gastrointestinal digestion

Once processed, beverages were digested following the *in vitro* methodology described by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a). This method consisted of two sequential stages: gastric (pH 2, containing pepsin) and small intestinal digestions with dialysis (pH 7, containing a pancreatin-bile mixture). Aliquots of digested beverages were collected from the dialyzed fraction at the end of the digestive process and immediately placed in a cold water bath during 10 minutes. All samples were frozen (-45 ºC) until analysis.

2.5. Bioactive compounds analysis

2.5.1. Vitamin C

Vitamin C was extracted, separated, identified and quantified by HPLC according to the methodology reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a). Vitamin C identification was carried out by comparison of its retention time and spectra with the standard (ascorbic acid) and using a calibration curve (R^2=0.9989, concentration in the range of 10 to 1000 mg/L). Results were expressed as mg of ascorbic acid /100 mL of sample.

2.5.2. *Phenolic compounds analyzed by HPLC*

Extraction, separation, identification and quantification of phenolic compounds by HPLC were performed following the methodology of Rodríguez-Roque, Rojas-Graü,

Elez-Martínez, & Martín-Belloso (2013b). Individual phenolic compounds were identified by comparison of their retention time and spectra with those of the standards (caffeic, chlorogenic, ferulic, sinapic, p-coumaric and 4-hydroxybenzoic acids; hesperidin, naringenin, quercetin, rutin and (+)-catechin). Quantification was carried out by integration of the peak areas and using calibration curves (R^2 in the range of 0.9978 to 0.9999, concentration from 5 to 500 mg/L). Results were expressed as mg of phenolic compound/100 mL of sample. Total phenolic acids and total flavonoids were determined as the sum of individual compounds of each family of phenolic substances. The concentration of total phenolic compounds (TPC by HPLC) was the sum of total phenolic acids and total flavonoids determined as individuals by HPLC.

2.5.3. Total phenolic content analyzed by Folin-Ciocalteu (TPC by F-C) methodology

Total phenolic compounds were determined according to the methodology of Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b), which is based on the method previously described by Singleton, Orthofer, & Lamuela-Raventós (1998). A calibration curve of gallic acid (R^2=0.9990, concentration from 50 to 2000 mg/L) was used to quantify the concentration of total phenolic compounds in each sample. Results were expressed as mg of gallic acid/100 mL of sample.

2.6. Hydrophilic antioxidant activity (HAA)

Extraction of hydrophilic fraction of non-digested or digested beverages was carried out based on the procedure reported by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, (2013b). The antioxidant activity was evaluated through the colorimetric method ($DPPH^{\bullet}$ radical) reported by Brand-Williams, Cuvelier, & Berset (1995). Results were expressed as percentage of $DPPH^{\bullet}$ inhibition.

2.7. Bioaccessibility calculations

Bioaccessibility was determined using Eq. 1 and was expressed as percentage.

$$Bioaccessibility(\%) = 100x\left(\frac{BC_{digested}}{BC_{non-digested}}\right) \quad \text{Eq. 1}$$

where $BC_{digested}$ corresponded to the bioactive compound concentration in the digested beverage and $BC_{non\text{-}digested}$ was the bioactive compound concentration in non-digested beverage.

2.8. Statistical analysis

Each bioactive compound was extracted and analyzed two times from two independent experiments. Analysis of variance (ANOVA) followed by the least significant difference test (LSD) were applied to the results obtained to verify whether there were significant differences ($p < 0.05$) in the concentration and bioaccessibility of bioactive compounds from beverages in relation to the factors studied in this research (food matrix and processing). All analyses were carried out with the statistical program Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD, USA). Results were reported as the mean ± standard deviation.

3. Results and discussion

3.1. Vitamin C

The concentration of vitamin C from the three beverages analyzed (WB, MB and SB) is reported in Table 1. In untreated beverages, the concentration of vitamin C was between 29.5 and 30.8 mg of ascorbic acid/100 mL with no significant differences among beverages. These results were within the range reported in similar products, which were from 9.3 to 41.6 mg /100 mL (Morales-de la Peña et al., 2010; Zulueta, Esteve, Frasquet, & Frígola, 2007).

According to the results obtained in this study, the vitamin C concentration was significantly influenced by treatments (HIPEF, HPP and TT), but not for the food matrix ($p > 0.05$). HIPEF processing reduced the concentration of this compound in the range of 8 – 15% as compared with those untreated. Beverages treated by HPP did not change their content of vitamin C in comparison with untreated ones, with the exception of SB, where a decrease of 10.5% was observed. When both treatments (HIPEF and HPP) were compared, no significant changes in the concentration of vitamin C from WB and SB were observed. On the other hand, the greatest losses of this bioactive compound were observed in TT samples, reaching up to 31% compared with untreated beverages. In line to these results, Cilla et al. (2012) and Morales-de la Peña et al. (2010) reported that fruit juice-based beverages processed by HIPEF and HPP, respectively, decreased around 11 – 13% their vitamin C concentration. Other authors also showed that the content of vitamin C in HIPEF or HPP samples was very close to those of untreated beverages, whereas the losses of this bioactive were always higher in

TT beverages (Morales-de la Peña et al., 2010; Plaza et al., 2006; Sánchez-Moreno et al., 2005).

Table 1. Concentration and bioaccessibility of vitamin C in blended beverages.[a]

	Vitamin C		
	WB	**MB**	**SB**
	Concentration (mg/100 mL)		
Untreated	30.4 ± 2.0aA	30.8 ± 2.1aA	29.5 ± 1.7aA
HIPEF	25.8 ± 1.3bA	27.9± 1.6bA	27.1 ± 1.5bA
HPP	28.0 ± 1.8abAB	29.5 ± 2.0abB	26.4 ± 1.5bA
TT	25.7 ± 1.1bA	21.3 ± 0.9cB	23.9 ± 1.0cC
	Bioaccessibility (%)		
Untreated	13.3 ± 0.8aA	11.3 ± 0.5aB	23.2 ± 1.2aC
HIPEF	14.2 ± 0.9aA	11.9 ± 0.5aB	21.3 ± 1.0abC
HPP	13.7 ± 0.6aA	13.1 ± 0.8bA	23.0 ± 1.6aB
TT	11.1 ± 0.6bA	10.9 ± 0.7aA	20.5 ± 1.4bB

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column show significant differences ($p < 0.05$) within treatments. Different capital letters in the same row indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.

Bioaccessibility of vitamin C. Vitamin C bioaccessibility of untreated and treated (HIPEF, HPP and TT) beverages was in the range of 10.9 and 23.2% (Table 1). Previous studies have shown a vitamin C bioaccessibility of 13% and 15% in a beverage based on fruit juice and soymilk and in a blended fruit juice, respectively (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013a; 2014). Similarly, this compound was between 7.2% and 12.58% bioaccessible in HPP treated and untreated fruit juice-beverages containing soymilk (Cilla et al., 2012).

The treatment by non-thermal technologies (HIPEF and HPP) did not modify the bioaccessibility of vitamin C as compared with untreated beverages, except for MB treated by HPP which increased 8%. In contrast, significant losses in the vitamin C bioaccessibility were observed in TT beverages, being reduced 16.5% and 11.6% in WB and SB, respectively. It has been reported that thermal treatment of food promotes the nutrient release through cell rupture (breakage) or cell separation (Parada & Aguilera, 2007) and can also enhance the bioavailability of several nutrients by releasing them

from the food matrix (Hotz and Gibson, 2007). However, vitamin C is a thermo-labile compound, which is very susceptible to chemical and enzymatic oxidation during processing (Yeom, Streaker, Zhang, & Min, 2000). The catalyzed oxidative pathway of ascorbic acid degradation is the most important reaction pathway for losing vitamin C in foods (Ball et al. 2006). Therefore, oxidative reactions of vitamin C in TT beverages could explain why in this research the worst bioaccessibility of vitamin C was obtained in TT beverages. On the other hand, vitamin C is also vulnerable to enzyme catalyzed oxidation, specifically to ascorbate oxidase and peroxidase (Davey et al. 2000). HIPEF and HPP are able to inactivate some of these enzymes (Sánchez-Moreno et al., 2005), avoiding the oxidation of vitamin C and thus, maintaining its active form and bioaccessibility in beverages treated by these technologies.

Similar results were reported in a study carried out with twelve healthy subjects, in which an orange juice treated by HIPEF or HPP preserved the *in vivo* bioavailability and the antioxidant characteristics of vitamin C of the fresh product (Sánchez-Moreno et al., 2003, 2004). Conversely, Cilla et al. (2012) reported that HPP diminished significantly the bioaccessibility of vitamin C in both milk and soymilk beverages, while it increased in TT beverages.

In overall, the food matrix had a significant influence ($p < 0.05$) on the bioaccessibility of vitamin C. The highest bioaccessibility of this compound was observed in SB products, followed by WB and MB. It is well known that the stability of vitamin C is influenced by several factors, such as oxygen availability, temperature, light, pH, metal catalyst, the presence of another antioxidants and reducing agents, as well as the possible presence of ascorbic acid oxidase (Eitenmiller, Ye, & Landen, 2008). Soymilk is a rich source of phenols and isoflavones, thus, it could be hypothesized that these antioxidant compounds could avoid the vitamin C oxidation in SB beverage. In contrast, antagonistic interactions between vitamin C and other food constituents (i.e. proteins, vitamins, metal ions) could occur, mainly in products containing milk (MB). Milk contains vitamin-binding proteins that could afford the formation of complexes (Claeys et al., 2013). In addition, other vitamins types (B_1, B_2 and B_{12}) and metal ions (Fe, Cu and Zn) contained in milk are able to interact with vitamin C increasing its degradation rate (Ball, 2006; Berry, 2009).

In our knowledge, there is really scarce literature concerning the food matrix effect on the bioaccessibility of bioactive compounds, including vitamin C, from mixed

beverages. Cilla et al. (2012) reported that the highest bioaccessibility of vitamin C was obtained in beverages containing 16.5% of whole milk, while the lowest was in that made with 42.5% of soymilk. Differences in the results obtained in the present research and in that reported by Cilla et al. (2012) could be explained by the proportion of juices, milk or soymilk utilized to prepare the beverages. Additionally, these authors (Cilla et al., 2012) determined the vitamin C bioaccessibility in the supernatants obtained after centrifugation of the intestinal digesta (3300g/1h at 4ºC) instead of the dialyzed fraction (as in this research). In a previous study carried out in our laboratory, it was found that a beverage made with a similar blend of juices and 42.5% of soymilk (2.4 times higher than in this research) led to 13% of vitamin C bioaccessibility (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). Taking into account the behaviour of vitamin C in similar beverages with different amount of soymilk, it could be speculated that the proportion in which beverages are made is very important in terms of synergistic and antagonistic interactions, which also affect the bioaccessibility of the biologically active compounds in foods and beverages.

3.2. Phenolic compounds

The concentration of individual phenolic compounds, total phenolic acids, total flavonoids, total phenolic compounds determined by HPLC as the sum individuals (TPC by HPLC) and total phenolic compounds determined by Folin-Ciocalteu method (TPC by F-C) is presented in Tables 2 and 3.

In untreated beverages, the concentration of TPC by HPLC was in the range of 25.4 and 34.4 mg/100 mL, and between 76 and 125 mg of gallic acid/100 mL by F-C method. Flavonoids were the predominant phenolic compounds of all the analyzed beverages, while sinapic acid and (+)-catechin were not detected. It has been reported that other non-phenolic substances (i.e. sugar, amines, organic acids and proteins) could be reduced by the Folin-Ciocalteu reagent (Prior, Wu, & Schaich, 2005), which could explain why the content of TPC by F-C was higher than that of those determined by HPLC.

Table 2. Concentration of phenolic compounds in blended beverages.[a]

Beverages	Treatments	Phenolic compounds concentration (mg/100 mL)								
		Caffeic acid	Chlorogenic acid	Ferulic acid	p-coumaric acid	4-hydroxibenzoic acid	Hesperidin	Naringenin	Quercetin	Rutin
WB	Untreated	0.570 ± 0.22aA	1.95 ± 0.04aA	0.61 ± 0.03aA	1.58 ± 0.07aA	4.8 ± 0.3aA	9.2 ± 0.3aA	3.13 ± 0.20abA	1.90 ± 0.08aA	1.64 ± 0.06aA
	HIPEF	0.64 ± 0.04bA	1.63 ± 0.08bA	0.656 ± 0.024bA	1.37 ± 0.03bA	3.31 ± 0.21bA	8.2 ± 0.5bA	2.90 ± 0.15aA	1.47 ± 0.10bA	1.30 ± 0.07bA
	HPP	0.63 ± 0.03bcA	1.87 ± 0.09aA	0.63 ± 0.04abA	1.44 ± 0.04bA	3.96 ± 0.25cA	8.9 ± 0.4aA	3.23 ± 0.17bA	1.61 ± 0.03cA	1.27 ± 0.04bA
	TT	0.586 ± 0.018acA	1.31 ± 0.08cA	0.602 ± 0.18aA	1.23 ± 0.04cA	3.09 ± 0.12bA	7.7 ± 0.3bA	2.36 ± 0.10cA	1.27 ± 0.06dA	1.26 ± 0.04bA
MB	Untreated	0.66 ± 0.04aB	2.40 ± 0.08aB	0.813 ± 0.019aB	1.66 ± 0.10aA	4.4 ± 0.3aA	11.0 ± 0.3aB	4.55 ± 0.25aB	1.71 ± 0.04aB	1.85 ± 0.08aB
	HIPEF	0.767 ± 0.022bB	3.25 ± 0.16bB	0.728 ± 0.012bB	1.84 ± 0.09bB	5.4 ± 0.3bB	13.8 ± 0.3bB	5.83 ± 0.24bB	2.17 ± 0.11bB	1.67 ± 0.06bB
	HPP	0.80 ± 0.04bB	3.43 ± 0.22bB	0.73 ± 0.05bB	1.86 ± 0.05bB	5.7 ± 0.4bB	15.0 ± 0.4cB	6.4 ± 0.3cB	2.30 ± 0.16bB	1.47 ± 0.04cB
	TT	0.70 ± 0.04aB	2.77 ± 0.11cB	0.66 ± 0.03cB	1.750 ±0.011abB	4.3 ± 0.3aB	13.0 ± 0.3dB	5.33 ± 0.13dB	1.93 ± 0.11cB	1.38 ± 0.08cB
SB	Untreated	0.585 ± 0.023aA	2.65 ± 0.10aC	1.16 ± 0.07aC	1.94 ± 0.04aB	4.8 ± 0.3aA	13.2 ± 0.3aC	6.5 ± 0.4aC	2.13 ± 0.11aC	1.54 ± 0.05aA
	HIPEF	0.66 ± 0.04bA	3.72 ± 0.18bC	0.95 ± 0.03bC	2.21 ± 0.15bC	5.6 ± 0.3bB	14.4 ± 0.4bB	7.4 ± 0.3bC	2.67 ± 0.12bC	1.77 ± 0.10bB
	HPP	0.65 ± 0.03bA	3.81 ± 0.12bC	0.94 ± 0.03bC	2.43 ± 0.11cC	5.78 ± 0.16bB	15.5 ± 0.9cB	8.9 ± 0.5cC	3.0 ± 0.14cC	2.27 ± 0.09cC
	TT	0.60 ± 0.03aA	3.28 ± 0.23cC	0.767 ± 0.023cC	1.956 ± 0.013aC	5.04 ± 0.25aC	14.6 ± 0.5bC	7.5 ± 0.3bC	2.45 ± 0.06dC	1.90 ± 0.04dC

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column for each beverage show significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.

Table 3. Concentration of total phenolic compounds in blended beverages.[a]

Beverages	Treatments	Phenolic compounds concentration (mg/100 mL)			
		Total phenolic acids	Total flavonoids	TPC by HPLC	TPC by F-C
WB	Untreated	9.5 ± 0.4aA	15.9 ± 0.3aA	25.4 ± 0.6aA	76 ± 4aA
	HIPEF	7.61 ± 0.22bA	13.8 ± 0.6bA	21.5 ± 0.5bA	88 ± 5bA
	HPP	8.53 ± 0.25cA	14.97 ± 0.21cA	23.5 ± 0.3cA	90 ± 6bA
	TT	6.82 ± 0.14dA	12.6 ± 0.3dA	19.4 ± 0.4dA	73 ± 5aA
MB	Untreated	9.9 ± 0.4aA	19.1 ± 0.4aB	29.1 ± 0.3aB	82 ± 4aA
	HIPEF	12.0 ± 0.4bB	23.5 ± 0.5bB	35.4 ± 0.8bB	90 ± 6aA
	HPP	12.5 ± 0.3cB	25.2 ± 0.3cB	37.7 ± 0.4cB	85 ± 5aA
	TT	10.22 ± 0.13aB	21.6 ± 0.3dB	31.8 ± 0.4dB	92 ± 6aB
SB	Untreated	11.1 ± 0.4aB	23.3 ± 0.6aC	34.4 ± 0.9aC	125 ± 9aB
	HIPEF	13.1 ± 0.3bC	26.2 ± 0.7bC	39.3 ± 0.8bC	118 ± 5aB
	HPP	13.6 ± 0.3bC	29.7 ± 0.7cC	43.3 ± 0.7cC	102 ± 5bB
	TT	11.6 ± 0.5aC	26.4 ± 0.5bC	38.0 ± 0.5dC	92 ± 5bB

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column for each beverage show significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.

The concentration of most phenolic acids increased from 10 to 44% after applying HIPEF or HPP, with the exception of ferulic acid from both MB and SB, p-coumaric acid from WB and 4-hydroxybenzoic acid from WB, which diminished their concentration (from 9 to 31%). In general, phenolic acids from TT beverages were reduced (up to 36%) or did not change their concentration as compared with untreated beverages. Hesperidin, naringenin and quercetin from both MB and SB, as well as rutin from SB were the flavonoids that increased their concentration (in the range of 9 to 47%) after been treated by any of the technologies analyzed herein. The increment in the phenolic concentration suggests that phenols linked to the food matrix or to other food constituents are released due to the influence of treatment, improving their extractability and therefore their content. Zobel, Lynch, & Jeffrey (1997) reported that the stress provoked in the food by some environmental or processing conditions (i.e. thermal treatment) could improve the phenolic content of food. Additionally, it is possible that these treatments inactivate enzymes related to the loss of phenolic substances (such as the polyphenol oxidase) or increase the activity of enzymes that

participate in the biosynthesis of phenols (i.e. the phenylalanine ammonia-lyse) (Morales-de La Peña et al., 2011). Some studies have reported a similar trend in fruit juice-soymilk beverages treated by HIPEF or TT (Morales-de La Peña et al., 2011); in HPP orange juice-milk beverages (Barba et al., 2012) and in orange juice treated by HIPEF or HPP (Sánchez-Moreno et al., 2005).

The food matrix had a significant influence on the concentration of phenolic compounds ($p < 0.05$). SB was the beverage which contained the highest concentration of total phenolic acids and flavonoids, as well as TPC (by HPLC and by F-C), followed by MB and WB. However, TPC by F-C were not statistically different in MB and WB products. Results suggest that the addition of soymilk or milk to blended fruit juices favoured the concentration of these constituents, in spite of the interactions between phenolic compounds and proteins.

There are really few reports evaluating the effect of the food matrix on the concentration of phenolic compounds from liquid food. However, in contrast to the results obtained in this study, Sharma, Vijay Kumar, & Jagan Mohan Rao (2008) observed that the greatest content of phenols was observed in black tea brew, followed by black tea with sugar, black tea with milk and sugar, and finally in black tea with milk. On the other hand, Barba, Esteve & Frígola (2012) reviewed the influence of HPP on the nutritional properties of different fluid foods, such as juices, purees/pastes based on fruits and vegetables. They concluded that although some general trends were observed, the effect of HPP depends on both the treatment intensity and the food matrix. Therefore, the effect of food processing must be separately studied in each food matrix.

Bioaccessibility of phenolic compounds. The bioaccessibilities of individual and total phenolic compounds are shown in Tables 4 and 5. In overall, the bioaccessibility of phenolic compounds in untreated beverages was in the range of 10.1 to 28.3%, except for rutin from MB which was not recovered in the dialyzed fraction. In line to these results, Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a,b) reported that the bioaccessibility of these compounds was in the range of 0 to 29% in an untreated blended fruit juice, as well as in soymilk. In addition, flavanones from orange juice were around 11 to 36% bioaccessible (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001).

Table 4. Bioaccessibility of phenolic compounds in blended beverages.[a]

		Bioaccessibility of phenolic compounds (%)								
Beverages	**Treatments**	**Caffeic acid**	**Chlorogenic acid**	**Ferulic acid**	**p-coumaric acid**	**4-hydroxibenzoic acid**	**Hesperidin**	**Naringenin**	**Quercetin**	**Rutin**
WB	Untreated	18.4 ± 1.0aA	24.1 ± 1.5aA	28.3 ± 1.0aA	17.2 ± 0.7aA	17.8 ± 1.0aA	15.6 ± 1.0aA	16.2 ± 1.0aA	13.3 ± 0.8aA	12.5 ± 0.7aA
	HIPEF	23.6 ± 1.5bA	21.6 ± 1.4bA	23.7 ± 1.9bA	19.4 ± 0.9bcA	15.9 ± 1.0bA	16.7 ± 0.3bA	16.4 ± 0.4aA	14.9 ± 0.4bA	15.4 ± 1.0bA
	HPP	22.2 ± 0.8bcA	23.0 ± 1.0abA	24.4 ± 1.6cA	20.0 ± 0.8bA	16.3 ± 0.8abA	17.5 ± 0.7bA	17.1 ± 0.8aA	16.9 ± 0.6cA	14.5 ± 0.7bcA
	TT	21.6 ± 1.5cA	21.4 ± 0.9bA	19.4 ± 1.3bA	18.6 ± 1.0cA	13.6 ± 0.3cA	15.5 ± 0.6aA	16.8 ± 1.1aA	15.2 ± 1.0bA	13.9 ± 0.8cA
MB	Untreated	11.7 ± 0.7aB	13.7 ± 0.6aB	13.8 ± 0.6aB	10.7 ± 0.7aB	15.6 ± 0.5aB	13.1 ± 0.4aB	14.4 ± 0.9aB	12.9 ± 0.8aA	0.0 ± 0.0aB
	HIPEF	13.9 ± 0.6bB	15.0 ± 1.0bB	18.6 ± 1.3bB	13.2 ± 0.9bB	16.8 ± 0.3aA	16.6 ± 0.7bA	13.2 ± 0.5abB	14.3 ± 0.7bA	7.2 ± 0.4bB
	HPP	13.5 ± 0.8bcB	15.4 ± 0.5bB	18.0 ± 0.3bB	14.8 ± 0.7cB	17.2 ± 0.8aA	17.3 ± 0.5bA	13.4 ± 0.7abB	16.02 ± 0.13cB	8.4 ± 0.5bcB
	TT	12.6 ± 0.5acB	14.5 ± 0.8abB	16.6 ± 1.0cB	11.9 ± 0.7dB	16.9 ± 2.0aB	14.7 ± 0.6cA	12.3 ± 1.2abB	12.8 ± 0.9aB	0.0 ± 0.0aB
SB	Untreated	13.8 ± 0.4aC	16.7 ± 0.5aC	19.7 ± 0.8aC	13.9 ± 0.7aC	18.8 ± 0.8aA	13.9 ± 0.7aB	15.2 ± 0.5aAB	12.6 ± 0.9aA	10.1 ± 0.6aC
	HIPEF	14.4 ± 1.0aB	17.5 ± 0.6aC	19.9 ± 1.3aB	12.6 ± 0.8bB	18.9 ± 0.9aB	15.0 ± 0.4bB	16.6 ± 1.1abA	14.4 ± 0.5bA	12.4 ± 0.6bcC
	HPP	14.4 ± 0.3aB	17.6 ± 0.8aC	20.4 ± 0.3aC	13.1 ± 0.9abC	20.1 ± 0.5bB	16.4 ± 1.0cA	17.0 ± 1.2bA	13.0 ± 0.6aC	13.1 ± 0.20cC
	TT	14.3 ± 0.7aC	17.2 ± 1.1aC	17.6 ± 0.7bB	13.3 ± 0.8abC	18.9 ± 0.6aC	14.6 ± 0.6abA	16.9 ± 0.9bA	13.1 ± 0.7aB	12.1 ± 0.4bC

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column for each beverage show significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.

Table 5. Bioaccessibility of total phenolic compounds in blended beverages.[a]

		Bioaccessibility of phenolic compounds (%)			
Beverages	**Treatments**	**Total phenolic acids**	**Total flavonoids**	**TPC by HPLC**	**TPC by F-C**
WB	Untreated	19.7 ± 0.5aA	15.1 ± 0.4aA	16.8 ± 0.3aA	25.9 ± 1.5aA
	HIPEF	19.0 ± 0.6aA	16.33 ± 0.12bA	17.29 ± 0.22aA	21.6 ± 1.7bA
	HPP	19.3 ± 0.5aA	17.1 ± 0.6bA	17.9 ± 0.4bA	18.0 ± 0.9cA
	TT	17.20 ± 0.18bA	15.5 ± 0.7aA	16.1 ± 0.4cA	23.2 ± 1.8abA
MB	Untreated	13.9 ± 0.3aB	12.1 ± 0.4aB	12.7 ± 0.3aB	21.2 ± 1.5abB
	HIPEF	15.70 ± 0.20bcB	14.9 ± 0.4bB	15.2 ± 0.3bB	22.5 ± 1.9abA
	HPP	16.1 ± 0.3cB	15.7 ± 0.3cB	15.84 ± 0.20cB	23.6 ± 1.4bB
	TT	15.0 ± 0.8bB	13.0 ± 0.4dB	13.6 ± 0.3dB	19.5 ± 1.4aB
SB	Untreated	17.3 ± 0.3aC	13.9 ± 0.3aC	15.0 ± 0.3aC	20.8 ± 0.3aB
	HIPEF	17.3 ± 0.3aC	15.2 ± 0.4bcB	15.9 ± 0.3bC	21.6 ± 0.9aA
	HPP	17.9 ± 0.3bC	16.0 ± 0.7cB	16.6 ± 0.5cC	30.9 ± 2.4bC
	TT	17.1 ± 0.4aA	14.9 ± 0.5bA	15.6 ± 0.4bA	29.0 ± 2.3bC

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column for each beverage show significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.

The processing has a variable influence on the bioaccessibility of phenolic compounds from beverages. An improvement up to 38% in the bioaccessibility of several phenolic substances (caffeic and p-coumaric acids from both WB and MB; chlorogenic and ferulic acids from MB; hesperidin and rutin from all beverages) was observed after applying treatments, mainly non-thermals (HIPEF and HPP). Processing (HIPEF, HPP and TT) did not change the bioaccessibility of caffeic and chlorogenic acids from SB, as well as naringenin from both WB and MB. On the contrary, all treatments diminished the bioaccessibility of ferulic acid from WB. The bioaccessibilities of chlorogenic and 4-hydroxybenzoic acids from WB were also significantly reduced by HIPEF (10 to 11%) and TT (11 to 24%). Among the treatments studied in this research, it was observed that the lowest bioaccessibility of phenolic compounds was found in TT beverages, specifically in ferulic acid from WB which was reduced 31% as compared with that untreated. It is also interesting to note that rutin from MB was not bioaccessible in untreated and TT product; however, it displayed bioaccessibilities of 7.2 and 8.4% in HIPEF and HPP beverages, respectively.

Processing is known to change some physicochemical features of phenolic compounds and thus, it may also modify the bioaccessibility of these compounds. For instance, several changes in the phenol structure (hydroxylation, methylation, isoprenylation, dimerization, glycosylation, among others) and/or the formation of phenolic derivatives (by partial degradation of the combined forms or by loosing the moieties between phenols and sugars) could occur during processing (Dugo et al., 2005; Fleuriet & Macheix, 1976; Rice-Evans, Miller, & Paganga, 1997).

On the other hand, the food matrix had a significant influence on the bioaccessibility of phenolic compounds ($p < 0.05$). In general, the highest bioaccessibilities of total phenolic acids, total flavonoids and TPC by HPLC were observed in WB, followed by SB and MB. According to these data, the combination of a blended fruit juice with milk or soymilk could decrease the bioaccessibility of some phenolic substances. Milk and soymilk contain important amounts of proteins which could reduce the bioaccessibility of the phenolic compounds contained in these beverages. Covalent or non-covalent interactions between proteins and phenolic compounds could produce the precipitation of proteins by two ways: multisite or multidentate interactions (Sharma, Vijay Kumar, & Jagan Mohan Rao, 2008). When several phenols are bound to one protein is known as multisite interactions. On the contrary, when one phenolic compound is bound to several protein sites/molecules is known as multidentate interactions. Protein precipitation could mask the phenolic compounds and therefore, reduce their bioaccessibility. It has been also proposed that the lipid content of milk could supply a favourable environment for protein-phenolic compound linkage (Serafini et al., 2009).

The results obtained in this research agreed with other studies. For instance, the *in vivo* absorption of some phenolic compounds was significantly reduced by the addition of milk to tea (Lorenz et al., 2007), blueberries (Serafini et al., 2009) and chocolate (Serafini et al., 2003). However, the impact of milk on the bioavailability of phenolic compounds is somewhat controversial, and other studies have not shown significant differences in the plasmatic concentration of tea flavonols and catechins (Hollman, Hof, Tijburg, & Katan, 2001; Van Het Hof, Kivits, Weststrate, & Tijburg, 1998) or may have a minimal impact on the bioavailability of coffee phenols (Ferruzzi, 2010; Renouf et al., 2010). Although the bioaccessibility of phenolic compounds could be reduced in matrices containing milk or soymilk, it is important to highlight that they are rich

sources of other bioactive substances, such as carotenoids and isoflavones, which can transferred other important biological properties to functional beverages.

3.3.Hydrophilic antioxidant activity (HAA)

The HAA of untreated and treated beverages is reported in Table 6, being in the range of 57.4 and 86.4% of $DPPH^{\bullet}$ inhibition in untreated beverages. Similarly, the HAA of a blend fruit juice showed 81% of $DPPH^{\bullet}$ inhibition and 66.3% in a beverage containing soymilk (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013a; (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014).

In overall, the HAA diminished significantly in WB and SB treated by any of the technologies studied in this research, but not in MB, which increased up to 30% their HAA. The decrease in the antioxidant activity of beverages after processing is usually explained by oxidation of certain bioactive compounds contained in foods, such as vitamin C and some phenolic constituents. However, it has been also suggested that processing could improve the concentration of antioxidant compounds through their release from the food matrix or by inactivation of degradative enzymes (Morales-de La Peña et al., 2011), which could explain the increase in the antioxidant activity.

Considering the food matrix influence, it was observed that WB showed the greatest HAA with average value of 77% of $DPPH^{\bullet}$ inhibition; meanwhile MB and SB, with around 68.5% of $DPPH^{\bullet}$ inhibition, did not show significant differences in the HAA value between them. Likely, the addition of milk or soymilk (containing proteins, minerals, metal ions, among others) could favour interactions with hydrophilic compounds (i.e. phenolic compounds and vitamin C) present in the fruit juices, resulting in a decrease in their antioxidant activity. Similarly, it has been reported that vitamin C is able to interact with proteins and minerals from milk (Claeys et al., 2013), whereas phenolic compounds could be non-covalently linked to caseins (Jöbstl, Howse, Fairclough, & Williamson, 2006). However, when these compounds (vitamin C and phenolic compounds) were individually analyzed (see previous sections), the food matrix did not follow the same trend than that observed in the HAA, which also suggest that antioxidant compounds from food could undergo synergistic and antagonistic interactions, depending on the food matrix.

Table 6. Hydrophilic antioxidant activity from blended beverages.[a]

	DPPH• inhibition (%)		
	WB	**MB**	**SB**
	Non-digested beverages		
Untreated	86.4 ± 1.0aA	57.4 ± 1.1aB	79 ± 3aC
HIPEF	76.0 ± 1.0bA	75 ± 3bA	65.2 ± 2.4bB
HPP	72 ± 4cA	73 ± 3bA	70.9 ± 1.1cA
TT	73.5 ± 1.1bcA	64 ± 3cB	65 ± 3bB
	Digested beverages		
Untreated	22.1 ± 1.5aA	12.3 ± 0.3aB	17.7 ± 0.7abC
HIPEF	18.0 ± 0.9bA	14.9 ± 1.0bB	16.8 ± 0.8aA
HPP	19.1 ± 2.2bA	14.1 ± 0.8bcB	17.9 ± 0.7bA
TT	14.5 ± 0.8cA	13.6 ± 0.8cA	14.3 ± 0.8cA

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column show significant differences ($p < 0.05$) within treatments. Different capital letters in the same row indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure processsing; TT, thermal treatment.

Digested beverages. As shown in Table 6, the HAA of digested and untreated beverages was in the range of 12.3 to 22.1% of DPPH inhibition.

Non-thermal technologies (HIPEF and HPP) increased up to 21% the HAA of digested MB in comparison with untreated beverages, but they did not modify the HAA of digested SB. In contrast, the HAA of digested WB was reduced 19% by HIPEF and 14% by HPP, as compared with untreated products. The lowest HAA value was reached in TT digested beverages, which showed up to 34% less HAA (in WB products) than those untreated. It is well known that the bioaccessibility of antioxidants is widely influenced by the structural properties of these compounds. Processing is able to modify some of these features, which could explain why the bioaccessibility of these compounds was changed after treatments.

The food matrix had a significant influence on the HAA of digested and untreated beverages. HAA displayed the highest value in WB and SB digested fractions, with no significant differences among them in treated beverages. In contrast, digested MB showed the lowest HAA, except for TT digested beverages where the HAA was the same in all beverages. Likely, the decrease in the antioxidant activity of hydrophilic compounds is due to the complexity of the food matrix. Milk contains certain amount of proteins, fat and minerals, which could be able to interact with water soluble

constituents of food, leading to the formation of complexes or aggregates. As a consequence, it is difficult that these compounds could be released from the food matrix by the digestive enzyme action and solubilised into the gastrointestinal tract, resulting in a decrease in their antioxidant activity.

4. Conclusion

The food matrix (water, milk and soymilk) and processing (high intensity pulsed electric fields (HIPEF), high-pressure processing (HPP) and thermal treatment (TT)) were able to modulate the bioaccessibility of hydrophilic constituents, such as vitamin C and phenolic compounds, from beverages based on a blend of fruit juices (orange, pineapple, kiwi and mango), as well as on their antioxidant activity.

The bioaccessibility of vitamin C was not modified by non-thermal processing technologies (HIPEF and HPP), but it diminished up to 16.5% by TT in comparison with untreated beverages. Total phenolic compounds (sum of individuals quantified by HPLC) were more bioaccessible after applying any of the treatments studied than in that untreated products. On the other hand, processing exerted a variable influence on the hydrophilic antioxidant activity (HAA) of digested beverages.

With respect to the food matrix, it was observed that the highest bioaccessibility of vitamin C was reached in beverages containing soymilk (SB). WB (beverages based on a blend of fruit juices + water) displayed the best bioaccessibility of phenolic compounds, as well as HAA. In contrast, milk beverages (MB) were not adequate for improving the bioaccessibility of hydrophilic constituents (vitamin C and phenolic compounds), at least under the assayed conditions.

These data suggest that the influence of processing on the bioaccessibility of bioactive compounds is dependent on the analyzed substance, as well as on the food matrix. Therefore, these factors (nutrient or bioactive compound type, food matrix and processing) should be considered when functional beverages are developed in order to avoid undesirable interactions that could reduce the bioavailability of these compounds. Simulated gastrointestinal digestion is a useful tool that allows assessing the influence of the food matrix and processing on the bioaccessibility of bioactive compounds. However, additional studies must be performed in order to clarify whether the results obtained by this *in vitro* methodology correlates with *in vivo* trials.

Acknowledgement

This research was supported by the Ministry of Education and Science (Spain), AGL2006-12758-C02-02/ALI and AGL2006-12758-C02-01/ALI projects. María Janeth Rodríguez-Roque thanks to the Comissionat per a Universitats i Recerca, del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya and European Social Fund for the predoctoral grant. Prof. Olga Martín-Belloso thanks to the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia Award 2008.

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CHAPTER VI

Food matrix and processing influence on the *in vitro* bioaccessibility of carotenoids and lipophilic antioxidant activity from blended beverages

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Abstract

Beverages made with a blend of fruit juices and water (WB), milk (MB) or soymilk (SB) were treated by high intensity pulsed electric fields (HIPEF) (35 kV/cm with 4 μs bipolar pulses at 200 Hz during 1800 μs), high pressure processing (HPP) (400 MPa at 40 ºC for 5 min) or thermal treatment (TT) (90 ºC during 1 min) in order to evaluate the influence of food matrix and processing on the bioaccessibility of carotenoids and on the lipophilic antioxidant activity (LAA). The bioaccessibility of carotenoids diminished after applying any treatment (HIPEF, HPP and TT), except of cis-violaxanthin+neoxanthin which increased up to 79% in HIPEF and HPP beverages. The lowest carotenoid bioaccessibility was always obtained in TT beverages (lossess up to 63%). The best food matrix for improving the bioaccessibility of carotenoids, as well as the LAA, was MB. Results demonstrate that treatment and food matrix are able to modulate the bioaccessibility of carotenoids as well as the lipophilic antioxidant potential of beverages. Additionally, HIPEF and HPP could be considered as promising technologies to obtain highly nutritional and functional beverages.

Keywords: Blended beverages; Bioaccessibility; Food matrix; Non-thermal and thermal processing; Carotenoids; Lipophilic antioxidant activity

1. Introduction

Functional beverages are becoming more popular because, in addition to their nutritional value, they help maintaining well-being and health (Howlett, 2008). For this reason, a variety of functional beverages are available in the market to suit different lifestyles of consumers, as well as to satisfy their preferences for tasty, nutritious, healthy and convenient products.

Carotenoids are a widespread family of fat-soluble plant pigments. They have shown to play an important role in human health by their powerful antioxidant potential and because some of them possess provitamin A activity. These compounds have been associated with immune system enhancement, antiaging, antiinflammation, antiulcer and anticancer properties (Fernández-García, Carvajal-Lérida, Jarén-Galán, Garrido-Fernández, Pérez-Gálvez, & Hornero-Méndez, 2012). The main food sources of carotenoids are yellow and orange fruits, dark green vegetables and dairy products (Maiani et al., 2009). Among the most utilized ingredients for producing beverages with functional properties stand out fruit juices and milk, which are considered as wholesome and nutrient-rich foods. Therefore, functional beverages based on these food stuffs could also contribute to carotenoids intake. In many cases, soymilk is utilized as surrogate of milk for consumers who experience lactose intolerance, protein milk allergy or galactosemia (Xu & Chang, 2009). Although soymilk does not contain carotenoids, it is an important source of other nutrients, such as phenolic compounds and isoflavones.

Thermal treatment (TT) has been widely used to preserve foods and beverages because of their excellent performance against microorganism (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013). Nevertheless, nutritional and sensorial features of food may be affected by the high temperatures reached during this treatment. In order to satisfy the increased demand of consumer for nutritious, healthy and tasty products, food technologist are looking for novel processing methods that did not compromise all these important characteristics. Non-thermal food processing technologies, such as high intensity pulsed electric fields (HIPEF) or high pressure processing (HPP), have been one of the main fields of research during the last decade due to there are considered as alternative to heat treatments (Barba, Cortés, Esteve, &

Frígola, 2012; Sanchez-Moreno, De Ancos, Plaza, Elez-Martinez, & Cano, 2009; Soliva-Fortuny, Balasa, Knorr, & Martín-Belloso, 2009).

Bioaccesibility is defined as the portion of the nutritients or bioactive compounds that are released from its food matrix into the gastrointestinal tract and thus become available for intestinal absorption (Fernández-García et al., 2009). Therefore, although functional beverages (processed or not) contain important amounts of nutrients, it does not mean that all these compounds can be absorbed. In particular, the availability of lipophilic constituents is limited because the hydrophobic nature of these compounds avoids their dispersion in the aqueous media of the digestive tract (Nagao, Kotake-Nara, & Hase, 2013). Carotenoids must be first released from the food matrix, dispersed in the digestive tract and solubilised into mixed micelles to be available for absorption. Thus, the formation of micelles is one of the most important factors that affect the absorption of carotenoids (Nagao, 2011). Bioaccessibility of nutrients is usually evaluated by *in vitro* gastrointestinal digestion (Nagao, Kotake-Nara, & Hase, 2013) and represents a useful and fast approach instead of *in vivo* trials which are expensive and long term studies.

Due to the fact that food processing involves changes on the microstructure of food (i.e. the disruption of cell walls and membranes), as well as on the release of carotenoids from carotenoid-protein complex, and their solubilisation (free and ester forms); the bioaccessibility of these nutrients could be affected (Maiani et al., 2009). In addition to processing, the surrounding environment in which carotenoids are contained also impacts on their bioaccessibility because interactions between carotenoids-carotenoid and/or carotenoids-food constituents (i.e. fiber and fat) could occur. As a result, it is important to know the concentration of bioactive compound that is accessible for absorption after digestion and the extent to which processing and food matrix may modify their bioaccessibility. There is a lack of information in the literature about these important topics that affect the bioaccessibility of bioactive compounds from beverages. Therefore, the aim of this research was to evaluate the influence of food matrix [milk beverage (MB), soymilk beverage (SB) and water beverage (WB)] and processing (HIPEF, HPP and TT) on the *in vitro* bioaccessibility of carotenoids and on the lipophilic antioxidant activity (LAA) of beverages based on a blend of fruit juices (orange, pineapple, kiwi and mango).

2. Material and methods

2.1. Reagents

Pepsin from porcine stomach, pancreatin from porcine pancreas, bovine bile, carotenoid standards (α-carotene, β-carotene, zeaxanthin, lutein, α-cryptoxanthin and β-cryptoxanthin) and 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Beverages

Three beverages were prepared by mixing 75% of a blended fruit juice (orange, kiwi, pineapple and mango); 17.5% of milk (MB, milk-fruit juice beverage), or soymilk (SB, soymilk-fruit juice beverage), or distilled water (WB, water-fruit juice beverage); and 7.5% of sugar. The pH of the beverages was adjusted to 3.30 ± 0.20 (Crison Instruments S.A., Alella, Barcelona, Spain) with citric acid and the soluble solid content was also assessed (Comecta S.A., Abrera, Barcelona, Spain), resulting in 18.0 ± 0.2, 18.5 ± 0.2, 19.3 ± 0.3 ºBrix for milk (MB), soymilk (SB) and water (WB) beverages, respectively. Beverages formulations were selected based on previous studies, where similar concentration of these fruit juices resulted in a high concentration of bioactive compounds (Salvia-Trujillo, Morales-de la Peña, Rojas-Graü, & Martín-Belloso, 2011).

Fruits (orange, kiwi, pineapple and mango) were purchased at commercial maturity in a local supermarket (Lleida, Spain). These fruits were washed, peeled and the juice extracted. Each fresh-squeezed juice was filtered with a cheesecloth using a vacuum pump. A blended fruit juice was obtained by mixing 40% of orange, 33% of kiwi, 13.5% of pineapple and 13.5% of mango juices.

Whole milk (Hacendado, Córdoba, Spain) and soymilk (Yosoy, Girona, Spain) were purchased at local supermarket. According to manufacturers, milk contained 3.6% of fat, 3.0% of protein and 4.5% carbohydrates; while 1.8% of fat, 3.6% of protein, 0.7% of carbohydrates and 1% of fiber were reported in soymilk.

2.3. Processing technologies

2.3.1. High Intensity Pulsed Electric Fields (HIPEF)

HIPEF treatment was carried out in a continuous-flow bench scale system (OSU-4F, The Ohio State University, Colombus, OH, USA), using square-wave pulses. Eight collinear chambers serially connected were used as treatment system. Each chamber consisted of two stainless steel electrodes separated by a gap of 0.29 cm. The flow rate was adjusted to 60 mL/min and controlled by a variable speed pump (model 752210-25, Cole Palmer Instrument Company, Vermon Hills, IL, USA). Treated beverages were refrigerated through an ice-water bath in a space provided between the chambers. HIPEF processing conditions for beverages were 35 kV/cm field strength in bipolar mode, 4 µs pulse width, 200 Hz pulse frequency and 1800 µs total treatment time. Temperature was always kept below 35 ºC. These conditions were selected based on previous studies performed in our laboratory, where the nutritional and microbiological stability of similar beverages was achieved (Morales-de La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2011; Salvia-Trujillo, Morales-de la Peña, Rojas-Graü, & Martín-Belloso, 2011).

2.3.2. High-Pressure Processing (HPP)

HPP was performed in a hydrostatic pressure unit with a vessel of 2925 mL capacity, a maximum pressure of 900 MPa, and a maximum temperature of 100 ºC (High Pressure Iso-Lab System, Model FPG7100:9/2C, Stansted Fluid Power LTD., Essex, UK). Beverages were vacuum packed in flexible Doypack® bags (Polyskin XL, Flexibles Hispania, S.L.) (300 mL) and introduced in the pressure unit filled with pressure medium (water). Samples were processed at 36 ºC with a holding time of 5 min at 400 MPa. The rates of compression and decompression were both 3 MPa/s. Because of adiabatic compression, the maximum temperature in the vessel was 40 ºC at 400 MPa. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the process. These conditions were selected based on previous studies where the nutritional and microbiological stability of fruit juices and similar beverages were achieved (Sánchez-Moreno et al., 2005; Muñoz, De Ancos, Sánchez-Moreno, & Cano, 2007)

2.3.3. Thermal Treatment (TT)

Beverages were thermally processed (90 ºC during 1min) in a tubular stainless-steel heat exchanger coil immersed in a hot water shaking bath (University of Lleida, Spain). A gear pump was used to maintain the beverage flow rate of 40.5 mL/min. After heating, the beverage was immediately cooled down to 5 ± 1 ºC in an ice-water bath.

2.4. In vitro gastrointestinal digestion

Once beverages were prepared and processed, they were digested through the *in vitro* methodology described by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a). This procedure consisted of two digestive stages: gastric (pH 2, containing pepsin) and small intestinal digestions (pH 7, containing a pancreatine-bile mixture). Aliquots of digested samples were collected from the micellar fraction, which was obtained by centrifugation of the small intestinal digesta at 5000 rmp for 20 minutes. Afterwards, the micellar fraction was immediately placed in a cold water bath during 10 minutes. All samples were frozen (-45 ºC until analysis).

2.5. Carotenoids analysis

Carotenoids of non-digested or digested samples were extracted following the methodology described by Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013a). Individual carotenoids were identified by comparing their retention time and spectrum with the standards and/or those reported in the literature. Carotenoid quantification was carried out integrating the peak areas and using calibration curves (R^2 in the range of 0.9961 to 0.9995; concentration from 0.1 to 50 mg/L). Results were expressed as µg of carotenoid/100 mL of sample.

2.6. Lipophilic antioxidant activity (LAA)

Extraction of lipophilic fraction of non-digested or digested beverages, as well as the antioxidant activity were performed according to the procedure of Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso (2013b). The antioxidant activity was evaluated using the colorimetric method reported by Brand-Williams, Cuvelier, & Berset (1995), which is based on the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) assay. Results were expressed as percentage of $DPPH^{\bullet}$ inhibition.

2.7. Bioaccessibility calculations

Bioaccessibility was determined as the ratio of carotenoid concentration in the digested beverage ($BC_{digested}$) with respect to non-digested beverage ($BC_{non\text{-}digested}$) (Eq. 1). Results were expressed as percentage.

$$Bioaccessibility(\%) = 100x\left(\frac{BC_{digested}}{BC_{non-digested}}\right) \quad \text{Eq. 1}$$

2.8. Statistical analysis

The *in vitro* gastrointestinal digestion was conducted in duplicated. Each bioactive compound was extracted and analyzed two times (n = 4). Results were reported as the mean ± standard deviation. Analysis of variance (ANOVA) of the results followed by the least significant difference test (LSD) was carried out to determine significant differences ($p < 0.05$) in the concentration and bioaccessibility of bioactive compounds from beverages in relation to the factors studied in this research (food matrix and processing). Multifactorial analysis of variance (ANOVA) was performed to study separately the main effects (food matrix and treatment) and the interaction effect (food matrix × treatment). As a significant interaction effect was observed in most of the variables, ANOVA, comparing the means within the same food matrix for different treatments and within the same treatment for different food matrix, was performed. All statistical analyses were performed with the program Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD, USA). Results were reported as the mean ± standard deviation.

3. Results and discussion

3.1. Carotenoids

Carotenoid profile in untreated, HIPEF, HPP and TT beverages is presented in Table 1. The concentration of total carotenoids (determined as the sum of individuals) was in the range of 322 and 426 μg/100 mL in untreated beverages, being xanthophylls up to 3.3 times higher than carotenes. A similar concentration of carotenoids (between 223 and 540 μg/100 mL) was reported in mixed fruit juices and beverages, where xanthophylls were also the predominant forms (Morales-de La Peña, Salvia-Trujillo,

Rojas-Graü, & Martín-Belloso, 2011; Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013a, 2014).

Processing had a significant influence on the concentration of carotenoids contained in the three beverages analyzed in this study ($p < 0.05$). The concentration of some carotenoids increased after applying HIPEF treatment with respect to untreated beverages, such as cis-violaxanthin+neoxanthin from both WB (9%) and MB (16%); cis-anteraxanthin from WB (8%); anteraxanthin (10%), lutein (23%) and zeaxanthin (28%) from MB. In the same way, HPP improved the concentration of cis-violaxanthin, anteraxanthin, lutein and zeaxanthin from MB (12-37%) as compared with untreated ones. TT enhanced up to 6% the concentration of certain carotenoids, mainly that of MB; however, that increment was not statistically significant ($p > 0.05$). An explanation of this trend could be attributed to greater stability of these products, the inactivation of both hydrolytic and oxidative enzymes, as well as the disruption of cell membranes and proteins due to processing, resulting in an increase of some individual carotenoids (Maiani et al., 2009; Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013). Torregrosa, Cortés, Esteve, & Frígola (2005) also observed a rise (from 111 to 160%) in the concentration of 9-*cis*-violaxanthin+neoxanthin, antheraxanthin, lutein, zeaxanthin, β-cryptoxanthin, when an orange-carrot juice was HIPEF treated at 35 kV/cm for 150 μs. Similarly, Cilla et al. (2012) reported that processing improved the concentration of some carotenoids in beverages made with fruit juices and milk or soymilk. These authors (Cilla et al., 2012) showed that lutein, zeaxanthin, and neoxanthin + 9–cis–violaxanthin increased from 53 to 99% their concentration in HPP beverages (400 MPa/40ºC/5 min), whereas lutein and neoxanthin + 9–cis–violaxanthin increased up to 61% in those TT treated (90 ºC during 30 s).

Other carotenoids did not change their concentration in HIPEF (mainly β-cryptoxanthin from the three samples) and HPP beverages (α- and β-cryptoxanthin of all samples) with respect to untreated ones. However, losses of some of these compounds were observed in beverages treated by HIPEF (7-38%), HPP (7-29%) and TT (8-48%). Carotenoid denaturalization is dependent on their chemical structure (Bitton & Hornero-Mendez, 2001) and most of them are molecules that easily oxidized and isomerized due to the double bounds of their chemical structure (Hedrén, Diaz, & Svanberg, 2002). Thus, carotenoids could undergo several changes during processing, resulting in the degradation of these constituents (Morales-de La Peña, Salvia-Trujillo, Rojas-Graü, &

Martín-Belloso, 2011). Zulueta, Esteve, & Frígola (2007) reported that treatment may affect the carotenoids concentration and their isomeric features. In addition, similar results have been reported in the literature, where orange juice, orange-carrot juice, and fruit juices and milk/soymilk beverages have been processed by these technologies (Barba, Cortés, Esteve, & Frígola, 2012; Cilla et al., 2012; Cortés, Torregrosa, Esteve, & Frígola, 2006; Morales-de La Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2011; Sánchez-Moreno et al., 2005; Torregrosa, Cortés, Esteve, & Frígola, 2005).

On the other hand, it was observed that the food matrix exert a significant influence ($p < 0.05$) on the concentration of carotenoids extracted from beverages. MB displayed the highest concentration of all individual carotenes and xanthophylls, indicating that this beverage had higher total carotenoid concentration than WB and SB (Table 1).

The concentration of total carotenoids from WB and SB was very similar in untreated and HPP beverages and not significant statistically differences were found. However, SB displayed the lowest concentration of total carotenoids in HIPEF and TT samples. Therefore, these results indicated that the composition of the food matrix exert an important effect on the stability and concentration of carotenoids extracted from food and beverages. In fact, it has been reported that the presence of dietary fiber, as well as the amount and type of fat are among the main dietary factors that may affect the carotenoids extraction and in consequence the carotenoid profile of food (Fernández-García, Carvajal-Lérida, Jarén-Galán, Garrido-Fernández, Pérez-Gálvez, & Hornero-Méndez, 2012; Van Het Hof, West, Weststrate, & Hautvast, 2000).

3.1.1. Carotenoid bioaccessibility. Table 2 shows the bioaccessibility of carotenoids from the beverages analyzed in this study. These compounds were between 9.2 and 31.4% bioaccessible in untreated beverages. Similar results were reported in a blend of fruit juices and in a fruit juice-soymilk beverage, where carotenoids were around 6.5 to 17.4% bioaccessible (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013a, 2014). In citrus juices, β-cryptoxanthin and β-carotene displayed bioaccessibilities from 16 to 33% (Dhuique-Mayer et al., 2007).

Table 1. Concentration of carotenoids in blended beverages.[a]

Beverages	Treatments	Carotenoid concentration (µg/100 mL)											
		Cis-violaxanthin+ neoxanthin	Cis-antheraxanthin	Antheraxanthin	Lutein	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	Total xanthophylls	Total carotenes	Total carotenoids
WB	Untreated	57.0 ± 2.2aA	82 ± 4aA	12.6 ± 0.5aA	43 ± 3aA	25.9 ± 1.3aA	8.2 ± 0.3aA	12.1 ± 0.8aA	4.7 ± 0.3aA	77 ± 5aA	240 ± 6aA	81 ± 4aA	322 ± 4aA
	HIPEF	62 ± 4bA	89 ± 3bA	11.1 ± 0.4bA	37.4 ± 1.5bA	20.5 ± 1.0bA	7.3 ± 0.5bcA	11.9 ± 0.7aA	3.59 ± 0.12bA	67.5 ± 1.6bA	238 ± 5aA	71.1 ± 1.7bA	309 ± 3bA
	HPP	63 ± 3abA	85 ± 5abA	11.5 ± 0.4bA	40.8 ± 0.7aA	24.1 ± 1.2cA	7.9 ± 0.4acA	12.3 ± 0.5aA	3.8 ± 0.3bA	66.5 ± 2.1bA	244.4 ± 1.7aA	70.4 ± 2.2bA	315 ± 3cA
	TT	58.6 ± 1.8abA	81.3 ± 2.5aA	9.8 ± 0.4cA	35.5 ± 1.4bA	17.4 ± 1.2dA	6.7 ± 0.3bA	10.9 ± 0.6bA	3.20 ± 0.10cA	60 ± 3cA	220 ± 4bA	63 ± 3cA	283 ± 3dA
MB	Untreated	66 ± 4aB	122 ± 3aB	18.3 ± 1.1aB	57 ± 4aB	34.3 ± 1.8aB	9.2 ± 0.3aB	15.3 ± 0.7aB	7.5 ± 0.3abB	96 ± 4aB	322 ± 10aB	104 ± 4aB	426 ± 12aB
	HIPEF	76.7 ± 2.2bB	109.2 ± 1.8bB	20.2 ± 1.0bB	70 ± 4bB	44 ± 3bB	8.7 ± 0.5aB	16.0 ± 0.6abB	7.1 ± 0.4bcB	89 ± 4bB	345 ± 4bB	97 ± 4bB	441.8 ± 1.3bB
	HPP	80 ± 4bB	110 ± 7bB	20.5 ± 1.4bB	75 ± 3bB	47 ± 3bB	9.2 ± 0.4aB	16.3 ± 0.5abB	7.9 ± 0.4aB	102 ± 4aB	358 ± 7cB	110 ± 4aB	467 ± 7cB
	TT	70 ± 4aB	99 ± 4cB	18.9 ± 0.6abB	57.4 ± 2.4aB	32.3 ± 2.1aB	7.7 ± 0.3bB	15.7 ± 0.6abB	6.88 ± 0.12cB	85 ± 5bB	302 ± 5dB	92 ± 5bB	393 ± 10dB
SB	Untreated	58 ± 3aA	87 ± 5aA	13.3 ± 0.9aA	48 ± 3aC	28.3 ± 1.9aA	7.2 ± 0.3abC	14.2 ± 0.6abB	5.1 ± 0.3abA	72 ± 2aA	256 ± 11aC	77.5 ± 2.1aA	334 ± 10aA
	HIPEF	53 ± 3aC	71 ± 3bC	11.3 ± 0.5bcC	29.7 ± 1.0bC	20.8 ± 1.4bA	6.69 ± 0.21acA	14.0 ± 0.7aC	4.8 ± 0.3aC	76 ± 3aC	206.7 ± 1.7bC	80 ± 3abC	287 ± 4bC
	HPP	56 ± 4aC	75 ± 4bC	11.9 ± 0.3cA	33.9 ± 1.4cC	21.4 ± 1.1bA	7.3 ± 0.5bA	14.9 ± 0.6bC	5.5 ± 0.4abC	78 ± 5aC	220 ± 6cC	83 ± 5bC	303 ± 11cA
	TT	43 ± 3bC	57.5 ± 1.7cC	10.8 ± 0.4bC	25.2 ± 1.2dC	15.3 ± 0.7cA	6.5 ± 0.3cA	12.4 ± 0.5cC	5.30 ± 0.23abC	61 ± 4bA	170 ± 4dC	66 ± 4cA	237 ± 6dC

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column and beverage indicate significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure procesing; TT, thermal treatmen

Table 2. Bioaccessibility of carotenoids in blended beverages.[a]

Beverages	Treatments	Bioaccessibility of carotenoids (%)											
		Cis-violaxanthin+ neoxanthin	Cis-antheraxanthin	Antheraxanthin	Lutein	Zeaxanthin	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	Total xanthophylls	Total carotenes	Total carotenoids
WB	Untreated	15.8 ± 0.8aA	14.0 ± 0.6aA	9.2 ± 0.6aA	16.0 ± 0.9aA	17.5 ± 1.2aA	17.5 ± 1.1aA	17.8 ± 1.1aA	17.7 ± 0.9aA	16.9 ± 0.7aA	15.19 ± 0.12aA	17.0 ± 0.7aA	15.63 ± 0.17aA
	HIPEF	17.2 ± 0.5bA	10.4 ± 0.7bA	7.05 ± 0.17bA	14.8 ± 0.6bA	13.5 ± 0.6bA	12.1 ± 0.7bA	9.2 ± 0.4bA	13.2 ± 0.8bA	12.2 ± 0.8bA	12.93 ± 0.19bA	12.3 ± 0.8bA	12.8 ± 0.3bA
	HPP	19.0 ± 1.2cA	10.0 ± 0.4bA	7.5 ± 0.3bA	13.8 ± 0.4cA	12.3 ± 0.4bcA	13.4 ± 0.8bA	9.8 ± 0.5bA	12.9 ± 0.5bA	13.1 ± 0.7bA	13.12 ± 0.19bA	13.1 ± 0.7bA	13.12 ± 0.21cA
	TT	8.9 ± 0.5dA	9.8 ± 0.3bA	6.5 ± 0.4cA	12.1 ± 0.5dA	11.8 ± 0.7cA	10.4 ± 0.7cA	7.8 ± 0.3cA	8.5 ± 0.3cA	10.2 ± 0.3cA	9.85 ± 0.21cA	10.1 ± 0.3cA	9.91 ± 0.12dA
MB	Untreated	21.6 ± 1.4aB	17.5 ± 0.8aB	14.6 ± 1.0aB	28.9 ± 1.4aB	30.0 ± 0.8aB	29,8 ± 1.3aB	20.0 ± 1.1aB	31.2 ± 1.4aB	31.4 ± 2.2aB	22.0 ± 0.7aB	31.4 ± 2.0aB	24.3 ± 0.6aB
	HIPEF	38.7 ± 2.5bB	15.5 ± 0.6bB	13.0 ± 0.6bB	38.1 ± 1.7bB	29.1 ± 1.6abB	30.1 ± 1.2aB	19.8 ± 1.0aB	28.5 ± 1.8bB	29.6 ± 2.0aB	27.4 ± 0.5bB	29.5 ± 1.8aB	27.8 ± 0.3bB
	HPP	33.8 ± 1.8cB	15.8 ± 1.1bB	13.9 ± 0.9abB	25.2 ± 1.0cB	27.6 ± 1.7bB	26.8 ± 1.8bB	13.7 ± 0.6bB	30.4 ± 1.6abB	29.0 ± 1.3aB	23.4 ± 1.1cB	29.1 ± 1.1aB	24.8 ± 0.7aB
	TT	15.9 ± 0.8dB	12.2 ± 0.5cB	10.6 ± 0.7cB	19.8 ± 0.9dB	23.3 ± 1.6cB	14.3 ± 1.0cB	12.8 ± 0.7bB	23.1 ± 0.7cB	22.9 ± 1.2bB	15.68 ± 0.17dB	22.6 ± 1.1bB	17.3 ± 0.3cB
SB	Untreated	13.9 ± 0.4aC	12.2 ± 0.8aC	11.1 ± 0.6aC	22.7 ± 1.4aC	24.1 ± 1.6aC	15.1 ± 1.0aC	18.6 ± 0.8aAB	22.0 ± 0.7aC	20.1 ± 1.3aC	16.3 ± 0.6aC	20.2 ± 1.3aC	17.2 ± 0.7aC
	HIPEF	17.1 ± 1.2bA	13.5 ± 0.6bC	9.4 ± 0.4bC	26.3 ± 1.4bC	17.3 ± 1.1bC	7.84 ± 0.22b	11.9 ± 0.6bC	16.5 ± 1.1bC	15.6 ± 1.1bC	16.0 ± 0.4aC	15.7 ± 1.1bC	15.9 ± 0.3bC
	HPP	21.5 ± 0.9cC	14.0 ± 0.8bC	9.66 ± 0.07bC	37.6 ± 1.5cC	20.7 ± 0.7cC	8.5 ± 0.6bC	15.9 ± 0.9cC	15.3 ± 0.5cC	16.1 ± 0.5bC	19.89 ± 0.20bC	16.1 ± 0.5bC	18.84 ± 0.22cC
	TT	9.2 ± 0.5dA	7.8 ± 0.4cC	7.2 ± 0.4cA	23.6 ± 1.0aC	14.5 ± 0.5dC	5.6 ± 0.4c	10.5 ± 0.6dC	11.8 ± 0.6dC	12.9 ± 0.9cC	11.21 ± 0.23cC	12.8 ± 0.8cC	11.65 ± 0.06dC

[a] Values are expressed as the mean ± standard deviation. Different lower case letters in the same column for each beverage show significant differences ($p < 0.05$) within treatments. Different capital letters in the same column and treatment indicate significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high-pressure processing; TT, thermal treatment.

Both food matrix and processing exerted a significant influence ($p < 0.05$) on the bioaccessibility of carotenoids. In overall, the bioaccessibility of individual carotenoids diminished after applying any type of treatment, mainly in TT beverages where the biaccessibility declined to 63%. HIPEF treatment decreased the bioaccessibility of carotenoids in the range of 7.6 to 48.2%, with respect to untreated beverages. In the same way, carotenoids were less bioaccessible in HPP beverages (between 8.2 and 45.1%) than in those untreated. β-cryptoxanthin from WB after HIPEF and HPP treatments and α-cryptoxanthin from SB after TT were the carotenoids that showed the lowest bioaccessibility.

As far as we know, really few reports have determined the influence of non-thermal (HIPEF and HPP) or thermal (TT) technologies on the bioaccessibility of carotenoids. In one such report, Cilla et al. (2012) observed that some carotenoids were less bioaccessible in HPP-milk (from 15 to 58%), TT-milk (from 30 to 52%) and TT-soymilk (from 50 to 90%) than in their respective untreated beverages. Stinco et al. (2012), reported that heat treatment reduced the bioacessibility of α-carotene and β-cryptoxanthin in orange juice as compared with fresh industrially squeezed juice.

On the other hand, in some cases HIPEF processing improved the bioaccessibility of carotenoids, in comparison to their respective untreated beverages, such as cis-violaxanthin+neoxanthin from the three beverages (from 9 to 79%), cis-antheraxanthin from SB (10%), and lutein from both MB (32%) and SB (16%). Total xanthophylls and total carotenoids from MB also increased 24.5% and 15%, respectively, when HIPEF treatment was applied. A similar trend was observed in beverages treated by HPP, where cis-violaxanthin+neoxanthin from the three beverages; cis-antheraxanthin and lutein from SB, total xanthophylls from both MB and SB, and total carotenoids from SB were more bioaccessible in HPP beverages (from 6.5 to 65%) than in untreated samples. On the contrary, no increases in the bioaccessibility of carotenoids were observed in TT beverages. The improvement in the bioaccessibility of some carotenoids in HIPEF and HPP beverages could be justified by changes in the structure of the food matrix due to processing effect, such as the breakdown of cell walls and membranes in which carotenoids are embebed. Thus, interactions between carotenoids and digestive enzymes could be enhanced, as well as their release from the food matrix and their solubilisation into micelles. This hypothesis is supported by Stinco et al. (2012), who reported that the food matrix structure is one of the most important factors that affect the bioaccessibility

of carotenoids. Additionally, Maiani et al. (2009) found that some types of food processing can improve the carotenoid bioavailability. Cilla et al., 2012 also reported significant increases (from 39 to 264%) in the bioaccessibility of carotenoids in milk (neoxanthin+9-cis-violaxanthin) and soymilk (neoxanthin+9-cis-violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene) based beverages treated by HPP with respect to untreated products.

The food matrix had a significant influence ($p < 0.05$) on the bioaccessibility of carotenoids. Total carotenoids from MB displayed the highest bioaccessibility with averages value of 23.5%, followed by SB (15.9%) and WB (12.9%). These results suggest that the greater fat content of milk (3.6%) with respect to soymilk (1.6%) and water (0%) could favour the incorporation of carotenoids into micelles and thus, increase their bioaccessibility in MB. In accordance to this hypothesis, it has been reported that dietary fat enhance the bioaccessibility of carotenoids from food (Fernández-García, Carvajal-Lérida, Jarén-Galán, Garrido-Fernández, Pérez-Gálvez, & Hornero-Méndez, 2012; Van Het Hof et al., 2000). Granado-Lorencio, Herrero-Barbudo, Blanco-Navarro, Pérez-Sacristán, & Olmedilla-Alonso (2009) also found that the addition of milk to blended fruit juices improve the bioaccessibility of carotenoids.
Other food constituent that could affect the bioaccessibility of carotenoids is fiber. Dietary fiber could augment the viscosity of the intestinal content (Hoffmann, Linseisen, Riedl, & Wolfram, 1999), leading to entrap bioactive compounds, such as carotenoids, and decrease the activity of digestive enzymes. Thus, the micellization and bioaccessibility of carotenoids are reduced due to the fiber content of food. In this sense, it could be expected that SB beverages contain more amount of fiber than MB, explaining why the bioaccessibility of carotenoids diminished in SB beverages. In contrast to these results, Cilla et al. (2012) found that the bioaccessibility of carotenoids was improved in soymilk than in milk based beverages. However, these authors (Cilla et al., 2012) made the beverages with different proportions of milk and soymilk (16.5% versus 41.5%, respectively), which could elucidate the differences in the results.

Considering the effect of both food matrix and processing, it was observed that HIPEF processing in combination with a milk matrix (MB) increased the bioaccessibility of total carotenoids (15%) in comparison to untreated beverages. Carotenoids from MB were equally bioaccesible in HPP and untreated beverages. In SB, the technology that improved the bioaccessibility of total carotenoids was HPP

(10%), whereas HIPEF slightly decrease them (7%). Both non-thermal technologies (HIPEF and HPP) decreased the bioaccessibility of total carotenoids in WB (around 17%). The lowest bioaccessibility was achieved in the three beverages treated by TT (losses up to 37%).

3.2. Lipophilic antioxidant activity (LAA)

The LAA from non-digested beverages is displayed in Figure 1, resulting in 5.3–16.7% of $DPPH^{\bullet}$ inhibition in untreated products. Similar results were previously reported in blended fruit juices (from 15.2 to 17% of $DPPH^{\bullet}$ inhibition) and in a beverage based on fruit juices and soymilk (11.9% of $DPPH^{\bullet}$ inhibition) (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013a, 2014).

Thermal treatment (TT) exerted a significant influence ($p < 0.05$) on the LAA of the all beverages analyzed in this study (WB, MB and SB), where the percentage of $DPPH^{\bullet}$ inhibition diminished in the range of 7 to 27% as compared with untreated beverages. SB beverages treated by HIPEF and HPP also exhibited a decrease of 22% and 17%, respectively, in the LAA in comparison to untreated products. In contrast, the LAA from WB and MB treated by both non themal technologies (HIPEF and HPP) remained unchanged with respect to untreated samples ($p > 0.05$). When the three treatments (HIPEF, HPP, TT) were compared, it was observed that the lowest LAA was obtained in beverages thermally treated while the highest was in HIPEF (WB) and HPP (MB). In our knowledge, this is the first study addressing the influence of non-thermal technologies comparing with thermal treatments on the lipophilic antioxidant activity of beverages. However, there is available information about the influence of HIPEF, HPP and TT on total antioxidant activity of food. In this sense, Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso (2010) observed that HIPEF treatment (35 kV/cm, 4μs bipolar pulses at 200 Hz for 1400 μs) did not affect the total antioxidant activity of a blended fruit juice-soymilk beverage in comparison to untreated juice. Elez-Martínez & Martín-Belloso (2007) did not find significant differences in the antioxidant activity of HIPEF (15 – 35 kV/cm, 20 – 10μs mono or bipolar pulses at 50 – 450 Hz for 100 – 1000 μs), TT (90 ºC/ 1 min) and untreated orange juice. Plaza, Sánchez-Moreno, Elez-Martínez, De Ancos, Martín-Belloso, & Cano (2006) also showed that the antioxidant activity of orange juice was not affected by HIPEF (35 kV/cm, 4μs bipolar pulses at 800 Hz for 750 μs) and thermal treatment (70 ºC during

30s) as compared to that untreated juice. On the other hand, Patras, Brunton, Da Pieve, & Butler (2009) observed that the antioxidant activity of liquid food could increase or not due to HPP processing. These authors (Patras, Brunton, Da Pieve, & Butler, 2009) reported that TT (70 ºC /2 min) and HPP (400 MPa/20 ºC/15 min) decrease the anti-radical power of strawberry pure (25 and 19%, respectively), but not in blackberry pure treated by these technologies. Significant reductions (between 7.5 and 11.5%) in the antioxidant activity of an orange juice-milk beverage thermally treated (90 or 98 ºC for 21s) were observed (Barba, Cortés, Esteve, & Frígola, 2012). However, the antioxidant activity remained unchanged in HPP samples (400 MPa /5 min) as compared with that untreated (Barba, Cortés, Esteve, & Frígola, 2012).

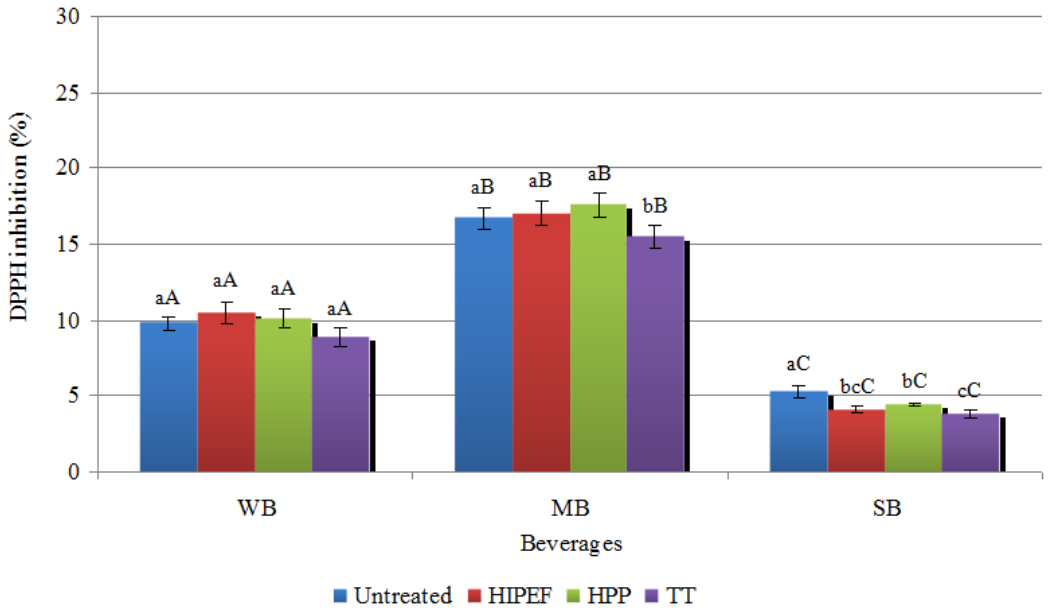

Figure 1. Lipophilic antioxidant activity (LAA) of non-digested beverages. Different lower case letters in the same beverage indicate significant differences ($p < 0.05$) within treatments. Different capital letters in the same treatment for WB, MB and SB beverages show significant differences ($p < 0.05$) within beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high pressure processing; TT, thermal treatment.

Considering the food matrix influence, it was observed that the LAA of all beverages were statistically different ($p < 0.05$), where SB displayed the lowest percentage of $DPPH^{\bullet}$ inhibition (4%) and MB the highest (17%). Likely, the higher fat content of milk

with respect to SB and WB matrices could improve the antioxidant activity of lipophilic constituents. Additionally, these results were in accordance to those found in carotenoids, where the greatest concentration of these compounds was found in MB (see previous sections). On the other hand, some protein and fiber types could mask the antioxidant activity of food (Sharma, Vijay Kumar, & Jagan Mohan Rao, 2008) and soymilk contains fiber and greater amounts of proteins (up to 20%), explaining why the lowest LAA was found in SB. In fact, a strong correlation between the LAA and total xanthophyll concentration (r^2 = 0.8495, p = 0.0000) from SB, as well as between LAA and total carotenoid concentration (r^2 = 0.7257, p = 0.0015) was observed.

3.2.1. Digested beverages. In Figure 2 are presented the lipophilic antioxidant activity (LAA) of digested beverages. The $DPPH^{\bullet}$ inhibition was in the range of 3.3 to 12.67% in untreated beverages, where MB showed the highest LAA.

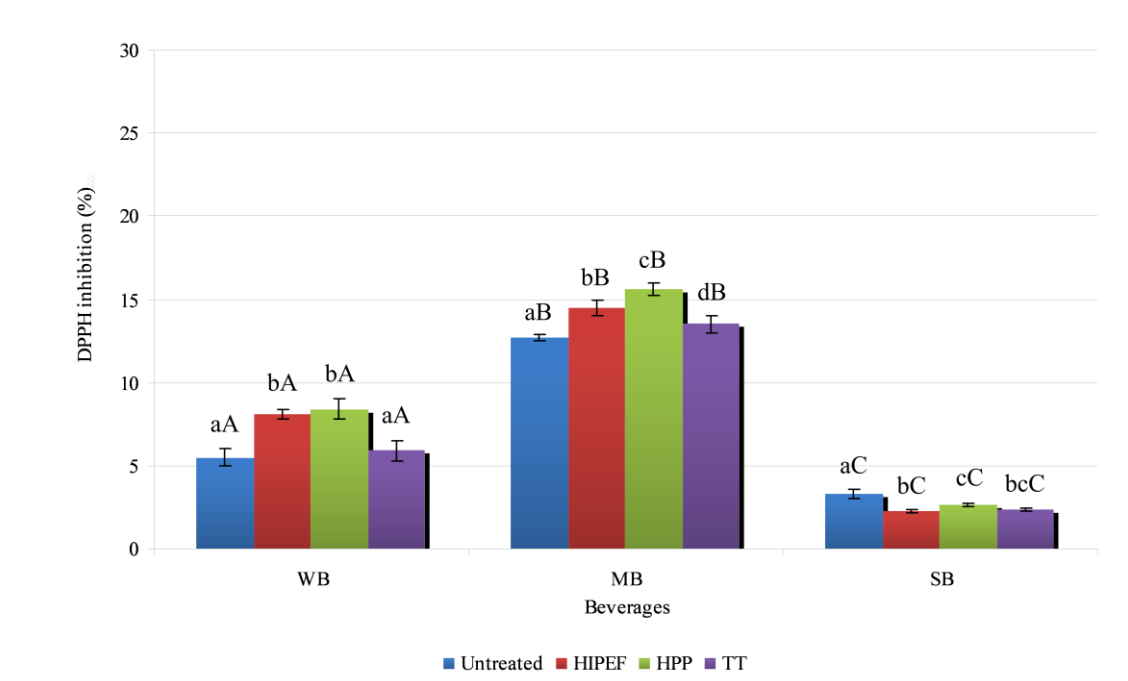

Figure 2. Lipophilic antioxidant activity from digested beverages. Different lower case letters in the same beverage indicate significant differences (p < 0.05) between treatments. Different capital letters in the same treatment for WB, MB and SB beverages show significant differences (p < 0.05) between beverages. WB, water-fruit juice beverage; SB, soymilk-fruit juice beverage; MB, milk-fruit juice beverage. HIPEF, high intensity pulsed electric fields; HPP, high pressure procesing; TT, thermal treatment.

All treatments (HIPEF, HPP and TT) increased from 7 to 17% the LAA of digested MB with respect to untreated beverages. Non-thermal technologies (HIPEF and HPP) also enhanced the LAA of digested WB (from 47 to 53%), while no significant differences were observed in the digested fraction of WB-TT. In contrast, the LAA of digested SB was reduced by any type of treatment, with losses between 21 and 30% as compared with untreated products. The LAA correlates well with the bioaccessibility of cis-violaxanthin+neoxanthin from MB ($r^2 = 0.7533$ $p = 0.0047$) and WB ($r^2 = 0.6487$, $p = 0.0225$), which was the carotenoid that increased its bioaccessibility after non-thermal processing. Therefore, the increment in the LAA of non-thermally treated beverages could be linked to the improvement in the solubilisation, digestibility and bioaccessibility of some lipophilic compounds with antioxidant activity, such as carotenoids.

The food matrix exerted a significant influence on the LAA of digested beverages. The lowest LAA was observed in digested SB, with around 2.30 and 3.3% of $DPPH^{\bullet}$ inhibition. On the other hand, digested MB displayed the highest LAA (from 12.67 to 15.6%). An explanation of these results could be attributed to the fact that the bioaccessibility of carotenoids was improved in matrices containing certain amount of fat (such as milk), as well as in beverages treated by non-thermal technologies (in the case of certain carotenoids). Therefore, the antioxidant potential and the bioaccessibility of these compounds could be modulated by both food matrix and processing.

4. Conclusion

Processing and food matrix exerted a significant influence on the bioaccessibility of carotenoids, as well as on the lipophilic antioxidant activity (LAA) of beverages. Non-thermal technologies (HIPEF and HPP) were more effective than TT to preserve the concentration and bioaccessibility of carotenoids and other lipophilic compounds with antioxidant activity from beverages based on a blend of fruit juices (orange, pineapple, kiwi and mango) and water (WB), milk (MB) or soymilk (SB). The best food matrix that allows obtaining higher bioaccessibility of total carotenoids (determined as the sum of individual compounds) was that containing milk (MB), followed by that made with soymilk (SB) and finally that of water (WB). HIPEF processing in combination with a milk matrix (MB) increased 15% the bioaccessibility of carotenoids as compared with that untreated. In SB beverages, HPP increased 10% the bioaccessibility of these

compounds, while all technologies (HIPEF, HPP and TT) diminished it in WB. Results demonstrate that both, treatment and food matrix, are able to modulate the bioaccessibility of carotenoids as well as the antioxidant potential of beverages, therefore these issues should be taken in consideration when developing functional food and beverages. In addition, HIPEF and HPP could be considered as promising technologies to obtain highly nutritional and functional beverages. Further studies should be carried out in order to evaluate the influence of food matrix and processing on the *in vivo* bioavailability of carotenoids.

Acknowledgement

This research was supported by the Ministerio de Ciencia e Innovación (Spain), reference AGL2006-12758-C02-02/ALI and AGL2006-12758-C02-01/ALI. María Janeth Rodríguez-Roque thanks to the Comissionat per a Universitats i Recerca, del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya and European Social Fund for the predoctoral grant. Prof. Olga Martín-Belloso thanks to the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia Award 2008.

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5. GENERAL DISCUSSION

5.1. Changes in the concentration of bioactive compounds throughout *in vitro* gastrointestinal digestion of soymilk-, milk-, and fruit juice-based beverages

Soymilk (SM), milk, two blended fruit juices (BFJ_1 containing orange, pineapple and kiwi juices; and BFJ_2 containing orange, pineapple, kiwi and mango juices), and two blended beverages (BFJ_1-SMB and BFJ_2-MB) were subjected to *in vitro* gastrointestinal digestion to evaluate the changes in the concentration of bioactive compounds (vitamin C, phenolic compounds, isoflavones, and carotenoids), as well as in the hydrophilic and lipophilic antioxidant activities.

5.1.1. Gastric digestion

The release of the bioactive compounds under acidic conditions was variable. The concentration of vitamin C from beverages displayed a significant reduction ($p < 0.05$) after gastric digestion. However, BFJ_1 was the product in which this compound showed the highest concentration under acidic conditions (recovery of 83%). It has been suggested that molecules of vitamin C are protonated at low pH, avoiding their interaction with oxygen (Ball, 2006) or other substances. In line with these results, other authors demonstrated that gastric digestion had little effect on the concentration of vitamin C, recovering 93% of this bioactive compound in broccoli inflorescences and 71% in pomegranate juice (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viquera, 2004). However, this bioactive compound diminished 32% in BFJ_2, 37% in BFJ_2-MB and 43% in BFJ_1-SMB, suggesting that the stability of vitamin C under gastric conditions could also depend on the surrounding environment in which this compound is immersed.

Total phenolic compounds determined as the sum of individual compounds (TPC by HPLC) and total phenolic content determined by Folin-Ciocalteu methodology (TPC by F-C) displayed higher concentration after gastric digestion than non-digested beverages. Quercetin from both SM and BFJ_1-SMB, rutin from BFJ_1, (+)-catechin from BFJ_2, 4-hydroxybenzoic acid from milk, and naringenin from BFJ_2-MB were the individual phenolic compounds that displayed the greatest recoveries after gastric digestion (with increases in their concentration between 38% and 163% as compared to non-digested beverages). A similar trend was followed by isoflavones from soymilk, where the

concentration of total glucosides and total aglycones augmented 20% and 31%, respectively, under acidic conditions. The increase in the concentration of these compounds suggest that the acidic pH and the digestive enzyme action hydrolyze the phenolic compounds bound to other food constituents, such as proteins and carbohydrates (Saura-Calixto, Serrano, & Goñi, 2007; Liyana-Pathirana & Shahidi, 2005; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). On the other hand, daidzein and genistein from BFJ_1-SMB were less affected by gastric conditions (recovery > 79%) than their glucoside form (recovery between 44% and 52%) with regard to non-digested product. The decrease in the concentration of isoflavones during gastric digestion could be attributed to the lack of solubility of these compounds under acidic conditions (Piskula, 2000).

All the carotenoids analyzed in this research diminished their concentration after gastric digestion. The most stable carotenoids from gastric digesta were lutein from both BFJ_1 and milk (recoveries of 90% and 67%, respectively), β-cryptoxanthin and α-carotene from BFJ_1-SMB (recoveries of 79% and 81%, respectively), α-cryptoxanthin from both BFJ_2-MB and BFJ_2 (recovery > 92%). The decrease in the concentration of carotenoids could be related to the fact that carotenoids are highly oxidized molecules due to the double bounds of their chemical structure (Hedrén, Diaz, & Svanberg, 2002). Other important factor to take into consideration is the change of pH during the *in vitro* gastrointestinal digestion, which could affect the carotenoid stability (Rao & Rao, 2007).

The gastric digesta of SM and milk showed higher hydrophilic antioxidant activity (HAA) (increases of 95% and 97%, respectively) than their corresponding non-digestive product. Phenolic compounds are among the most important BFJ bioactive compounds with hydrophilic antioxidant activity. Thus, the increase in the HAA of gastric digesta from both SM and milk could be attributed to the increment in the concentration of phenolic compounds under acidic conditions. In contrast, the HAA diminished in the range of 9% to 17% in the gastric digesta of BFJ_1, BFJ_2, BFJ_1-SMB and BFJ_2-MB. A similar trend was observed in the lipophilic antioxidant activity (LAA) of these beverages (BFJ_1, BFJ_2, BFJ_1-SMB and BFJ_2-MB), being reduced between 10.5% and 38%. The antioxidant activity in fruit juices and beverages depends on the composition and concentration of their antioxidants, such as vitamins, phenols, and carotenoids (Liu, 2003). Some of these constituents were significantly reduced after gastric digestion of beverages, explaining the results obtained in this research.

5.1.2. Small intestinal digestion

During small intestinal digestion, all the analyzed compounds displayed significant losses ($p < 0.05$). Vitamin C diminished in the range of 39% to 53% in the small intestinal digesta of beverages with respect to gastric digesta, being BFJ_2-MB the product in which the greatest losses were achieved. Intestinal environment, such as pH > 4, dissolved oxygen, heat and the enzyme activity, particularly favour the oxidation of this compound (Ball, 2006; Jeney-Nagymate & Fodor, 2008). The vitamin C oxidation in the gastrointestinal tract could also be related to the fact that this compound keeps metal ions in a reduced state (i.e. iron), or regenerate the active form of other dietary constituents (i.e. vitamin E) by donating an electron (Ball, 2006; Schlueter & Johnston, 2011).

Phenolic compounds decreased between 24% and 95% in the small intestinal digesta in comparison with gastric digesta, with the exception of caffeic acid and quercetin both from BFJ_2 (remained unchanged), 4-hydroxybenzoic acid from SM (increased 8%), and hesperidin from BFJ_2 (increased 13%). Isoflavones were reduced in the range of 11% to 39%, being daidzin from BFJ_1-SMB the lowest recovered. The instability of these bioactive substances under alkaline conditions suggests that these compounds undergo several changes (i.e. oxidation, polymerization and epimerization), affording the formation of other bioactive derivatives with different physicochemical properties. For instance, phenolic compounds are sensitive to mild alkaline conditions and a great portion of flavanones from orange juice (50% - 60%) were transformed into chalcones under intestinal conditions (Gil-Izquierdo, Gil, Ferreres & Tomás-Barberán, 2001). Furthermore, the reduction in the concentration of phenolic substances under alkaline conditions was related to the complexes formation between these compounds and other food constituents, mainly metal ions, proteins and fiber (Argyri, Komaitis, & Kapsokefalou, 2006; Saura-Calixto, Serrano, & Goñi, 2007).

A similar trend was followed by carotenoids, where the losses reached up to 70% in the small intestinal digesta with regard to gastric digesta. Cis-antheraxanthin and antheraxanthin from the small intestinal digesta of BFJ_1-SMB were the carotenoids less affected by intestinal conditions (both carotenoids showed losses of 11%) as compared to gastric digesta. In contrast, cis-violaxanthin+neoxanthin from BFJ were the lowest recovered carotenoids. Carotenoids are known to be highly sensitive molecules to pH changes (Rao & Rao, 2007), therefore the alkaline pH of intestinal environment could affect the stability of these compounds.

Both HAA and LAA significantly diminished after intestinal digestion. The HAA decreased in the range of 37% to 89%, whereas LAA was reduced between 57% and 74% when the small intestinal digesta of beverages was compared with gastric digesta. An explanation of these results could be attributed to the fact that most of the hydrophilic and lipophilic compounds with antioxidant activity were unstable under intestinal conditions.

5.1.3. Dialyzed fraction

The lowest concentration of hydrophilic bioactive substances was observed in the dialyzed fraction. Vitamin C, phenolic compounds and isoflavones, were the dialyzable constituents of the beverages analyzed in this research.

The recovery of vitamin C varied in the range of 30% and 42% in the dialyzed fraction as compared to the small intestinal digesta of beverages. On the other hand, some phenolic acids, such as gallic, 4-hydroxybenzoic, p-coumaric, ferulic and sinapic acids, were not detected in the dialyzed fraction of SM, BFJ_1, BFJ_2, BFJ_1-SMB and BFJ_2-MB. Rutin from SM was the flavonoid that was not dialyzable. Quercetin from BFJ_1 was the most recovered flavonoid (92%) in the dialyzed fraction with respect to the small intestinal digesta. Glycitein from SM was the isoflavone with the lowest concentration (recovery of 14%) in the dialyzed fraction as compared to the small intestinal digesta, whereas genistein from BFJ_1-SMB was the most recovered isoflavone (59%). The HAA of the analyzed beverages diminished in the range of 47% (for SM) to 93% (for milk) when the dialyzed fraction was compared with the small intestinal digesta.

Because of the changes in the physicochemical features of bioactive compounds caused by gastrointestinal digestion, most of these substances could increase their molecular weight (complexes formation) or reduce their solubility, making their dializability difficult and thus, their absorption into the gut. In fact, it has been found that a compound linked to other molecules is not absorbed as easily as its free form and it must be hydrolized in the gut in order to be taken up by enterocytes (During, 2008). In line with these results, some phenolic acids from red wine were not dialyzable due to modifications in their chemical structure and solubility (Argyri, Proestos, Komaitis & Kapsokefalou, 2005). Other authors also reported that the molecular weight of phenolic compounds had a significant influence on their uptake into the gut (Scalbert & Williamson, 2000).

5.1.4. Micellar fraction

Losses between 49% and 77% in the concentration of total carotenoids were observed in the micellar fraction of beverages with respect to the small intestinal digesta. Among the individual carotenoids, α-carotene from the BFJ_1-SMB was the carotenoid with the lowest concentration (recovery of 11%) in the micellar fraction with respect to the small intestinal digesta. In contrast, cis violaxanthin+neoxanthin from BFJ_2 showed the highest concentration (recovery of 89%) when the micellar fraction was compared with the small intestinal digesta, followed by that of BFJ_2-MB (recovery of 77%). A similar trend was followed by the LAA of the micellar fraction of beverages, where the antioxidant activity was reduced in the range of 32% (for BFJ_2-MB) and 90% (for SM) as compared to that of the small intestinal digesta.

It is well known that carotenoids must be solubilised and incorporated into mixed micelles before being available for absorption. Micelles are molecular aggregates that transport the lipophilic material to the intestinal epithelium (Fernández-García et al., 2012). Micellization is affected by several factors, such as the physicochemical properties of carotenoids, food matrix, and the fat solubility of individual carotenoids (Van Het Hof, West, Weststrate & Hautvast, 2000), which could explain why the carotenoids displayed differences in their degree of micellization and thus, in their bioaccessibility.

5.2. Bioaccessibility of bioactive compounds from soymilk-, milk-, and fruit juices-based beverages

The vitamin C showed a bioaccessibility of 11.5% in BFJ_2-MB, followed by BFJ_1-SMB (bioaccessibility of 13%) and blended fruit juices (BFJ_1 and BFJ_2, with bioaccessibilities of 15% each one). A similar vitamin C bioaccessibility was observed in a soymilk-based beverage (13%) but in pomegranate juice it just showed 2.5% (Cilla et al. 2012; Pérez-Vicente et al. 2002). Vitamin C is a molecule susceptible to chemical or enzymatic oxidation. In the presence of oxygen and trace amounts of transition metals, a complex is formed reducing the bioaccessibility of vitamin C. The presence of fiber also reduces the vitamin C bioaccessibility due to entrapment by the fiber matrix (Ball, 2006). Additionally, vitamin C may acts as antioxidant, protecting other dietary

substances, such as carotenoids, from oxidation (Berry, 2009), resulting in a decrease in its bioaccessibility.

The bioaccessibility of some phenolic acids from beverages was 0%, as well as rutin from SM. Quercetin from BFJ_1 and (+)-catechin from SM displayed the highest bioaccessibility among the analyzed beverages (bioaccessibilities of 29% and 28%, respectively). Isoflavones showed bioaccessibilies in the range of 15% to 53%, being genistin from SM the most bioaccessible isoflavone. Results suggest that the bioaccessibility of phenolic compounds is widely influenced by modifications in their physicochemical properties (i.e. molecular weight and solubility) due to pH changes and interactions with other food constituents during *in vitro* gastrointestinal digestion.

In line with the results obtained in this research, Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberá (2001) obtained a similar bioaccessibility of flavanones (between 11% and 36%) in orange juice. Vallejo, Gil-Izquierdo, A., Pérez-Vicente, A., & García-Viguera (2004) reported bioaccessibilities of ferulic and sinapic acids of 8.2% and 1.8%, respectively.

Carotenoids were bioaccessible in the range of 6.5% (cis-violaxanthin+neoxanthin from BFJ_1-SMB) to 27% (β-carotene from BFJ_2-MB). Changes in the molecular structure of carotenoids and a competitive inhibition between themselves could occur, affecting their incorporation into micelles and thus, their bioaccessibility. Moreover, the fat and fiber content of food significantly influenced the bioaccessibility of carotenoids (Hedrén et al., 2002; Maiani et al., 2009). The bioaccessibility of carotenoids was around 39.84% according to Cilla et al. (2012) in a similar blended beverage. Dhuique-Mayer et al. (2007) found that β-cryptoxanthin from citrus juices (orange, mandarin and lemon) was between 16% and 40% bioaccessible.

All these data showed that, in general, hydrophilic compounds displayed up to 2 times higher bioaccessibility than lipophilic substances. An explanation of this trend could be attributed to the fact that hydrophilic compounds are more soluble in the aqueous environment of gastrointestinal lumen with respect to lipophilic constituents (During, 2008). Additionally, bioaccessibility is widely influenced by differences in bioactive compound concentration within plant tissues, variations in cell wall structure, location of bioactive substances into the cells, and the binding of compounds within the food matrix (Balasundram et al., 2006), which could explain the differences in the bioaccessibilities of compounds analyzed in this research.

5.3. Influence of the food matrix on the *in vitro* bioaccessibility of bioactive compounds from beverages based on fruit juice, milk or soymilk

5.3.1. Vitamin C

The food matrix had a significant influence ($p < 0.05$) on the bioaccessibility of vitamin C from fruit juice-based beverages containing soymilk (SB), water (WB) and milk (MB). SB displayed the highest bioaccessibility of this bioactive compound, followed by WB and MB. Soymilk is a rich source of phenolic compounds and isoflavones, thus, it could be hypothesized that these antioxidant compounds could avoid the vitamin C oxidation in SB. In contrast, antagonistic interactions between vitamin C and other food constituents (i.e. proteins, vitamins, and metal ions) could be favoured in products containing milk (MB). It is well known that milk contains vitamin-binding proteins that could afford the formation of complexes (Claeys et al., 2013). In addition, other vitamin types (B_1, B_2 and B_{12}) and metal ions (Fe, Cu and Zn) contained in milk are able to interact with vitamin C increasing its degradation rate (Ball, 2006).

Another important factor to take into consideration, in terms of synergistic/antagonistic interactions, is the ratio in which the beverages are combined. In SB (containing 17.5% of soymilk) vitamin C was 23.3% bioaccessible, while in BFJ_1-SMB (containing 42.5% of soymilk) it just showed a bioaccessibility of 13%. On the other hand, BFJ_2-MB and MB, which were made using the same proportion of fruit juices and milk, displayed the same bioaccessibility of vitamin C (around 11.4%). In our knowledge, there is really scarce information concerning the food matrix effect on the bioaccessibility of bioactive compounds, including vitamin C, from mixed beverages. In contrast to these results, a study reported that the highest bioaccessibility of vitamin C was obtained in beverages containing 16.5% of whole milk, while the lowest was found in beverages made with 42.5% of soymilk (Cilla et al. 2012). Differences in the results obtained in the present research and in that reported by Cilla et al. (2012) could be explained by the proportion of juices, milk or soymilk utilized to prepare the beverages. Additionally, these authors (Cilla et al., 2012) determined the vitamin C bioaccessibility in the supernatants obtained after centrifugation of the intestinal digesta (3300g/1h at 4ºC) instead of the dialyzed fraction (as in this research).

Therefore, the proportion in which a beverage is made also affects the bioaccessibility of the biologically active compounds.

5.3.2. Phenolic compounds

The bioaccessibility of phenolic compounds was significantly influenced by the food matrix ($p < 0.05$). In general, the highest bioaccessibilities of total phenolic acids, total flavonoids, and TPC by HPLC were observed in WB (in the range of 15% and 20%), followed by SB (between 14% and 18%) and MB (in the range of 12% and 16%). This trend was also observed in some individual phenolic compounds, such as chlorogenic acid, p-coumaric acid, and rutin.

On the other hand, it was found that TPC by HPLC from blended beverages (BFJ_1-SMB and BFJ_2-MB) were less bioaccessible in comparison with their respective blend of fruit juices, with reductions in their bioaccessibility between 5% and 17%. For instance, the bioaccessibility of ferulic acid, sinapic acid, quercetin, hesperidin and rutin was higher in BFJ_1 with respect to that of BFJ_1-SMB. All individual phenolic compounds showed more bioaccessibility in BFJ_2 than in BFJ_2-MB. These results showed that the combination of a blended fruit juice with milk or soymilk reduced the bioaccessibility of some phenolic substances likely because the degree of digestibility and therefore, the degree of bioaccessibility is influenced by the complexity of the food matrix in which a bioactive compound is contained (During, 2008).

Phenolic substances could interact with dietary proteins, iron or fiber, leading to the formation of complexes (Argyri, Proestos, Komaitis & Kapsokefalou, 2005; Saura-Calixto, Serrano & Goñi, 2007). Milk and soymilk are known to be rich sources of proteins. Covalent or non-covalent interactions between proteins and phenolic compounds could produce the precipitation of proteins (Sharma, Vijay Kumar, & Jagan Mohan Rao, 2008), masking the phenolic compounds and thus, reducing their bioaccessibility. Additionally, it has been also proposed that the lipid content of milk could supply a favourable environment for protein-phenolic compound linkage (Serafini et al., 2009).

There is not available information about the effect of the food matrix on the bioaccessibility of phenolic compounds from blended beverages at this time. However, these results were in accordance with other studies where the *in vivo* absorption of some phenolic compounds was significantly reduced by the addition of milk to tea (Lorenz et al., 2007), blueberries (Serafini et al., 2009) and chocolate (Serafini et al., 2003). On the

contrary, other studies have not shown significant differences in the plasmatic concentration of flavonols and catechins in tea combined with milk (Hollman, Hof, Tijburg, & Katan, 2001); while the bioavailability of these compounds was slightly affected in a matrix containing coffee with milk (Ferruzzi, 2010; Renouf et al., 2010).

5.3.2.1. Isoflavones. Because of the fact that isoflavones are almost exclusively found in legumes, such as soy-beans and their derived products, the influence of the food matrix on the bioaccessibility of isoflavones was only compared in products containing soymilk (SM and BFJ_1-SMB).

The food matrix exerted a significant influence ($p < 0.05$) on the bioaccessibility of isoflavones, with the exception of daidzein which showed a similar bioaccessibility in SM and BFJ_1-SMB. The bioaccessibility of isoflavones was in the range of 15% to 53% in SM and between 15% and 26.5% in BFJ_1-SMB. Only the isoflavone genistein increased its bioaccessibility in a complex matrix (BFJ_1-SMB). In contrast, total isoflavones displayed 40% less bioaccessibility in BFJ_1-SMB than in SM.

The most affected compounds by the food matrix were the glucoside isoflavones daidzin and genistin, which decreased 45% and 51%, respectively, in BFJ_1-SMB in comparison with SM. Similarly, glycitein showed a bioaccessibility of 15% in SM but it was not detected in BFJ_1-SMB. SM is a rich source of proteins and iron (Jinapong, Suphantharika & Jamnong, 2008), while fruit juices contain certain amount of natural fiber and different phenolic profile with respect to SM. Some phenolic substances, including isoflavones, may bind to proteins and fiber resulting in insoluble aggregates that precipitate and make the digestibility of food difficult.

Additionally, the efficiency of the digestive process by which a bioactive compound becomes more bioaccessible in the gastrointestinal tract is inversely related to the degree of complexity of the food matrix (During, 2008). It is known that the sugar content of the food matrix hinders the extraction of isoflavones, thus decreasing their bioaccessibility after *in vitro* digestion (de Pascual-Teresa et al., 2006). In this case, BFJ_1-SMB contained higher concentration of sugar in comparison to SM (15.0 ºBrix in BFJ_1-SMB against 5.83 ºBrix in soymilk), which also could explain the reduced bioaccessibility of isoflavones in BFJ_1-SMB.

5.3.3. Carotenoids

Among all the beverages analyzed in this research, it was observed that food matrices containing milk improved the bioaccessibility of carotenoids. Total carotenoids from MB displayed the highest bioaccessibility with averages value of 23.5%, followed by SB (16%) and WB (13%). Particularly, the most bioaccessible carotenoid in MB was α-carotene (average bioaccessibility of 28%) and antheraxanthin the lowest (average bioaccessibility of 13%). In SB the highest and lowest bioaccessible carotenoids were lutein (around 27%) and antheraxanthin (around 9%), respectively. Cis-violaxanthin displayed the greatest bioaccessibility in WB (around 15%), whereas antheraxanthin showed the lowest (around 7.5%). A similar trend was observed when BFJ_2-MB and BFJ_2 were compared, where BFJ_2-MB showed 37% higher total carotenoid bioaccessibility than that of BFJ_2. These results suggest that the greater fat content of milk (3.6%) with respect to soymilk (1.6%) and water (0%) could favour the incorporation of carotenoids into micelles and thus, increase their bioaccessibility in beverages containing milk.

Other authors have also reported that dietary fat enhance the bioaccessibility of carotenoids from food (Fernández-García, et al., 2012; Van Het Hof et al., 2000). Similarly, Granado-Lorencio, Herrero-Barbudo, Blanco-Navarro, Pérez-Sacristán, & Olmedilla-Alonso (2009) showed that the addition of milk to blended fruit juices improve the bioaccessibility of carotenoids.

Fiber is another food constituent that could affect the bioaccessibility of carotenoids. Dietary fiber could increase the viscosity of the intestinal content (Maiani et al. 2009), leading to entrap bioactive compounds, such as carotenoids, and decrease the activity of digestive enzymes. Therefore, the micellization, bioaccessibility and absorption of carotenoids are reduced due to the fiber content of food, while their faecal excretion is increased. In this sense, it could be expected that SB beverages contain more amount of fiber than MB, explaining why the bioaccessibility of carotenoids diminished in SB beverages. In contrast to these results, Cilla et al. (2012) found that the bioaccessibility of carotenoids was better in soymilk-based beverages than in those containing milk. However, these authors (Cilla et al., 2012) made the beverages with different proportions of milk and soymilk (16.5% versus 41.5%, respectively), which could elucidate the differences in the results.

5.3.4. Antioxidant activity

5.3.4.1. Hydrophilic antioxidant activity (HAA). The food matrix also exerted a significant influence on the hydrophilic antioxidant activity of digested beverages. HAA displayed the highest value in digested WB and SB (in the range of 14% to 22% of $DPPH^{\bullet}$ inhibition), with no significant differences among them in treated beverages. In contrast, digested MB showed the lowest HAA (between 12% and 15% of $DPPH^{\bullet}$ inhibition), except for TT digested beverages where the HAA was the same for all beverages. A similar tendency was observed in digested blended fruit juices (BFJ_1 and BFJ_2), which also displayed higher HAA (between 15% and 18% of $DPPH^{\bullet}$ inhibition) in comparison with BFJ_1-SMB and BFJ_2-MB (around 11% of $DPPH^{\bullet}$ inhibition).

These results also suggest that the bioaccessibility of hydrophilic compounds with antioxidant activity decrease as the complexity of the food matrix increase. Milk and soymilk contain certain amount of proteins and minerals, which could be able to interact with water soluble constituents of food, leading to the formation of complexes or aggregates. As a consequence, it is difficult that these compounds could be released from the food matrix by the digestive enzyme action and solubilised into the gastrointestinal tract, resulting in a decrease in their antioxidant activity.

5.3.4.2. Lipophilic antioxidant activity (LAA). The antioxidant activity of lipophilic compounds was significantly influenced by the food matrix ($p < 0.05$). In overall, digested SB displayed the lowest LAA (in the range of 2% to 3% of $DPPH^{\bullet}$ inhibition), while digested MB showed the highest (between 13% and 16% of $DPPH^{\bullet}$ inhibition). Similarly, it was observed that the antioxidant activity of lipophilic compounds was improved in the digested beverage containing milk (BFJ_2-MB, with 6.5% of $DPPH^{\bullet}$ inhibition), as compared to that of the digested blend of fruit juices (BFJ_2, with 5% of $DPPH^{\bullet}$ inhibition).

Carotenoids are among the main bioactive compounds that contribute to the lipophilic antioxidant activity of food and their bioaccessibility was improved in matrices containing certain amount of fat (such as milk). In addition, the surrounding environment in which carotenoids are contained also impacts on their bioaccessibility because interactions between carotenoids-carotenoid and/or carotenoids-food

constituents (i.e. fiber and fat) could occur. As a result of these interactions, the antioxidant activity of liposoluble food constituents could be significantly affected.

5.4. Influence of processing on the *in vitro* bioaccessibility of bioactive compounds from beverages based on fruit juice, milk or soymilk

5.4.1. Vitamin C

The bioaccessibility of vitamin C was not modified in beverages treated by non-thermal technologies (HIPEF and HPP) in comparison with untreated beverages ($p > 0.05$), with the exception of MB treated by HPP, which increased 8%. Thermal treatment (TT) did not change the bioaccessibility of vitamin C in MB with respect to untreated and HIPEF treated products. On the contrary, TT reduced 16.5% and 11.6% the vitamin C bioaccessibility in WB and SB, respectively, as compared to untreated products.

Similar results were reported in a study carried out with twelve healthy subjects, in which a vegetable soup treated by HIPEF preserved the *in vivo* bioavailability of vitamin C of the fresh product (Sánchez-Moreno et al., 2005). Conversely, Cilla et al. (2012) reported that HPP diminished significantly the bioaccessibility of vitamin C in both milk and soymilk beverages, while it increased in beverages treated by heat (TT).

Thermal treatment of food promotes the nutrient release through cell rupture (breakage) or cell separation (Parada & Aguilera, 2007) and can also enhance the bioavailability of several nutrients (i.e. carotenoids, thiamine, vitamin B-6, niacin, and folate) by releasing them from the food matrix (Hotz and Gibson, 2007). However, vitamin C is a thermo-labile compound, which is very susceptible to chemical and enzymatic oxidation during processing. The catalyzed oxidative pathway of ascorbic acid degradation is the most important reaction pathway for losing vitamin C in foods (Ball et al. 2006). Therefore, oxidative reactions of vitamin C in TT beverages could explain why in this research the worst bioaccessibility of vitamin C was obtained in TT beverages.

5.4.2. Phenolic compounds

Processing exerted a variable influence on the bioaccessibility of phenolic compounds from SB, WB and MB beverages. The bioaccessibility of caffeic and p-coumaric acids from both WB and MB; chlorogenic and ferulic acids from MB; hesperidin and rutin from all three beverages was improved after applying treatments, mainly non-thermals (HIPEF and HPP). Processing (HIPEF, HPP and TT) did not change the bioaccessibility of caffeic and chlorogenic acids from SB, neither naringenin from WB or MB. On the contrary, all treatments diminished the bioaccessibility of ferulic acid from WB. Overall, the lowest bioaccessibility of phenolic compounds was obtained in TT beverages. It is also interesting to note that rutin from MB was not bioaccessible in untreated or TT products, however, it displayed bioaccessibilities of 7.2% and 8.4% in HIPEF and HPP beverages, respectively.

Processing is known to change some physicochemical features of phenolic compounds. For instance, several changes in the phenol structure (hydroxylation, methylation, isoprenylation, dimerization, glycosylation, among others) and/or the formation of phenolic derivatives (by partial degradation of the combined forms or by losing the moieties between phenols and sugars) could occur during processing (Dugo et al., 2005).

Because of the fact that the bioaccessibility of phenolic compounds is dependent on their chemical structure and solubility into the gut, the changes produced not only by the *in vitro* gastrointestinal digestion, but also by processing of food may modulate the bioaccessibility of these constituents.

5.4.3. Carotenoids

The bioaccessibility of carotenoids was significantly influenced by the processing ($p < 0.05$). Most of individual carotenoids diminished their bioaccessibility after applying any type of treatment, mainly in TT beverages where the bioaccessibility declined by 63%. HIPEF treatment decreased the bioaccessibility of carotenoids in the range of 7.6% to 48.2%, with respect to untreated beverages. In the same way, carotenoids were less bioaccessible in HPP beverages (between 8.2% and 45.1%) than in those untreated. β-cryptoxanthin from WB was the lowest bioaccessible carotenoids in the HIPEF and HPP beverages, whereas in those TT was α-cryptoxanthin from SB.

Really few reports have evaluated the effect of non-thermal (HIPEF and HPP) or thermal (TT) technologies on the bioaccessibility of carotenoids. However, in line with

these results, it was reported that some carotenoids were less bioaccessible in HPP-MB (between 15% and 58%), TT-MB (in the range of 30% and 52%) and TT-SB (between 50% and 90%) in comparison with their respective untreated product (Cilla et al., 2012). Heat treatment also reduced the bioaccessibility of α-carotene and β-cryptoxanthin in orange juice, with respect to fresh industrially squeezed juice (Stinco et al., 2012).

On the other hand, HIPEF processing improved the bioaccessibility of cis-violaxanthin+neoxanthin from the three beverages (between 9% and 79%), cis-antheraxanthin from SB (10%), and lutein from both MB (32%) and SB (16%). Total xanthophylls and total carotenoids from MB also increased 24.5% and 15%, respectively, when HIPEF treatment was applied. A similar trend was observed in beverages treated by HPP, where cis-violaxanthin+neoxanthin from the three beverages; cis-antheraxanthin and lutein from SB, total xanthophylls from both MB and SB, and total carotenoids from SB were more bioaccessible in HPP beverages (in the range of 6.5% to 65%) than in untreated samples. On the contrary, no increases in the bioaccessibility of carotenoids were observed in TT beverages.

The food matrix structure is one of the most important factors that affect the bioaccessibility of carotenoids (Stinco et al., 2012) and processing is able to modify it. The improvement in the bioaccessibility of some carotenoids in HIPEF and HPP beverages could be justified by changes in the structure of the food matrix due to processing effect, such as the breakdown of cell walls and membranes in which carotenoids are embebed. Therefore, interactions between carotenoids and digestive enzymes could be enhanced, as well as their release from the food matrix and their solubilisation into micelles. Maiani et al. (2009) also reported that some types of food processing can improve the carotenoid bioavailability. Cilla et al. (2012) found significant increases (between 39% and 264%) in the bioaccessibility of carotenoids in milk- (neoxanthin+9-cis-violaxanthin) and soymilk- (neoxanthin+9-cis-violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene) based beverages treated by HPP with respect to untreated beverages.

5.4.4. Antioxidant activity

5.4.4.1. Hydrophilic antioxidant activity (HAA). Processing exerted a variable influence on the hydrophilic antioxidant activity of digested beverages. Non-thermal technologies (HIPEF and HPP) increased up to 21% the HAA of digested MB in

comparison with untreated beverages, but they did not modify the HAA of digested SB. In contrast, the HAA of digested WB was reduced 19% by HIPEF and 14% by HPP, with respect to untreated products. The lowest HAA value was reached in TT digested beverages, which showed up to 34% less HAA (in WB products) than those untreated.

It is well known that the bioaccessibility of antioxidants is widely influenced by the structural properties of hydrophilic compounds. Among these bioactive compounds vitamin C and phenolic compounds stand out. Processing modifies the physicochemical features of these compounds, explaining why the bioaccessibility of these compounds after treatment changed.

5.4.4.2. Lipophilic antioxidant activity (LAA). Processing (HIPEF, HPP and TT) exerted a significant influence ($p < 0.05$) on the LAA of digested beverages, as compared with untreated products.

All treatments (HIPEF, HPP and TT) increased in the range of 7% to 17% the LAA of digested MB with respect to untreated beverages. Non-thermal technologies (HIPEF and HPP) also enhanced the LAA of digested WB (between 47% and 53%), while no significant differences were observed in the digested fraction of WB-TT. In contrast, the LAA of digested SB was reduced by any type of treatment, with losses between 21% and 30% as compared to untreated products.

Carotenoids, which are among the main contributors to the lipophilic antioxidant activity of foods and beverages, followed a similar tendency, at least in HIPEF and HPP beverages, explaining these results. This was the first study addressing the effect of the food processing on the bioaccessibility of lipophilic antioxidant compounds from the micellar fraction of digested beverages. Therefore, it was not possible to compare the results obtained with those of literature.

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6. CONCLUSIONS

CONCLUSIONS

Results obtained in this doctoral thesis allow obtaining the following conclusions:

- ✓ Gastric digestion improved the release from the food matrix of phenolic compounds and isoflavones in most of the analyzed beverages. However, vitamin C and carotenoids were unstable under acidic conditions. On the other hand, the concentration of all the analyzed compounds, as well as the antioxidant activity, significantly diminished during the small intestinal digestion, mainly in the dialyzed and micellar fractions.

- ✓ The bioaccessibility of hydrophilic bioactive compounds was generally higher (with bioaccessibilities in the range of 11% to 42%) than that of lipophilics (bioaccessibilities between 9% and 27%), except for milk and blended fruit juice-milk beverage (BFJ_2-MB), where the lipophilic compounds were the most bioaccessible (bioaccessibilities up to 56%).

- ✓ The bioaccessibility of vitamin C was higher in simple matrices (blended fruit juices BFJ_1 and BFJ_2) than in complexes matrices (blended beverages BFJ_1-SMB and BFJ_2-MB). However, soymilk beverages (SB) displayed the greatest bioaccessibility of vitamin C among the blended analyzed beverages (WB, SB and MB).

- ✓ The addition of milk or soymilk to blended fruit juices (MB and SB, respectively) reduced the bioaccessibility of phenolic compounds as compared to beverages containing water (WB).

- ✓ The bioaccessibility of lipophilic compounds, such as carotenoids was improved in matrices containing milk.

- ✓ The bioaccessibility of vitamin C was not modified by non-thermal processing technologies (high-intensity pulsed electric fields [HIPEF] and high-pressure processing [HPP]), but it diminished up to 16.5% by thermal treatment (TT) in comparison with untreated beverages. Phenolic compounds were more bioaccessible after applying any treatment with respect to untreated products.

- ✓ Non-thermal technologies (HIPEF and HPP) were more effective than TT to preserve the bioaccessibility of carotenoids and other lipophilic compounds with antioxidant activity from water (WB), milk (MB) or soymilk beverages (SB).

As a final conclusion it can be stated that both food matrix and food processing were able to modulate the bioaccessibility of hydrophilic and lipophilic constituents from beverages. Particularly, HIPEF and HPP could be considered promising technologies for obtaining highly nutritional and functional beverages. Moreover, *in vitro* gastrointestinal digestion is a useful tool for estimating physicochemical changes, release, stability, interactions and bioaccessibility of bioactive compounds from beverages, as well as for determining the influence of the food matrix and processing on the bioaccessibility of these substances. Therefore, *in vitro* gastrointestinal digestion can be used as a prior step toward *in vivo* studies.