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Facultad de Farmacia

Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal

TESIS DOCTORAL INTERNACIONAL

BIODISPONIBILIDAD Y EFECTOS BIOLÓGICOS DE ESTEROLES EN BEBIDAS FUNCIONALES

INTERNATIONAL DOCTORAL THESIS

BIOAVAILABILITY AND BIOLOGICAL EFFECTS OF STEROLS IN FUNCTIONAL BEVERAGES

Programa de Doctorado en
CIENCIAS DE LA ALIMENTACIÓN

Presentada por:
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Valencia, julio 2018

Las Dras. Reyes Barberá, Guadalupe García y el Dr. Antonio Cilla, Catedrática, Profesora Titular y Profesor Ayudante Doctor, respectivamente, del Área de Nutrición y Bromatología del Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València,

CERTIFICAN QUE:

La graduada en Ciencia y Tecnología de los Alimentos, Dña. Andrea Álvarez-Sala Martín, ha realizado bajo su dirección el trabajo que lleva por título **"Biodisponibilidad y efectos biológicos de esteroles en bebidas funcionales"**. El trabajo ha dado lugar a 6 artículos en los cuales firma como primera autora (3 publicados y 3 en revisión).

1. *Impact of lipid components and emulsifiers on plant sterols bioaccessibility from milk-based fruit beverages. Journal of Agricultural and Food Chemistry (2016), 64, 5686−5691.*
   Índice de impacto JCR (2016): 3,154 (17/130) Q1. Food Science & Technology.

2. *Safe intake of a plant sterol-enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage. Journal of Food Composition and Analysis, (2018), 68, 111−117.*
   Índice de impacto JCR (2017): 2,956 (32/133) Q1. Food Science & Technology.

3. *Effects of plant sterols or β-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death. Journal of Agricultural and Food Chemistry, (2018), 66, 1157−1166.*
   Índice de impacto JCR (2017): 3,412 (18/133) Q1. Food Science & Technology.

4. *Positive impact on serum lipid profile and cytokines after consumption of a plant sterol-enriched beverage with milk fat globule membrane: a clinical study (Food & Function, under review).*
   Índice de impacto JCR (2017): 3,289 (20/133) Q1. Food Science & Technology

5. *Anti-proliferative effect of phytosterol-ingredient and its main phytosterol (β-sitosterol) on human cancer cell lines (International Journal of Food Sciences and Nutrition, under review).*
Índice de impacto JCR (2017): 2,317 (44/133) Q2. Food Science & Technology

6. *Physiological concentrations of phytosterols enhance the apoptotic effects of 5-fluorouracil in colon cancer cells (Journal of Functional Foods, under review).*
Índice de impacto JCR (2017): 3,470 (16/133) Q1. Food Science & Technology

Para ello, autorizan su presentación para optar al Título de Doctor con Mención Internacional, por la Universitat de València, en Ciencias de la Alimentación.

Y para que conste a los efectos oportunos,

En Burjassot, julio de 2018

Fdo: Reyes Barberá Saez   Fdo: Guadalupe García Llatas   Fdo: Antonio Cilla Tatay

Las Dras. Reyes Barberá, Guadalupe García y el Dr. Antonio Cilla, Catedrática, Profesora Titular y Profesor Ayudante Doctor, respectivamente, del Área de Nutrición y Bromatología del Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València,

INFORMAN QUE:

La graduada en Ciencia y Tecnología de los Alimentos Dña. Andrea Álvarez-Sala Martín ha contribuido:

- En el trabajo que lleva por título *"Safe intake of a plant sterol-enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage"* a partes iguales con Dña. Virginia Blanco Morales.

- En el trabajo que lleva por título "*Effects of plant sterols or β-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death"* a partes iguales con D. Gabriel López García.

En ambos artículos, Dña. Andrea Álvarez-Sala Martín ha participado en la parte experimental, evaluación de resultados y discusión, así como en la redacción de los dos artículos. Los datos de estos trabajos no han sido publicados en tesis anteriores.

En Burjassot, julio de 2018

Fdo: Reyes Barberá Saez   Fdo: Guadalupe García Llatas   Fdo: Antonio Cilla Tatay

UNIVERSITÀ DEGLI STUDI DI PALERMO

CEBAS-CSIC

En Burjassot, julio de 2018

Fdo: Reyes Barberá Saez   Fdo: Guadalupe García Llatas   Fdo: Antonio Cilla Tatay

Y al final…, ¡hasta las tesis se acaban!. Ha llegado el momento más esperado durante el proceso de escritura… escribir sus últimas líneas. Es inevitable sentir emoción al no saber por dónde empezar, pues han pasado seis años desde que empecé la aventura en el laboratorio con un trabajo final de grado entre las manos, y ahora, estoy a punto de ser ¡Doctora!. Durante estos años he crecido tanto a nivel personal como profesional y han sido muchas las personas que han contribuido a ello. No hay palabras que puedan definir mi agradecimiento, pero espero poder transmitirlo lo mejor posible y no olvidarme de nadie.

Me gustaría agradecer, en primer lugar, a mis directores por haberme brindado la oportunidad de llevar a cabo este proyecto.

A Reyes, por ser mi gran referente a lo largo de los estudios de grado y doctorado, siempre a pie de cañón. Por los "no me gusta ni mucho ni poco" que hacían mejorar cada epígrafe de mi tesis, aunque cabe recalcar que en los últimos meses la frase se transformó en "me gusta". Gracias por todos los consejos que me has dado.

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A Toni, gracias por transmitirme tu entusiasmo por el mundo de la ciencia, por tu gran eficiencia y predisposición, por creer en mi capacidad desde el primer momento y darme la oportunidad de conocer a gente maravillosa durante mis estancias en otros laboratorios, sin ello la tesis no hubiese alcanzado la totalidad de su esencia.

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mondo delle cellule. Alle mie coinquiline e a Federica, per essere come sorelle e farmi sentire a casa.

A la "élite" murciana (Juan Carlos, Antonio, Pachi, María, Lola, Ángela, Adrián…) agradeceros la amabilidad y disponibilidad durante mi estancia. Especialmente a Juan Carlos por tu gran sentido de humor y sabiduría, y a Antonio, por compartir conmigo tu amplio conocimiento sobre el mundo de las células. No cabe duda que vuestra participación ha enriquecido el trabajo realizado en mi tesis doctoral.

No me puedo olvidar de "mi morada murciana" (Alicia, Jose y Luzma), pelis, cenas, viajes, estrella de levante…y más. ¡Os espero en Valencia!

Agradecer, también, a mis amigas de toda la vida (Ana, Sara, María, Andrea y Ali) y a las nuevas amistades que lo parecen (Isa, Jorge, Arias, Guille, Iván, Ernest, Dani, Lluch, Balma, Elena…), por aguantar mis momentos de saturación, permitirme desconectar del "mundo tesis" y repetirme que, aunque la tesis es un trabajo arduo "puedes con eso y más".

A Dani, mi compañero de viaje, por aguantarme, entenderme, quererme, exprimir de mi hasta mis más imperceptibles cualidades, gracias por tu apoyo, me haces sentir valiosa y grande, sin duda has sido un pilar fundamental en este recorrido. Gracias por hacerme ver las cosas con otra perspectiva, porque la vida está llena de retos y sé que contigo soy capaz de enfrentarme a todos. Y a ti Nati, porque conforme disfrutas de la vida me haces ver que, aunque las cosas no siempre son fáciles, la satisfacción final merece la pena.

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TODO PASA, TODO QUEDA, TODO SUMA

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Abreviaturas
Abbreviations

ABCG5: "ATP-cassette binding proteins G5"; co-transportador G5 dependiente de ATP

ABCG8: "ATP-cassette binding proteins G8"; co-transportador G8 dependiente de ATP

ACAT-2: "Acetyl-coenzyme A acetyltransferase"; Acil-coenzima A colesterol aciltransferasa

ATP: "adenosine triphosphate"; adenosin trifosfato

BA: "bioaccessibility"; bioaccesibilidad

BD: "bioavailability"; biodisponibilidad

CCAH: "Scientific Committee on Food"; Comité Científico de la Alimentación Humana

β-Cx: "β-cryptoxanthin"; β-criptoxantina

c-HDL: colesterol ligado a lipoproteínas de alta densidad

c-LDL: colesterol ligado a lipoproteínas de baja densidad

COPs: "cholesterol oxidation products"; productos de oxidación del colesterol

CRP: "C-reactive protein"; proteína C reactiva

DGM: "dynamic gastric model"; modelo gástrico dinámico

EAS: "European Atherosclerosis Society"; Sociedad Europea de Aterosclerosis

EPIC: "European Prospective Investigation of Cancer"; Estudio Prospectivo Europeo sobre Dieta y Cáncer

ESPEN: "European Society for Clinical Nutrition and Metabolism"; Sociedad Europea de Nutrición Clínica y Metabolismo

EV: esteroles vegetales

FB: "bioaccessible fraction"; fracción bioaccesible

FSC: "forward-scatter"

5-FU: "5-fluorouracil"; 5-fluorouracilo

GSH: "glutathione"; glutatión

HDL: "high density lipoprotein"; lipoproteínas de alta densidad

HGS: "gastric human simulator"; simulador gástrico humano

HMGCoA: "hydroxy-methyl-glutaryl coenzyme-A reductase"; hidroxi-metil-glutaril coenzima A reductasa

IARC: "International Agency for Research on Cancer"; Agencia Internacional de Investigación del Cáncer

ICAM-1: "intercellular adhesion molecule 1"; molécula 1 de adhesión intracelular

IL: "interleukin"; interleuquina

LDL: "low density lipoprotein"; lipoproteínas de baja densidad

LXR: "liver X receptor"; receptor X del hígado

MCP-1: "monocyte chemoattractant protein-1"; proteína 1 quimiotáctica de monocitos

MFGM: "milk fat globule membrane"; membrana del glóbulo graso de la leche

MTP: "microsomal triglyceride transfer protein"; proteína microsomal de transferencia de triglicéridos

NAFLD: "nonalcoholic fatty liver disease"; enfermedad del hígado graso no alcohólico

NASH: "nonalcoholic steatohepatitis"; esteatohepatitis no alcohólica

NF-kB: "nuclear factor kappa-B cells"; factor nuclear-kB

NOEL: "no observed effect level"; nivel sin efecto observable

NPC1L1: "Niemann-Pick C1-like protein 1"

POPs: "phytosterol oxidation products"; productos de oxidación de los esteroles vegetales

PS: "plant sterols"

RONS: "reactive oxygen nitrogen species"; especies reactivas de oxígeno y nitrógeno

ROS: "reactive oxygen species"; especies reactivas de oxígeno

SHIME: "simulator of human intestinal microbial ecosystem"; simulador del ecosistema microbiano intestinal humano

SOPs: "sterol oxidation products"; productos de oxidación de esteroles SREBP: "sterol regulatory element-binding proteins"; proteína de unión de esteroles

tBOOH: *"tert*-butyl hydroperoxide"; terc-butilo hidroperóxido

TG: "trygliceride"; triglicéridos

TICE: "transintestinal cholesterol excretion"; eflujo transintestinal del colesterol

TNF-α: "tumor necrosis factor α"; factor de necrosis tumoral α

TNO: "The Netherlands Organization"

TRAIL: "TNF-related apoptosis-inducing ligand"; ligando inductor de la apoptosis relacionado con el TNF

UE: "European Union"; Unión Europea

VCAM-1: "vascular cell adhesion molecule 1"; molécula 1 de adhesión de células vasculares
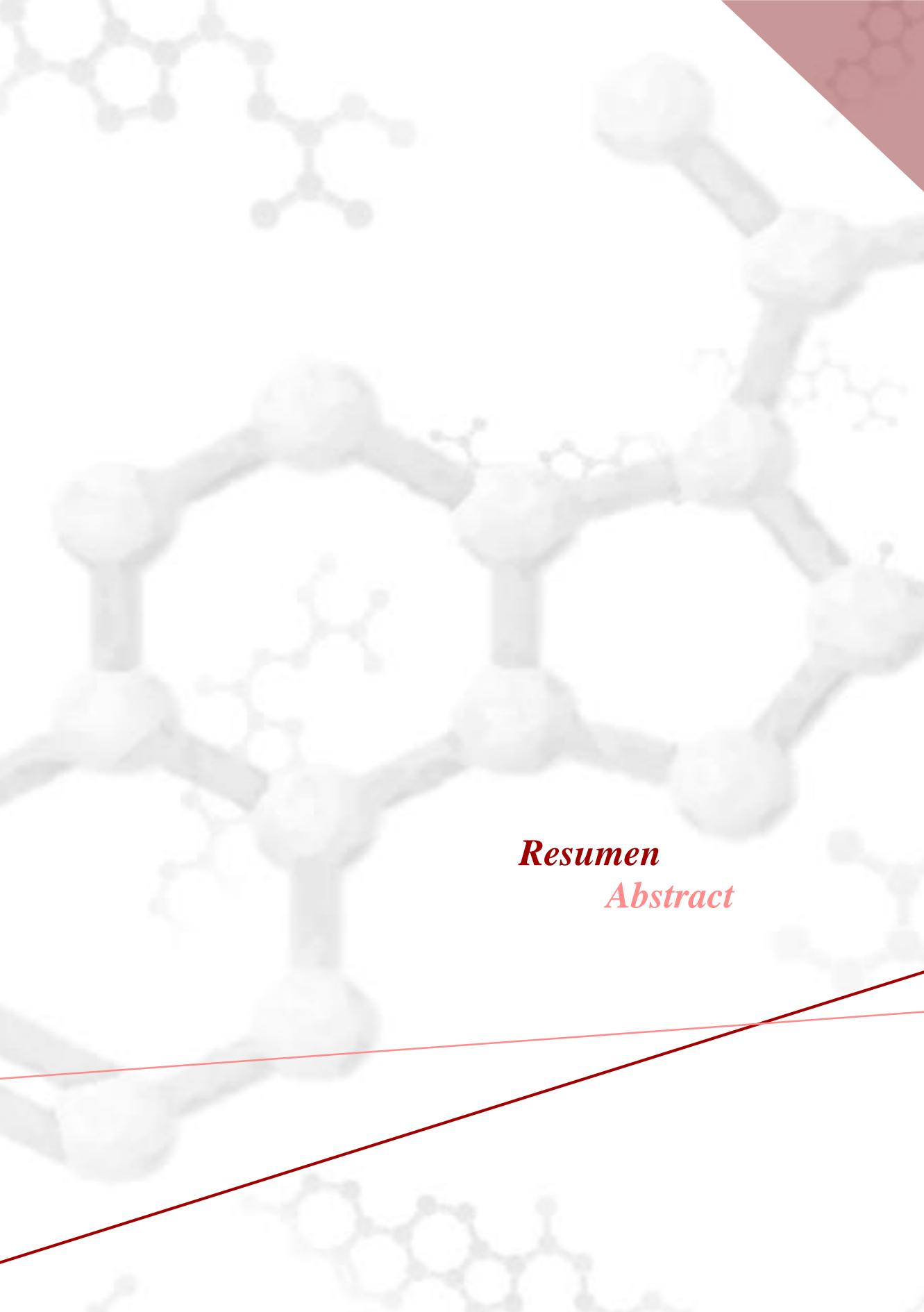
Resumen
Abstract

La Sociedad Europea de Aterosclerosis (EAS), basándose en la evidencia de la capacidad de los esteroles vegetales (EV) para disminuir los niveles de c-LDL y a la ausencia de efectos adversos, ha considerado beneficioso el consumo de 2 g EV/día, en personas con hipercolesterolemia y con riesgo cardiovascular moderado y bajo que no precisen tratamiento farmacológico, así como en personas que requieren de tratamiento farmacológico pero no alcanzan los objetivos terapéuticos o tienen intolerancia al mismo. Dado que con la dieta habitual no se alcanza dicha ingesta de EV, en la actualidad, diversos alimentos se enriquecen en EV. Las bebidas a base de zumo de frutas y leche (en las que la UE permite la adición de EV), son una matriz adecuada para obtener las recomendaciones dietéticas de EV. Además, estos productos se consideran alimentos saludables por su aporte en antioxidantes (carotenos y β-critoxantina (β-Cx), principalmente). Asimismo, aunque el interés por los EV se ha debido principalmente al efecto hipocolesterolemiante, éstos se asocian con otros efectos beneficiosos, tales como antiinflamatorio y anticarcinogénico.

El objetivo de la presente Tesis Doctoral es seleccionar y evaluar la funcionalidad de una bebida a base de zumo de frutas y leche desnatada enriquecida en EV y conteniendo β-Cx, utilizando un triple abordaje mediante estudios *in vivo*, *ex vivo* e *in vitro*.

Se estudian tres bebidas (MFb: sin adición de grasa; MFbM: con adición de grasa láctea y concentrado de proteínas de suero lácteo enriquecido con membrana de glóbulo graso de leche (MFGM); MFbO: con adición de aceite de oliva virgen extra y lecitina de soja), de las cuales, se selecciona la bebida MFbM para llevar a cabo el ensayo clínico, en base a la BA obtenida de los EV (31,4%) y a los posibles efectos beneficiosos que se le atribuye al MFGM. El almacenamiento (20-25ºC, 6 meses) de la bebida, no produce cambios ni del contenido ni de la BA de los EV y el porcentaje de oxidación de los mismos es bajo (0,022-0,023%).

La ingesta de la bebida MFbM, aportando 2 g EV/día, durante 6 semanas por mujeres postmenopáusicas, produce un efecto hipocolesterolemiante (reducción del colesterol total (2,9%) y el c-LDL (5,1%)), así como un efecto antiinflamatorio (reducción de la citoquina IL-1β proinflamatoria (6,7%) y aumento de la IL-10 antiinflamatoria (22,5%)).

Se constata que los EV, a concentraciones séricas tras la ingesta de una bebida a base de zumo de frutas y leche enriquecida en EV y conteniendo β-Cx, previenen, en eritrocitos, los efectos hemolíticos y, parcialmente, los efectos pro-eriptóticos inducidos por β-Cx con o sin estrés oxidativo.

El ingrediente fuente de EV (*tall oil*) utilizado para el enriquecimiento de la bebida MFbM y su principal EV (β-sitosterol), muestran un efecto antiproliferativo sobre células de cáncer de mama (MCF-7), colon (HTC116) y cuello uterino (HeLa). Además, en dos modelos de células de cáncer de colon (Caco-2 y HT-29), los EV pueden actuar como coadyuvantes del fármaco quimioterapéutico 5-fluorouracilo (5-FU).

Aunque son necesarios más estudios *in vivo*, los resultados obtenidos demuestran la idoneidad y funcionalidad de la bebida diseñada y evaluada con EV, β-Cx y MFGM.

Based on the evidence of the ability of plant sterols (PS) to reduce LDL-cholesterol levels and the absence of adverse effects on human health, the European Society of Atherosclerosis (EAS) recommends daily consumption of PS. EAS considers a daily dose of 2 g of PS to be beneficial for individuals with hypercholesterolemia and moderate or low cardiovascular risk who do not qualify for pharmacotherapy, as well as for individuals under pharmacologic therapy who fail to reach therapeutic targets or display an intolerance to the therapy. Given that a usual diet is unable to offer the effective dose currently, several foods have been enriched with PS. The milk-based fruit beverages (where the addition of PS is allowed in the EU) are a convenient matrix to reach the daily recommended amount of PS. Moreover, these beverages are also considered healthy foods for their contribution in antioxidants (mainly carotenoids and β-critoxanthin (β-Cx)). Likewise, although the main interest for PS is their hypocholesterolemic effect, other functions such as antiinflammatory and anticarcinogenic effects have also been attributed to PS.

The general objective of this Doctoral Thesis is to select and evaluate the functionality of a skimmed milk-based fruit beverage enriched with PS and containing β-Cx, through *in vivo*, *ex vivo* and *in vitro* studies.

Three beverages (MFb: without fat addition; MFbM: with addition of milk fat and whey proteins enriched with milk fat globule membrane (MFGM); MFbO: with extra virgin olive oil and soy lecithin addition) have been studied, of which the MFbM is selected to carry out the clinical trial, based on the bioaccessibility (BA) of the PS (31.4%), and the possible health benefits attributed to MFGM. Under storage conditions (20-25ºC, 6 months), this beverage is stable, since no modifications were observed in the content and BA of PS, and their oxidation rate is low (0.022-0.023%).

The consumption of the MFbM beverage, providing 2 g of PS, during 6 weeks by postmenopausal women, produces a hypocholesterolemic effect (reduction of total cholesterol (2.9%) and the LDL-cholesterol (5.1%)), as well as an antiinflammatory effect (reduction of proinflammatory cytokine IL-1β (6.7%) and increase of antiinflammatory IL-10 (22.5%)).

It was demonstrated that the PS, at serum concentrations obtained after the intake of PS-enriched milk-based fruit beverage containing β-Cx, prevents hemolytic and partially pro-eryptotic effects induced by β-Cx with or without oxidative stress conditions in erythrocytes.

The PS-ingredient (*tall oil*) used for enrichment of the MFbM beverage and its main PS (β-sitosterol), show an antiproliferative effect in breast (MCF-7), colon (HTC116) and cervical (HeLa) cancer cells. In addition, in two models of colon cancer cells (Caco-2 and HT-29), it is shown that PS can act as coadjuvants of the chemotherapeutic agent 5-fluorouracil (5-FU).

Although more *in vivo* studies are necessary, the results obtained demonstrate the suitability and functionality of the designed and evaluated beverage with PS, β-Cx and MFGM.
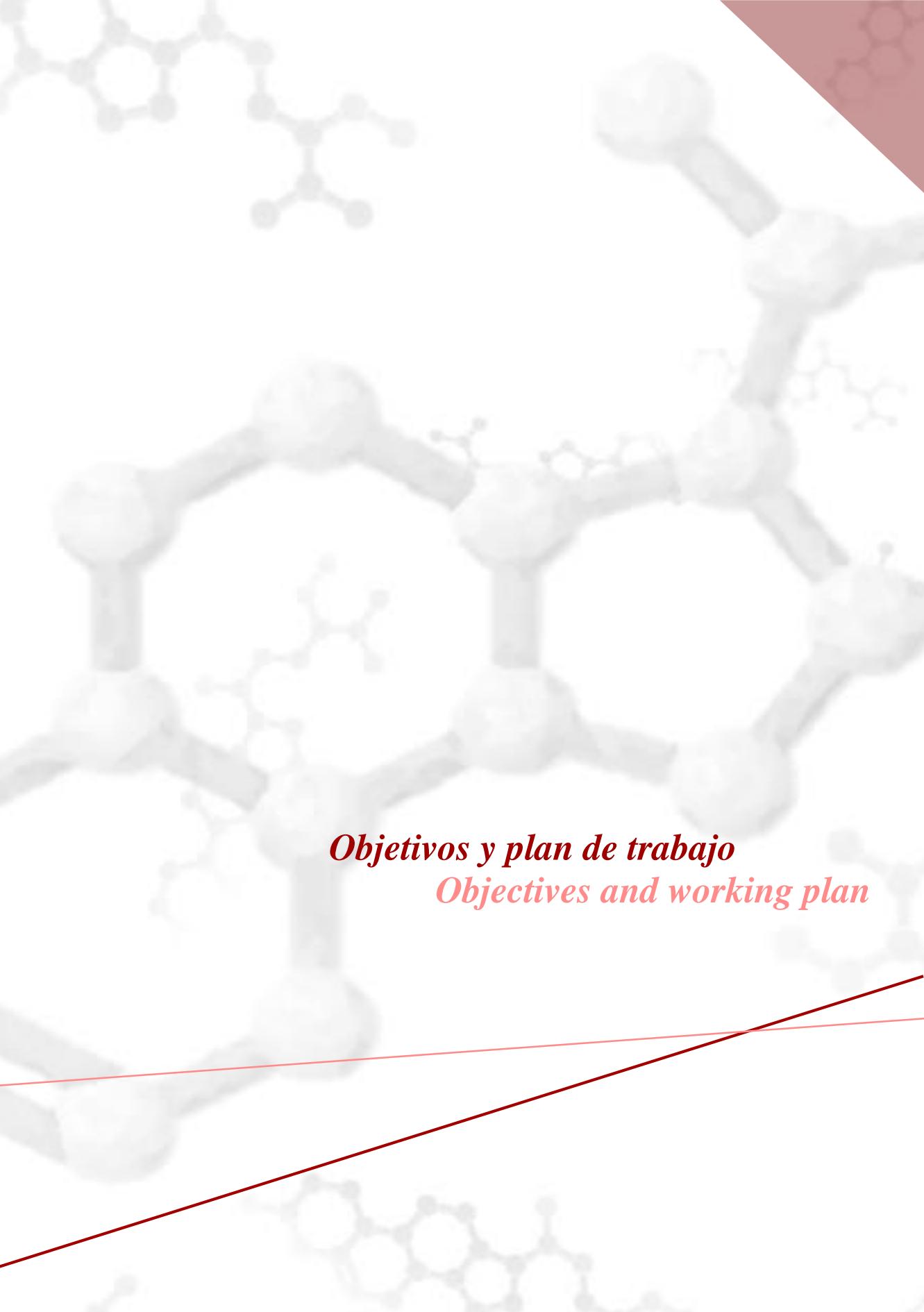
Objetivos y plan de trabajo
Objectives and working plan

El objetivo global de la presente Tesis Doctoral es seleccionar y evaluar la funcionalidad de una bebida a base de zumo de frutas y leche desnatada enriquecida en EV y conteniendo β-Cx, utilizando un triple abordaje mediante estudios *in vivo*, *ex vivo* e *in vitro*.

Los objetivos específicos son:

♦ Evaluar la influencia de cambios en la matriz alimentaria en bebidas enriquecidas en EV tales como *(i)* fuente de la fracción grasa (sin adición de grasa o con adición de grasa láctea o aceite de oliva virgen extra), *(ii)* contenido graso (1,1-2,4%) y *(iii)* presencia de emulgentes (MFGM o lecitina), sobre la BA de los esteroles. Seleccionar posteriormente la bebida más idónea para su utilización en un estudio de intervención clínica.

♦ Determinar la estabilidad y BA de los esteroles y sus productos de oxidación en la bebida seleccionada a lo largo de su vida útil (6 meses), periodo que incluye el estudio de intervención.

♦ Estudiar *in vivo* los efectos biológicos derivados del consumo regular de la bebida seleccionada, evaluando marcadores séricos de exposición de EV y sistémicos relacionados con la inflamación.

♦ Estimar *ex vivo* el efecto pro- o anti-eriptótico/hemolítico de los compuestos bioactivos (EV y β-Cx) presentes en la bebida objeto de estudio.

♦ Analizar *in vitro* el efecto antiproliferativo de los EV y evaluar su papel como coadyuvantes del fármaco 5-FU en quimioterapia frente al cáncer de colon.

The general objective of this Doctoral Thesis is to select and evaluate the functionality of a skimmed milk-based fruit beverage enriched with PS and containing β-Cx, through *in vivo*, *ex vivo* and *in vitro* studies.

The specific objectives are the following:

♦ To evaluate the influence of changes in the dietary matrix in PS-enriched beverages such as *(i)* source of the fat fraction (without fat added or with milk fat or extra virgin olive oil added), *(ii)* fat content (1.1-2.4%) and *(iii)* presence of emulsifiers (MFGM or lecithin) on sterol BA. Afterwards, to select the most suitable beverage to carry out an intervention trial.

♦ Determine the stability and BA of the sterols and their oxidation products in the selected beverage throughout its useful life (6 months, which include the period of clinical intervention).

♦ *In vivo* study of the biological effects from regular consumption of the selected beverage, evaluating serum PS exposure markers and systemic biomarkers related to inflammation.

♦ To estimate the pro- or anti-eriptotic/hemolytic effect of the bioactive compounds (PS and β-Cx) present in the beverage in an *ex vivo* model.

♦ To evaluate through *in vitro* studies, the PS antiproliferative effect and its role as coadjuvants of the 5-FU agent in the chemotherapy against colon cancer.

Para llevar a cabo los objetivos mencionados se propone el siguiente plan de trabajo:

1) Revisión de los antecedentes bibliográficos relativos a los EV y sus productos de oxidación, en relación a su biodisponibilidad y efectos biológicos.

2) Determinación del contenido de esteroles y estimación de su BA, en tres bebidas a base de zumo de frutas y leche desnatada enriquecidas en EV y conteniendo β-Cx. Selección de la bebida a utilizar en ensayos posteriores.

3) Evaluación de la estabilidad (contenido y BA) de esteroles y sus productos de oxidación, presentes en la bebida seleccionada.

4) Estimación de los efectos biológicos derivados del consumo regular, por mujeres postmenopáusicas, de la bebida seleccionada mediante un ensayo de intervención clínica: determinación sérica de EV y precursores del colesterol y de citoquinas pro- y anti-inflamatorias.

5) Análisis *ex vivo* (eritrocitos humanos) del efecto pro o anti-eriptótico/hemolítico, sin o con estrés oxidativo, de los EV y/o β-Cx presentes en la bebida seleccionada.

6) Evaluación *in vitro* (células MCF-7, HCT116 y HeLa) del efecto antiproliferativo del ingrediente fuente de EV y su principal EV (β-sitosterol), utilizados en el enriquecimiento de la bebida seleccionada, así como el posible efecto coadyuvante de los EV con el agente quimioterapéutico 5-FU en células HT-29 y Caco-2.

7) Evaluación y difusión de los resultados obtenidos y redacción de la Tesis Doctoral.

To achieve these objectives, the following work plan has been proposed:

1) Review of the background literature relating to bioavailability and biological effects of the PS and their oxides.

2) Determination of sterol content and its BA, in three PS-enriched skimmed milk-based fruit beverages containing β-Cx. Selection of the beverage for use in the subsequent studies.

3) Stability (content and BA) study of sterols and their oxidation products, present in the selected beverage.

4) Evaluation of the biological effects derived from the selected beverage consumption by postmenopausal women through a clinical intervention trial: determination of serum PS, cholesterol precursors and pro- and anti-inflammatory cytokines.

5) *Ex vivo* analysis (human erythrocytes) of the pro or anti-eriptotic/hemolytic effect, with or without oxidative stress, of the PS and/or β-Cx present in the selected beverage.

6) *In vitro* evaluation (MCF-7, HCT116 and HeLa cells) of the antiproliferative effect of a PS-ingredient and its main PS (β-sitosterol), used in the enrichment of the selected beverage, as well as the possible coadjuvant effect of the PS with the chemotherapeutic 5-FU agent in HT-29 and Caco-2 cells.

7) Evaluation of the results obtained and drafting of the Doctoral Thesis.
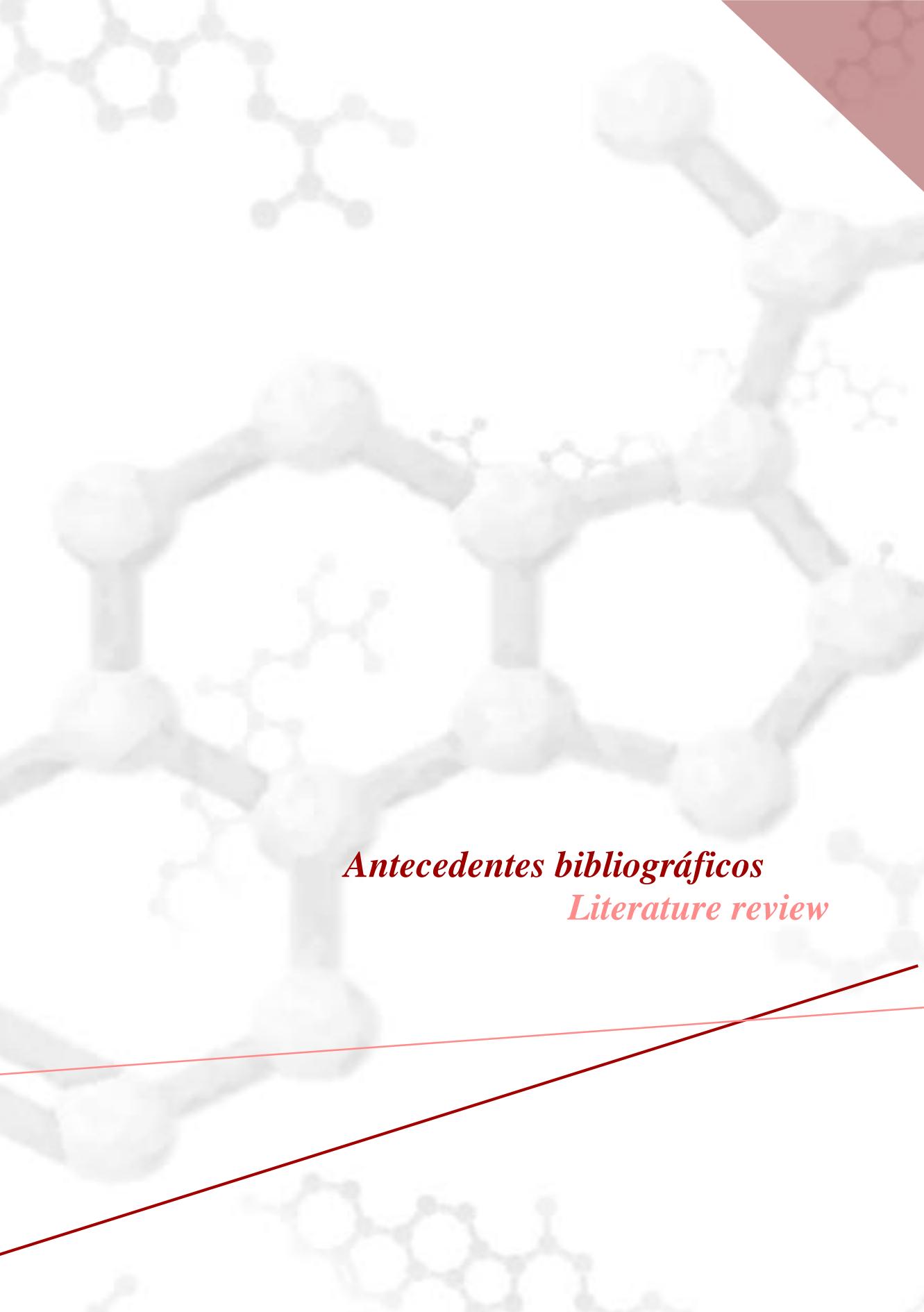
Antecedentes bibliográficos
Literature review

1.- Esteroles vegetales

1.1.- Concepto y estructura

Los esteroles son componentes lipídicos que forman parte de la fracción insaponificable. La base estructural de los esteroles se compone de un núcleo tetracíclico (ciclopentanoperhidrofenantreno) con un grupo hidroxilo en el carbono 3 (C-3) y una cadena lateral con 8-10 carbonos en posición C-17. Según su origen, los esteroles se clasifican en esteroles animales, siendo el colesterol el más predominante, y en vegetales (EV). Los EV, a su vez, se subdividen en fitoesteroles y fitoestanoles, de acuerdo con la presencia o ausencia de un doble enlace en la posición C-5, respectivamente (Figura 1). Los fitoesteroles pueden convertirse en fitoestanoles por hidrogenación química (Garcia-Llatas y Rodríguez-Estrada, 2011; López et al., 2016). Más de 200 tipos de EV se encuentran en la naturaleza, siendo los mayoritarios los fitoesteroles (95-98%), como el β-sitosterol, campesterol y estigmasterol, y los fitoestanoles (sitostanol y campestanol). Ambos tipos de EV se hallan en forma libre, o como conjugados, en los que el grupo 3β-hidroxilo está esterificado a un ácido graso o un ácido hidroxicinámico, o glicosilado con una hexosa (generalmente glucosa) o una acil-6 hexosa esterificada (Lagarda et al., 2006; Moreau et al., 2018).

Estructuralmente, los EV son muy similares al colesterol (Figura 1), pero a diferencia de éste, incluyen en la cadena hidrocarbonada lateral, un grupo alquilo (metilo o etilo) en posición C-24, y con menos frecuencia una insaturación en el C-22. La mayoría de las cadenas laterales de los EV, contienen 9-10 átomos de carbono, en lugar de 8 como en el colesterol (Lagarda et al., 2006; García-Llatas y Rodríguez-Estrada, 2011).
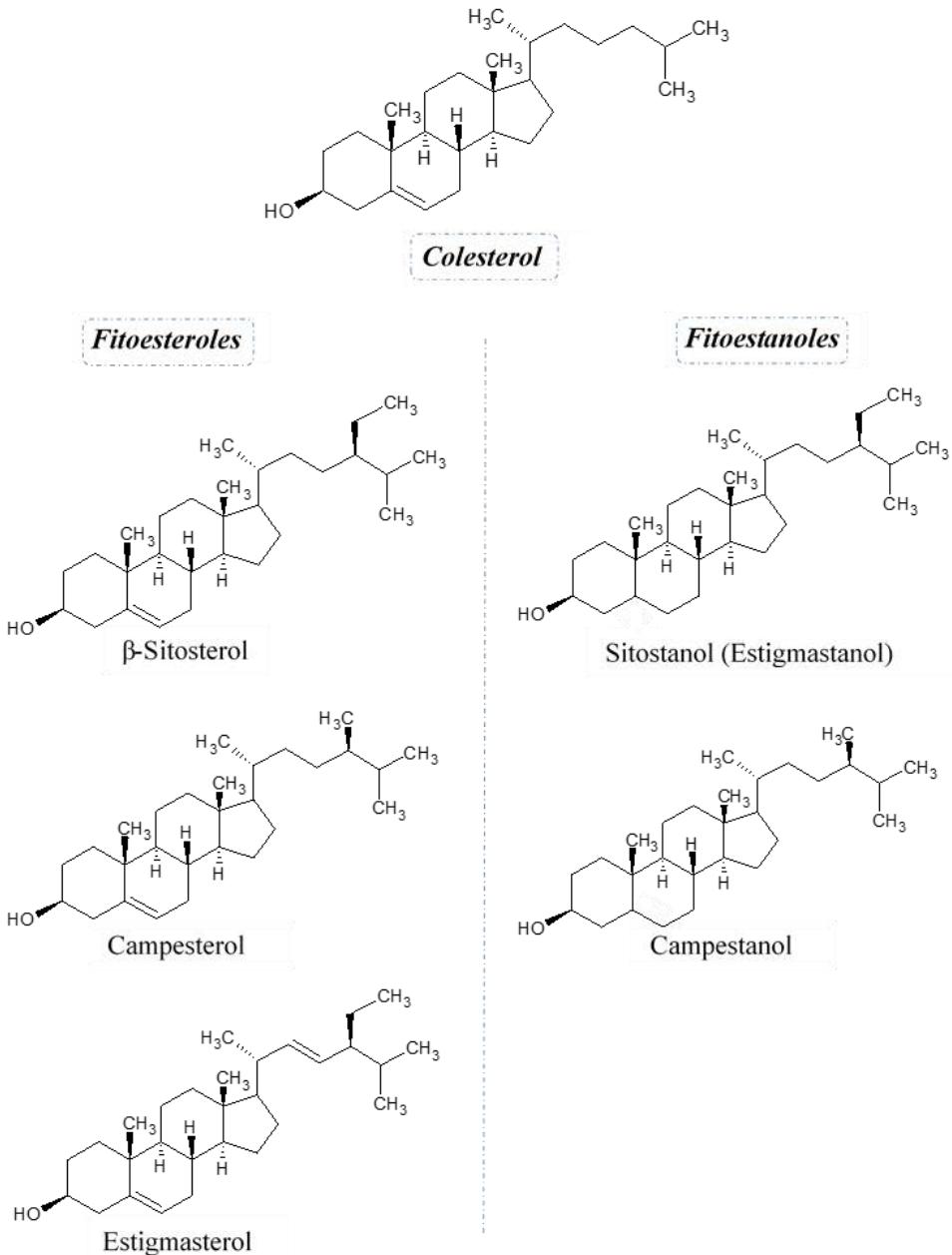
Figura 1. Estructuras de los principales esteroles

1.2.- Fuentes naturales e ingesta dietética

Los EV son aportados exclusivamente a través de la dieta. Las fuentes naturales más ricas en EV son los aceites vegetales y derivados, en particular los aceites no refinados. En general, durante el proceso de refinado de los aceites disminuye el

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contenido de EV totales (10-70%) (Ellegärd et al., 2007). Los frutos secos, semillas, cereales, frutas y legumbres son también buenas fuentes de EV. En la Tabla 1 se muestran los contenidos de EV (mg/100g) en diferentes alimentos. Los aceites son los más abundantes en EV (39-1190) seguidos por los frutos secos y semillas (22-714), cereales (22-178) y legumbres (22-161). En general, las frutas (1-75) y las hortalizas (2-49) son los grupos de alimentos que menos EV contienen.

Se ha estimado que el contenido de EV en los alimentos tiene una gran variabilidad genética dentro de una misma especie, y que las condiciones ambientales pueden modificar el contenido de los mismos; duplicándose en condiciones de sequía y altas temperaturas (Silva et al., 2016).

En general, los EV más abundantes en la dieta son los fitoesteroles, constituyendo el 95% del total de EV, siendo el mayoritario el β-sitosterol (79,7%), seguido de campesterol (9,5%) y estigmasterol (6,8%) (Jiménez-Escrig et al., 2006). Los fitoestanoles están presentes en pequeñas cantidades en los alimentos, principalmente en cereales (trigo y arroz). Los más comunes en la dieta son el sitostanol y el campestanol, los cuales en conjunto representan un 5% del total de EV ingeridos (Andersson et al., 2004).

Tabla 1. Contenido de esteroles vegetales (mg/100 g) en alimentos

Alimentos *Aceites y grasas*	Contenido	Alimentos *Frutos secos y semillas*	Contenido
Algodón[2,7]	324-327	Almendra[1-3,7-9,11,12]	114-199
Amaranto[13]	637-715	Anacardo[1,2,7-9]	135-158
Amapola[12]	276	Avellana[1,3,9,12]	116-138
Cacahuete[6-9,12]	207-353	Cacahuete[1-3,7-9,11]	104-220
Cártamo[12]	444	Castaña[14]	22
Coco[2,9]	91-97	Nuez[2,3,8,9]	127-131
Colza[2,6,8,9]	668-704	Nuez de Brasil[1,9]	95-111
Germen de trigo[2,8,9,12]	553-919	Nuez de macadamia[1,12]	187-116
Girasol[3-6,8,9,12]	100-493	Piñón[1]	147
Lino[2,9]	338-387	Pipas[2,3,6,8,9,12]	227-534
Maíz[2,5-9]	763-968	Pistacho[1-3,8,9,12]	214-279
Germen de maíz[9]	799	Sésamo[1,2,7-9,12]	360-714
Oliva refinado[2,3,5-9]	154-236	Colza[6]	307
Oliva virgen[3]	260	Lino[9]	187
Palma[2,8,9]	39	*Cereales*	
Soja[2,3,6,8,9,12]	250-335	Amaranto[12,13]	22-178
Avellana[6,12]	145-120	Arroz[2,3,8,12]	28-30
Sésamo[7,9]	472-865	Arroz (harina)[2,8,10]	23
Nuez[9,12]	162-176	Avena[4,5,10]	34-52
Salvado de arroz[2,7,12]	1055-1190	Cebada[4,15]	50-83
Avena[9]	525	Centeno[2,4,6,8,10,12,13]	69-110
Almendra[12]	266	Centeno (harina)[2,8,10]	86
Margarina[2,8,5,9,12]	92-721	Cuscús[10]	58
Mantequilla[5]	153	Maíz[4,6,13]	44-178
Mantequilla de cacahuete[9]	140-182	Maíz (harina)[2,8,10]	52
Grasa de coco[9]	56	Trigo[2,3,4,6,8]	41-69
Chocolate negro[9]	122	Trigo (germen)[1,2,6,8,10]	344-413
Chocolate con leche[9]	91	Trigo (harina)[2,5,8,10]	28-29
Lino[9]	187	Trigo (salvado)[2,5,6,8,10]	178-200
Legumbres			
Frijoles[2,7]	127		
Garbanzo[3,7,12]	35-121		
Guisante[2,5,11,12]	22-135		
Habas[2,3]	108-124		
Lentejas[3,12]	57-117		
Soja[7,12]	50-161		
Alubias[12]	76		

Tabla 1 (continuación). Contenido de esteroles vegetales (mg/100 g) en alimentos

Alimentos	Contenido	Alimentos	Contenido
Frutas		*Hortalizas*	
Aceitunas negras[2,8]	50	Acelga[3]	17
Aceitunas verdes[2,3,8]	35-38	Alcarchofas[3]	49
Albaricoque[3,12]	15-18	Apio[2,3]	8-17
Banana[2,3,7,11,12]	12-20	Batata (boniato)[2]	10
Cereza[2,3]	12-20	Berenjena[3,12]	6-7
Ciruela[3,11,12]	7-19	Brócoli[2-5,12]	18-44
Fresa[3,12]	11-12	Calabacín[3]	2
Higo[2,7,8,12]	22-31	Calabaza[12]	12
Kiwi[2,3,8,11]	7-18	Cebolla[2,3,7,8,11,12]	7-15
Limon[2,3,8]	3-18	Champiñón[2,8]	18
Mandarina[2,3,8]	16-22	Coles de Bruselas[2,4,8,11,12]	24-43
Naranja[12]	24	Coliflor[2,3,4,8,12]	
Manzana[2-5,7,8,11,12]	12-18	Endivia[3]	17
Melocoton[2,3,8,12]	10-15	Escarola[3]	18
Melon[2,3,8,12]	2-10	Esparrago[3,12]	11-24
Naranja[2-4,8,11,12]	23-30	Espinacas[3,8,12]	9-16
Níspero[12]	2	Hinojo[2,8]	10
Pera[2,3,7,8,12]	8-12	Judía verde[3]	19
Persimon[12]	4	Lechuga[3,7,11,12]	9-38
Piña[2,8,12]	6-17	Patata[2-4,7,8,11,12]	4-5
Pomelo[2,8,12]	17-18	Pepino[3,5,12]	6-14
Sandía[2,3,8,12]	1-5	Perejil[3]	7
Uva blanca[3,11,12]	17-33	Pimiento rojo[3]	7
Uva roja[12]	4	Pimiento verde[2,3,8,9,12]	7-9
Aguacate[11]	75	Puerro[2,3,8]	8-12
		Tomate[2,3,5,7,8,11,12]	5-10
		Zanahoria[2-4,7,8,11]	12-19

1: Phillips et al. (2005); 2: Bacchetti et al. (2011); 3: Jiménez-Escrig et al. (2006); 4: Piironen et al. (2000); 5: Ellegärd et al. (2007); 6: Plumb et al. (2011); 7: Abidi (2001); 8: Marangoni y Poli (2010); 9: Normén et al. (2007); 10: Normén et al. (2002); 11: Piironen et al. (2003); 12: https://ndb.nal.usda.gov

La contribución de los distintos alimentos a la dieta es un factor importante en la ingesta total de EV. La ingesta diaria promedio de EV (mg/día) a la dieta de los estadounidenses se estima que es de 154-363 (De Vries et al., 1997; Racette et al., 2015; Jaceldo-Siegl et al., 2017), 258-474 en China (Wang et al., 2018), 237-305 en Finlandia (Valsta et al., 2004), 215-272 en Croacia (De Vries et al., 1997), 208-373 en Japón (Hirai et al., 1986, De Vries et al., 1997), 400 en México (Cerqueira et al., 1979), 146-307 en Países Bajos (De Vries et al., 1997; Normén et al., 2001), 179-

220 en Italia (De Vries et al., 1997), 91-186 en Gran Bretaña (Morton et al., 1995) y 219-257 en Suecia (Klingberg et al., 2013). Recientemente, López et al. (2016) indican que la ingesta media diaria de fitoesteroles a partir de la dieta habitual en la sociedad occidental, varía de 167 a 437 mg, llegando a alcanzar con dietas vegetarianas los 600 mg/día. La cantidad de fitoestanoles que se ingieren es inferior, del orden de 20 a 50 mg/día.

En España, según Jiménez-Escrig et al. (2006), la ingesta media diaria de EV es de 276 mg, siendo la contribución relativa de los distintos grupos de alimentos a la ingesta total de EV, del 39% para los aceites, 30% cereales, 12% frutas, 9% legumbres, 7% hortalizas y alrededor del 2,5% frutos secos. Concretamente, los principales alimentos consumidos (mg/día) son el aceite de girasol (52,7), aceite de oliva refinado (35,2), naranja (12,7), garbanzos (8,9), aceite de oliva virgen (8,1), lentejas (8,0), alubias (7,0), patata (5,0), arroz (4,7), manzana (4,4) y tomate (4,2). Además, en un estudio prospectivo europeo sobre dieta y cáncer (EPIC) que incluye un análisis transversal de cohorte en población española (Asturias, Navarra, Gipuzkoa, Murcia y Granada), se estima que la ingesta de EV en hombres es superior que en mujeres (309-349 y 241-259 mg/día, respectivamente). La ingesta de EV está directamente relacionada con la ingesta energética, siendo ésta superior en hombres, e inversamente proporcional con la edad (Escurriol et al., 2009). En particular en la Comunidad Valenciana, se constata que durante los años 2005 y 2013-2014 la ingesta de EV es de 282 y 274 mg/día respectivamente, siendo acorde a la ingesta media previamente mencionada para España. En cuanto a la contribución relativa de los distintos grupos de alimentos a la ingesta total de EV, en ambos años, predominan las legumbres (30-35%) seguido de las verduras y hortalizas (20-19%). Según el año estudiado, como tercer o cuarto contribuyente están los aceites y grasas (7-16%) o las frutas (11-18%), y les siguen los cereales (7-10%). Los autores indican que la contribución de EV a la dieta puede estar influenciada por diversos parámetros como el género, las estaciones y la región geográfica, entre otros (Garcia-Llatas et al., 2015a).

1.3.- Alimentos enriquecidos

1.3.1.- Legislación

Dado que con la dieta habitual no se alcanza la dosis recomendada de EV (1,5-2 g/día) para lograr el efecto hipocolesterolemiante (Gylling et al., 2014), algunos alimentos se enriquecen en dichos compuestos.

La comercialización en la Unión Europa (UE) de alimentos enriquecidos en EV, se lleva a cabo en base al Reglamento del 2015/2283 sobre nuevos alimentos o ingredientes alimentarios. En la Tabla 2 se muestran los alimentos enriquecidos con EV y permitidos para su comercialización en la UE. Todos los reglamentos que se citan en dicha tabla establecen porcentajes máximos de EV permitidos para el enriquecimiento de los distintos alimentos: β-sitosterol < 80%, sitostanol < 15%, campesterol < 40%, campestanol < 5%, estigmasterol < 30%, brasicasterol < 3%, otros fitoesteroles y fitoestanoles < 3%. Además, especifican que los productos se presentarán de forma que puedan dividirse fácilmente en porciones que contengan 3 g como máximo (en el caso de una porción diaria), o bien 1 g como máximo (en el caso de tres porciones diarias) de fitoesteroles o fitoestanoles añadidos. Como excepción, en la Decisión 2000/500/CE relativo a las grasas amarillas para untar, se establece un contenido máximo de 8% (p/p) de fitoesterol, equivalente a 14% (p/p) de ésteres de fitoesterol, y se especifica que la composición de los fitoesteroles sea: campesterol 10-40%, estigmasterol 6-30%, β-sitosterol 30-65% y otros 0-5%.

Tabla 2. Legislación relativa a la comercialización de alimentos enriquecidos en esteroles vegetales

Alimento	Forma de química de EV	Decisión
- Grasas amarillas para untar	Ésteres de fitoesterol	2000/500/CE
- Grasas amarillas para untar - Aliños para ensaladas que contengan mayonesa - Bebidas tipo leche (semidesnatada y desnatada), bebidas de frutas, cereales o soja, y productos tipo leche fermentada (yogur y queso)	Fitoesteroles o fitoestanoles	2004/333/CE
- Grasas amarillas para untar - Productos tipo leche (semidesnatada y desnatada) y yogur - Salsas aromáticas	Fitoesteroles o fitoestanoles	2004/334/CE
- Productos tipo leche (semidesnatada y desnatada), tipo yogur y tipo leche/yogur.	Ésteres de fitoesterol	2004/335/CE
- Grasas amarillas para untar - *Bebidas de fruta a base de leche*, productos tipo yogur y queso.	Fitoesteroles o fitoestanoles	2004/336/CE
- Bebidas a base de leche (semidesnatada y desnatada)	Fitoesteroles o fitoestanoles	2004/845/CE
- Pan de centeno con harina que contiene: ≥ 50 % de centeno (harina integral de centeno, granos de centeno enteros o fragmentados y copos de centeno) y ≤ 30 % de trigo; con ≤ 4 % de azúcar añadido pero sin grasa añadida	Fitoesteroles o fitoestanoles	2006/58/CE y 2006/59/CE
- Aceite para su uso en alimentos como: Grasas para untar; Productos lácteos, como los productos a base de leche semidesnatada y leche desnatada, con posible adición de frutas o cereales, productos a base de leche fermentada (yogur y queso); Bebidas de soja; Salsas aromáticas y aliños de ensalada, incluida la mayonesa	Fitoesteroles o fitoestanoles	2007/343/CE
- Bebidas de arroz	Fitoesteroles o fitoestanoles	2008/36/CE

EV: Esteroles vegetales; CE: Comisión Europea

El etiquetado de alimentos e ingredientes alimentarios con fitoesteroles, ésteres de fitoesterol, fitoestanoles y/o ésteres de fitoestanoles añadidos se regula en la UE por el Reglamento 1169/2011. En dicho reglamento se detalla que las etiquetas de los alimentos, deben incluir: "1) «Con esteroles vegetales añadidos» o «Con estanoles vegetales añadidos» en el mismo campo visual que la denominación del alimento; 2) En la lista de ingredientes se indicará el contenido de fitosteroles, ésteres de fitosterol, fitostanoles y ésteres de fitostanol añadidos (expresado en % o en g de esteroles o estanoles vegetales libres por 100 g o 100 mL de alimento); 3) Se indicará que el alimento está destinado exclusivamente a las personas que desean reducir su nivel de colesterol en la sangre; 4) Se indicará que los pacientes que toman medicación para reducir su colesterolemia sólo deben consumir el producto bajo control médico; 5) Se indicará de forma fácilmente visible que el alimento puede no ser nutricionalmente adecuado para las mujeres embarazadas o que amamantan y los niños menores de cinco años; 6) Se aconsejará el alimento como parte de una dieta equilibrada y variada, que incluya un consumo regular de fruta y verdura para ayudar a mantener los niveles de carotenoides; 7) En el mismo campo de visión que la declaración contemplada en el punto 3, se indicará que debe evitarse un consumo superior a 3 g/día de esteroles o estanoles vegetales añadidos; 8) Una definición de una porción del alimento o del ingrediente alimentario en cuestión (preferentemente en g o en mL), que precise la cantidad de esteroles o estanoles vegetales que contiene cada porción".

Los EV pueden tener declaraciones de propiedades saludables. En el Reglamento 1924/2006/CE las declaraciones de propiedades saludables se definen como cualquier declaración que afirme, sugiera o dé a entender que existe una relación entre una categoría de alimentos, un alimento o uno de sus constituyentes, y la salud. Existen distintos tipos de declaraciones saludables: 1) relativas a la reducción del riesgo de enfermedad; 2) relativas al desarrollo y salud de los niños; 3) distintas de las relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños.

En base al citado reglamento, existen declaraciones de propiedades saludables autorizadas por la UE referentes a los EV y relativas a la reducción de un factor de

riesgo de enfermedad (983/2009/CE; 376/2010/UE; 384/2010/UE). Las condiciones de uso relativas a la información del consumidor sobre la magnitud del efecto de reducción del colesterol son modificadas por el Reglamento (UE) 686/2014. Por otro lado, existen declaraciones de propiedades saludables, referentes a los EV, distintas de las relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños (432/2012/UE). En la Tabla 3, se muestran las declaraciones de propiedades saludables autorizadas sobre los EV.

De acuerdo con diversas Decisiones de la Comisión Europea, el Comité Científico de la Alimentación Humana (CCAH), en el informe general sobre los efectos a largo plazo de la ingesta de elevadas cantidades de fitoesteroles procedentes de diversas fuentes alimentarias, con especial atención a los efectos sobre los niveles de β-caroteno, indica que no existen pruebas de que ingestas superiores a 3 g/día de EV, produzcan beneficios adicionales y que, puesto que una ingesta elevada puede producir efectos adversos, es prudente evitar las ingestas de EV superiores a 3 g/día (2006/59/CE).

El Panel de Consenso de la Sociedad Europea de Aterosclerosis (EAS), basándose en la evidencia sobre la capacidad de los EV para disminuir tanto los niveles del colesterol ligado a lipoproteínas de baja densidad (c-LDL) como en la ausencia de efectos adversos, ha considerado beneficioso el consumo de alimentos enriquecidos con EV (2 g/día) junto con un de estilo de vida saludable, en personas con hipercolesterolemia o con riesgo cardiovascular moderado o bajo que no precisen tratamiento farmacológico, así como en pacientes que reciben tratamiento farmacológico pero no alcanzan los objetivos terapéuticos de c-LDL o tienen intolerancia a las estatinas u otros compuestos de similar acción. También se recomienda en adultos o niños (> 6 años) que sufren hipercolesterolemia familiar (Gylling et al., 2014).

Tabla 3. Declaraciones de propiedades saludables autorizadas sobre los esteroles vegetales

Declaraciones de propiedades saludables	Nutriente, sustancia, alimento o categoría de alimentos	Declaración	Condiciones o restricciones de uso del alimento
Relativas a la reducción del riesgo de enfermedad[1]	Fitoesteroles/ ésteres de fitoestanol	Se ha demostrado que los fitoesteroles y los ésteres de fitoestanol disminuyen/reducen el colesterol sanguíneo. Una tasa elevada de colesterol constituye un factor de riesgo en el desarrollo de cardiopatías coronarias.	«Debe informarse al consumidor de que el efecto beneficioso se obtiene con una ingesta diaria de 1,5 a 3 g de fitoesteroles/fitoestanoles. Sólo podrá hacerse referencia a la magnitud del efecto para los alimentos incluidos en las siguientes categorías: grasas amarillas para untar, productos lácteos, mayonesa y aliños para ensaladas. Cuando se haga referencia a la magnitud del efecto, deberá comunicarse al consumidor la variación "del 7 % al 10 %" para los alimentos que aporten una ingesta diaria de 1,5 a 2,4 g de fitoesteroles/fitoestanoles o la variación "del 10 % al 12,5 %" para los alimentos que aporten una ingesta diaria de 2,5 a 3 g de fitoesteroles/fitoestanoles, así como el período a partir del cual se obtiene el efecto: "de dos a tres semanas".»
Distintas de las relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños[2]	Fitoesteroles y fitoestanoles	Los fitosteroles y los fitostanoles contribuyen a mantener niveles normales de colesterol sanguíneo.	«Para que un producto pueda llevar esta declaración, se informará al consumidor de que el efecto beneficioso se obtiene con una ingesta diaria mínima de 0,8 g de fitosteroles o fitostanoles.»

1: Reglamento 686/2014/UE; 2: Reglamento 432/2012/UE

1.3.2.- Fuentes de esteroles vegetales para el enriquecimiento de alimentos

Una de las principales fuentes de EV para el enriquecimiento de alimentos son los aceites vegetales (soja, colza, girasol y maíz, entre otros). Para la obtención de EV procedentes de aceites vegetales, los aceites crudos sufren un proceso de refinado que incluye: desgomado (principalmente para eliminar los fosfolípidos), refinado (para eliminar ácidos grasos libres), blanqueo (para eliminar pigmentos), y desodorización (destinada a eliminar pesticidas y compuestos volátiles que causan olores y sabores desagradables, pero también se pierden algunos esteroles y tocoferoles vegetales). Los contenidos de fitoesteroles libres derivados del refinado de aceites vegetales oscilan entre 5-20%, y los esterificados entre 1-6%. En cuanto a los fitoestanoles, contienen una proporción de sitostanol/campestanol de aproximadamente 68:32 (Moreau, 2015). Las pérdidas de EV que pueden producirse durante la etapa de desodorización, pueden recuperarse de los subproductos del destilado. El método más eficiente para la extracción de EV es el tratamiento de los aceites vegetales con vapor y a vacío, obteniéndose un subproducto conocido como "destilado desodorizado". El 10-30% de este subproducto lo compone la fracción insaponificable, de la cual el 40% son EV. El proceso de extracción consta de tres etapas: hidrólisis o transesterificación de los EV esterificados con ácidos grasos para obtener EV libres; esterificación de los ácidos grasos libres con un alcohol, normalmente metanol, dando lugar a ésteres metílicos más volátiles; y, finalmente, recuperación de los EV mediante extracción física (por cristalización de los EV), extracción química (con disolventes orgánicos) o extracción físico-química (mediante la formación de aductos) (Rozner y Garti, 2006; García-Llatas y Rodríguez-Estrada, 2011).

El *tall oil*, subproducto de la pulpa de madera procedente del pino y otros árboles, es considerado otra fuente importante para la extracción de EV. El proceso de aislamiento de EV, generalmente, es similar al aplicado en aceites vegetales. Los fitoesteroles procedentes de *tall oil* se refinan y purifican, y posteriormente pueden ser esterificados con ácidos grasos y/o convertidos a fitoestanoles mediante hidrogenación química. Los contenidos de fitoesteroles varían entre 10-35%, siendo el β-sitosterol el mayoritario. A su vez, los fitoestanoles varían entre 1-4% y

contienen principalmente sitostanol y campestanol en una proporción de aproximadamente 92:8 (García-Llatas et al., 2011; Moreau, 2015).

Además del origen, los ingredientes fuente de EV pueden presentarse en diversas formas físicas (emulsionados, en polvo o como pasta oleosa) o químicas (esterificados o libres). En este sentido, González-Larena et al. (2011) tras estudiar 8 ingredientes utilizados para el enriquecimiento de alimentos con EV, ponen de manifiesto que la proporción entre los EV varía dependiendo del origen del ingrediente (*tall oil* o aceites vegetales). En general, en todos los ingredientes analizados, el β-sitosterol es el EV más abundante, seguido del campesterol y el estigmasterol. En ingredientes, cuya composición es exclusivamente aceite de soja, el contenido en estigmasterol es más de 10 veces superior que en los demás ingredientes. Sin embargo, los ingredientes que provienen de *tall oil*, o está presente en su composición, generalmente tienen menores contenidos de estigmasterol y mayores de sitostanol (Tabla 4).

Tabla 4. Contenido de esteroles vegetales en ingredientes (González-Larena et al., 2011)

Origen	Campesterol	Campestanol	Estigmasterol	β-sitosterol	Sitostanol	Formato
Soja	26,43	0,65	29,55	40,19	1,41	Emulsión líquida
Soja	25,03	0,76	26,26	44,03	2,39	En polvo
Tall oil	8,98	1,22	0,74	76,25	12,75	Emulsión líquida
Tall oil	7,13	1,20	0,82	78,86	11,95	En polvo
Mezcla[*#]	17,19	0,89	1,37	69,31	8,83	Polvo seco pulverizado
Mezcla[*]	12,58	1,04	1,08	73,50	9,81	Polvo seco pulverizado
Girasol +*tall oil*	7,28	1,15	0,86	79,66	11,04	Emulsión líquida
Girasol +*tall oil*	7,30	1,15	0,85	79,25	11,42	Pasta oleosa

Expresado como g/100 g EV. [*]a base de aceite de soja, colza, girasol y maíz; [#]Esteroles vegetales esterificados

1.4.- *Biosdisponibilidad (absorción y metabolismo)*

Para evaluar la funcionalidad de los EV hay que considerar su bioaccesibilidad (BA) y bioactividad. La BA se define como la fracción de un compuesto que se libera de la matriz en la que se encuentra presente en el tracto gastrointestinal, quedando disponible para su absorción intestinal e incorporación al torrente sanguíneo. La BA incluye todos los procesos que tienen lugar durante la digestión del alimento, absorción/asimilación a través de las células epiteliales y metabolismo presistémico. Por su parte, la bioactividad contempla todos los procesos por los que el nutriente o compuesto bioactivo es transportado hasta alcanzar el tejido diana, cómo interactúa con otras biomoléculas, el metabolismo o biotransformación que puede sufrir, la generación de un marcador biológico y la respuesta fisiológica que causa (Férnandez-García et al., 2009; Alegría et al., 2015). Ambos conceptos (BA y bioactividad) se engloban en el término biodisponibilidad (BD). La BD es clave para evaluar la funcionalidad de componentes alimentarios. Varios factores pueden influir en la BD: factores fisiológicos (intrínsecos al organismo) y dietéticos (extrínsecos) (Férnandez-García et al., 2009). En el caso de la BD de los EV, entre los factores dietéticos puede influir el tipo de EV (β-sitosterol, sitostanol, campesterol, campestanol y estigmasterol, entre otros), la forma en que se presenten (libres o conjugados), la fuente de la que procedan (aceites vegetales o *tall oil*), procesado del alimento, matriz alimentaria e interacción con otros componentes del alimento o la dieta (Racette et al., 2015). Los principales métodos para evaluar la BD de compuestos bioactivos son los métodos *in vitro* e *in vivo* (Cardoso et al., 2015) (Figura 2).
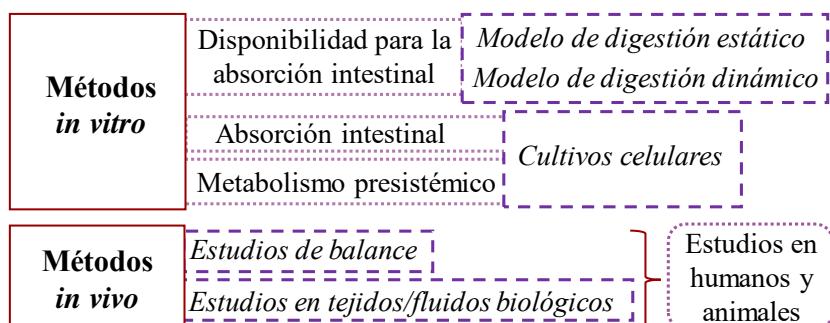
Figura 2. Principales métodos de determinación de la biodisponibilidad

1.4.1.- Métodos in vitro

Los ensayos *in vitro* simulan las condiciones fisicoquímicas del tracto gastrointestinal y evalúan las transformaciones en el lumen y las interacciones con los componentes de los alimentos. Se caracterizan por su rapidez, relativo bajo coste y el mejor control de las variables experimentales. A su vez, proporcionan estimaciones relativas de la BD por lo que pueden resultar predictivos de la situación *in vivo* (Cilla et al., 2017a).

Los modelos *in vitro* de simulación gastrointestinal para evaluar la BA incluyen modelos estáticos o dinámicos (Figura 2). Los modelos estáticos son los más ampliamente utilizados y consisten en un tratamiento secuencial enzimático simulado en dos o tres etapas (oral, gástrica e intestinal). De forma general, para adultos, en la etapa salivar se utiliza α-amilasa a pH 6,5-7,5 (de segundos a minutos), la etapa gástrica se lleva a cabo con HCl y/o pepsina a pH 1-2 (1-3 h) y la intestinal mediante pancreatina o enzimas individuales con o sin sales biliares como surfactantes a pH 6,5-7,5 (1-5 h) (Alegría et al., 2015; Cilla et al., 2017a). Concretamente, para compuestos lipofílicos (como los EV), es usual la formación de liposomas y fases micelares en el duodeno, por lo tanto, la adición de lipasa humana pancreática y otras enzimas específicas (colesterol esterasa, fosfolipasa A2 y colipasa, entre otras) se suelen utilizar para asemejar mejor una situación *in vivo*. Estos compuestos lipofílicos forman parte de las micelas hasta que son absorbidos por los enterocitos. Posteriormente, tras la digestión gastrointestinal simulada, se obtiene la fracción soluble (por centrifugación) denominada fracción bioaccesible (FB), que es la máxima fracción del componente ingerido disponible para ser absorbido, o la fracción dializada, que es la fracción soluble cuyos componentes tienen un tamaño molecular del tamaño de poro de la membrana utilizada (Cilla et al., 2017a). En el contexto de un consenso internacional (COST Action Infogest), se ha armonizado un método estático de digestión *in vitro* basado en las condiciones fisiológicas *in vivo* de los humanos (Minekus et al., 2014), validado posteriormente en un amplio estudio interlaboratorio (Egger et al., 2016). Recientemente, en base al protocolo armonizado de Infogest se han llevado a cabo propuestas para estandarizar modelos de digestión *in vitro* destinados a poblaciones específicas como lactantes,

ancianos y sujetos con desórdenes gastrointestinales (Shani-Levi et al., 2017). Los modelos estáticos, aunque simulan movimientos peristálticos, no reproducen los procesos dinámicos que ocurren durante la digestión humana, como el vaciado gástrico, los continuos cambios de pH y las velocidades de flujo de secreción. Sin embargo, estos sistemas proporcionan datos útiles que permiten establecer la influencia sobre la BA, de nutrientes y/o compuestos bioactivos, de las condiciones de digestión, de la estructura, composición y procesado de los alimentos (tamaño de partícula, adición de emulsionantes, enriquecimiento, tratamientos térmicos, etc.) y de factores dietéticos (interacciones entre componentes alimenticios), con el fin de establecer el valor nutricional de los alimentos y mejorar su formulación y diseño (Alegría et al., 2015).

Para una mejor aproximación a la situación *in vivo*, se han desarrollado sistemas combinados que incluyen las fracciones obtenidas de la digestión gastrointestinal simulada y la incorporación de cultivos celulares. Una de las líneas celulares más utilizadas son las células Caco-2 (modelo validado de epitelio intestinal), procedentes de adenocarcinoma de colon humano. Estas células, cuando se cultivan, se diferencian de forma espontánea, estructural y funcionalmente, asemejándose a células del intestino delgado con microvellosidades, uniones intracelulares estrechas y diversas actividades enzimáticas. Las células Caco-2 cultivadas como monocapas sobre soportes sólidos o en pocillos bicamerales (con compartimentos apical y basolateral) nos permiten evaluar la captación o captación y transporte a través de la monocapa celular, respectivamente (Cilla et al., 2017a). Otras líneas celulares similares a los enterocitos del intestino pero menos utilizadas son las células diferenciadas HT-29 y T84 (Christensen et al., 2012).

Los modelos dinámicos, incluyen procesos fisicoquímicos y mecánicos (peristaltismo, progresiva acidificación del contenido gástrico mediante la adición de HCl, flujo de pepsina y vaciado gástrico, entre otros) con el fin de asemejarse más a las condiciones *in vivo* que los modelos estáticos. Aun así, estos modelos no reproducen por completo el conjunto de procesos que ocurren en el organismo humano (Guerra et al., 2012). Dos de los modelos dinámicos diseñados para simular el estómago, son el modelo gástrico dinámico (DGM) y el simulador gástrico

humano (HGS) (Cilla et al., 2017a). Se ha indicado (Marze et al., 2017) entre los modelos dinámicos para simular el sistema gastrointestinal: el desarrollado por el TNO (The Netherlands Organization), llamado TIM-1, que consta de 4 compartimentos en serie que simulan el estómago y los 3 segmentos del intestino delgado (duodeno, yeyuno e íleon), y el SHIME (simulador del ecosistema microbiano intestinal humano), que consiste en 5 compartimentos; estómago, intestino delgado y las tres regiones del intestino grueso. Este último sistema suele utilizarse para estudiar las interacciones del alimento con la microbiota, y en menor medida para el estudio de digestión gastrointestinal de los alimentos. Cabe mencionar, que otro de los sistemas dinámicos que simula el intestino grueso del ser humano es el TIM-2, el cual, permite evaluar fermentación colónica (Venema, 2015). La combinación del digerido resultante de la fermentación con modelos celulares de intestino humano, es una buena plataforma para estudiar la respuesta del epitelio como resultado de un proceso fermentativo *in vitro*. A su vez, dado que para acceder al intestino grueso son necesarias técnicas médicas invasivas, en muchos casos, los estudios se han limitado al análisis de heces (Payne et al., 2012). Recientemente, nuestro grupo de investigación (Cuevas-Tena et al., 2018), a través de una fermentación colónica *in vitro* con el residuo procedente de una digestión gastrointestinal simulada de una bebida a base de zumo de frutas y leche enriquecida con EV, y heces que aportan la microbiota intestinal, constata que durante la fermentación, los esteroles se metabolizan, siendo el metabolismo por la microbiota intestinal mayor para los EV frente al colesterol, especialmente en β-sitosterol generando etilcoprostanol.

♦ *Efecto de la matriz alimentaria sobre los esteroles vegetales: estudios de bioaccesibilidad*

Como se ha comentado anteriormente, la determinación de la BA es un primer paso en la evaluación de la BD y diversos factores pueden influir en la BA de un compuesto bioactivo, entre ellos, la matriz alimentaria. En sistemas modelo utilizando distintas sales biliares (gliocodeoxicolato, taurodeoxicolato o taurocolato sódico) para evaluar la solubilidad de los esteroles, se ha indicado, en general, que el colesterol presenta mayor solubilidad en las micelas mixtas, seguido del

campesterol y β-sitosterol, siendo el estigmasterol el menos soluble. A su vez, la estructura juega un papel importante en la solubilidad de los esteroles, favoreciéndose ésta cuando la cadena lateral no presenta dobles enlaces y tiene un menor número de átomos de carbono (Armstrong y Carey, 1987; Goncalves et al., 2011; Matsuoka et al., 2008, 2010, 2015).

Se ha constatado que la BA de compuestos hibrófobos (como los EV) puede mejorarse utilizando emulsiones que son capaces de formar micelas mixtas, facilitando su incorporación al endotelio durante la digestion gastrointestinal (Zhang y McClements, 2016). Recientemente (He et al., 2017), ha sido estudiada la contribución de diferentes emulgentes sobre la emulsificación de los EV en leche, siendo el orden: monoestearato de tripoliglicerol > éster de sacarosa > monoglicérido > Span 80 > Tween 80 > estearoil lactilato de sodio > lecitina de soja. Sin embargo, mientras que la adición de Tween 80, tripoliglicerol y lecitina de soja mejora la estabilidad de la emulsión de los EV en leche, los demás emulgentes contribuyen de forma negativa a la emulsion de los EV en este tipo de matriz alimentaria. En soluciones micelares modelo, la solubilidad de diversas soluciones patrón de esteroles (β-sitosterol o sitostanol y/o colesterol), se ha visto favorecida en presencia de lecitina (Ikeda et al., 1989; Ostlund et al., 1999; Matsuoka et al., 2012) o de ácido oleico y monoleina (Matsuoka et al., 2012). A su vez, a la membrana del glóbulo graso de la leche (MFGM) se le atribuye carácter emulsionante, considerándola un tensioactivo eficiente ya que disminuye la tensión superficial y además, también puede desempeñar un papel en el transporte de compuestos liposolubles en el tracto gastrointestinal (Contarini y Povolo, 2013).

La influencia de la matriz sobre la BA de los EV, ha sido evaluada previamente por nuestro grupo de investigación. A través de una digestión gastrointestinal simulada que incorpora una fase micelar intestinal, se ha constatado que los EV presentan mayor BA en matrices enriquecidas con EV como son las bebidas a base de zumo de frutas y leche desnatada (4,4-6,5%), frente a bebidas de frutas (3,1%) o solo leche desnatada (2,6%), siendo en general, la BA mayor para campesterol (3,8-8,3%) y campestanol (3,5-6,8%), seguida de β-sitosterol (2,5-6,4%), sitostanol (2,7-6,1%) y estigmasterol (2,9-5,9%). En todas las matrices, el colesterol es el esterol

que mayor BA presenta (53-99%) (Alemany et al., 2013a). La presencia de leche en las bebidas a base de frutas, favorece la BA de los esteroles, ya que los lípidos de ésta podrían actuar favoreciendo la incorporación de los esteroles a la micela mixta. A su vez, se indica que los carotenoides podrían reducir la incorporación de los EV a la micela mixta al competir con los mismos, reduciendo su BA (Alemany et al., 2013a). En un posterior estudio con cuatro bebidas de leche fermentada enriquecida en EV, se indica que la BA de los EV (9-17%) no depende del contenido de proteínas, lípidos o contenido de EV presentes en las bebidas, sin embargo, la presencia de carbohidratos, incluida la fibra (oligofructosa o inulina), se relaciona con una menor BA de EV (Vaghini et al., 2016). Por el contrario, recientemente Blanco-Morales et al. (2018), en bebidas a base de zumo de frutas y leche desnatada enriquecidas con EV y MFGM, con o sin galactooligosacáridos en su composición, observan que la adición de este tipo de fibra soluble a las bebidas no influye en la BA de los EV, siendo similar en todas las bebidas: campestanol (≈43%) > sitostanol/campesterol (≈40%) > β-sitosterol (≈37%) > estigmasterol (≈33%). Sin embargo, la BA del colesterol se ve favorecida con el incremento de galactooligosacáridos (85 *versus* 80%). Además, en este estudio se compara por primera vez, la BA de los EV obtenida tras una digestión micelar y el método armonizado de Infogest, mostrando bioaccesibilidades dos veces superiores tras la aplicación de la digestión micelar, y poniendo en evidencia la importancia del contenido en sales biliares durante la digestión. Con todo ello, se corrobora la importancia de la matriz alimentaria sobre la BA de los EV, sin embargo, dada la controversia sobre la influencia de determinados componentes sobre la BA de los EV, son necesarios más estudios al respecto.

En este contexto, en esta Tesis Doctoral se ha evaluado la influencia, sobre la BA de los EV, de cambios en la matriz alimentaria en tres bebidas a base de zumo de frutas y leche enriquecidas con EV y con diferente contenido y tipo de fracción grasa (leche desnatada, grasa láctea o aceite de oliva) y emulgentes (lecitina o MFGM).

Este estudio ha dado lugar a la publicación: Alvarez-Sala, A., Garcia-Llatas, G., Cilla, A., Barberá, R., Sánchez-Siles, L. M., Lagarda, M. J. (2016). *Impact of lipid*

components and emulsifiers on plant sterols bioaccessibility from milk-based fruit beverages. Journal of Agricultural and Food Chemistry, 64, 5686−5691.

1.4.2.- Métodos in vivo

Los métodos *in vivo* para la determinación de la BD proporcionan datos directos, pero presentan inconvenientes como coste, variabilidad interindividual, requerimientos éticos y, dadas las diferencias en la capacidad de digestión y absorción entre animales y humanos, los estudios con animales no siempre son extrapolables. Entre las distintas metodologías utilizadas se encuentran: los estudios de balances químicos (donde se determina la cantidad absorbida del analito a partir de la cantidad ingerida y de la excretada) o el empleo de isótopos (radioactivos o estables), y los estudios en tejidos o fluidos biológicos, como plasma y suero, entre otros (Guerra et al., 2012; Cilla et al., 2017a) (Figura 2).

♦ *Absorción de los esteroles*

En el organismo humano, la absorción del colesterol se sitúa entre 30-60%, mientras que la de los EV es más baja debido al aumento en la longitud de la cadena lateral. La absorción del campesterol (9,4-14,8%) es aproximadamente 3 veces mayor que la del β-sitosterol (3,1-4,5%) y estigmasterol (~4%). A su vez, la absorción de los fitoestanoles (campestanol y sitostanol), es menor que la de los fitoesteroles debido a la ausencia de doble enlace en el anillo del esterol, variando entre 0,1-2% (Jain y Bathla, 2015).

El colesterol se puede obtener de dos fuentes distintas: a partir de la dieta o de la síntesis endógena, principalmente en los hepatocitos. La síntesis de colesterol celular tiene lugar a través de una cadena de reacciones enzimáticas que comienza con la acetil-coenzima A, dando lugar a través de isoprenoides, a precursores cíclicos que se modifican aún más para alcanzar el producto final, el colesterol. Los precursores de colesterol más comunes son escualeno, latosterol y desmosterol, cuyas cantidades en el sistema circulatorio se correlacionan positivamente con la actividad de la ruta sintética del colesterol, por lo que se utilizan como indicadores de su biosíntesis. Las concentraciones séricas de los mismos en promedio (y rango) son: escualeno 1,7 (1,2-2,1) mmol/L, latosterol 10 (2-20) mmol/L, y desmosterol 5 (2-7) mmol/L, aproximadamente, 1/1000 respecto del colesterol (Olkkonen et al., 2017).

Sin embargo, los EV no se pueden sintetizar en el organismo humano y se obtienen exclusivamente a través de la dieta. Una vez liberados del alimento en el organismo humano, los EV siguen una ruta de absorción similar a la del colesterol (Trautwein et al., 2003; Marangoni y Poli, 2010) (Figura 3). El colesterol de la dieta y los EV que están en forma esterificada, al llegar a la luz intestinal por la acción de la enzima carboxil éster lipasa pancreática (colesterol esterasa), se escinden los ácidos grasos y se genera el esterol libre. Para poder ser absorbidos en el intestino, los esteroles, al ser moléculas muy hidrófobas, deben ser solubilizados, lo que se consigue integrándolos en estructuras lipídicas llamadas micelas mixtas (formadas por ácidos biliares, fosfolípidos, monoglicéridos, ácidos grasos y colesterol), las cuales actúan como emulsionantes (Pujol, 2014; De Boer et al., 2018). Los esteroles en forma libre incorporados a las micelas mixtas, se absorben en el enterocito por difusión pasiva o a través de un transportador activo denominado Niemann-Pick C1-like protein 1 (NPC1L1), presente en la membrana plasmática del borde en cepillo. Una vez en el enterocito, una parte de los esteroles libres se re-esterifican de nuevo por acción de la enzima acil-coenzima A colesterol aciltransferasa (ACAT-2), siendo menos eficiente para los EV que para el colesterol y, por lo tanto, solo una pequeña parte de los EV absorbidos se re-esterifican en el enterocito, lo que puede explicar la menor absorción y concentración en el torrente sanguíneo de los mismos (De Smet et al., 2012; López et al., 2016). Una vez los esteroles son re-esterificados, se incorporan a los quilomicrones (aproximadamente 70-80% del colesterol y 12% de EV) por la acción de la proteína microsomal de transferencia de triglicéridos (MTP) (Trautwein et al., 2003). Los quilomicrones primero se exportan a la circulación linfática y posteriormente al torrente circulatorio como constituyentes de las partículas de lipoproteínas, principalmente en partículas de LDL (65-70%) (Gylling et al., 2014). Los esteroles que no son re-esterificados en las células epiteliales intestinales son secretados desde los enterocitos al lumen del intestino, a través de transportadores ABC dependientes de adenosin trifosfato (ATP) "binding casette", concretamente los co-transportadores G5 y G8 (ABCG5 y ABCG8), situados en la parte apical del enterocito (Figura 3).
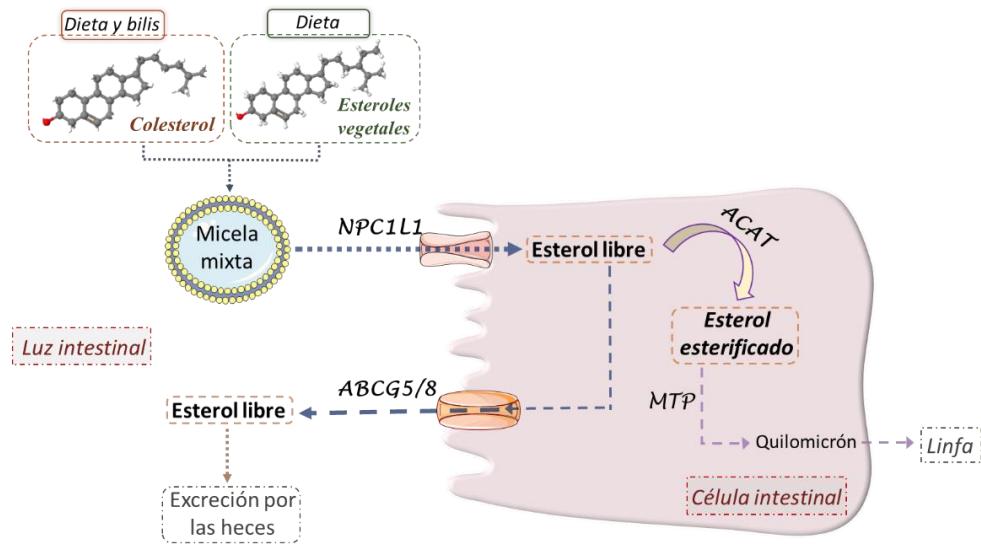
Figura 3. Mecanismo de absorción de los esteroles. ABCG5/8: binding casette G5/8; ACAT: acil-coenzima A colesterol aciltransferasa EV: esteroles vegetales; MTP: proteína microsomal de transferencia de triglicéridos; NPC1L1: Niemann-Pick C1-like protein 1

La principal ruta de excreción de los esteroles (EV y colesterol) es vía biliar, aunque una pequeña fracción de EV se excreta a través de la piel (Trautwein et al., 2003) y recientemente, se ha descrito que el colesterol también puede excretarse por la parte proximal del intestino delgado a través de un eflujo transintestinal (TICE) (De Boer et al., 2018). El cómputo global endógeno de los EV *versus* colesterol es del orden de 500 veces menor para los fitoesteroles y de 10000 veces para los fitoestanoles, debido a la menor absorción de los EV en el intestino y la más rápida excreción biliar en comparación con el colesterol (Gylling et al., 2014; Andrade et al., 2015).

♦ *Biodisponibilidad de esteroles vegetales: estudios clínicos*

El único meta-análisis, hasta el momento, que evalúa el efecto del consumo de alimentos enriquecidos con EV (en su mayoría margarinas bajas en grasa) sobre las concentraciones plasmáticas de los mismos, se ha llevado a cabo por Ras et al. (2013). En este meta-análisis se indica que la ingesta de estos alimentos con una dosis media de 1,6 g EV/día, produce un aumento medio en las concentraciones plasmáticas de β-sitosterol y de campesterol de 31% y 37%, respectivamente. Además, se pone de manifiesto que a mayor dosis de EV (2,0-3,2 g/día) el

incremento medio de β-sitosterol y campesterol es mayor, de hasta 42 y 48%, respectivamente, manteniéndose los EV totales por debajo del 1% de esteroles totales plasmáticos. Por otro lado, la composición de la mezcla de EV del alimento o la fuente de EV usada para el enriquecimiento, influye en la magnitud del incremento de estos compuestos en plasma. Así, estudios que utilizan EV procedentes de *tall oil*, los cuales contienen menos campesterol (∼ 5-10%) y más β-sitosterol (75-80%), observan mayores incrementos plasmáticos de β-sitosterol y menores de campesterol en comparación con el uso de aceite de soja como fuente de EV (20-30% campesterol y 45-50% β-sitosterol). Los autores, además, indican que las concentraciones plasmáticas de EV observadas tras ingerir alimentos enriquecidos en EV (2,0-3,2 g/día) son del orden de 20-45 veces más bajas que las indicadas en pacientes que sufren sitosterolemia (anomalía de los transportadores de excreción ABCG5/ABCG8 en los enterocitos) (Ras et al., 2013).

En un estudio clínico llevado a cabo por nuestro grupo de investigación en mujeres postmenopáusicas, se indica que, la ingesta durante 4 semanas de bebidas a base de zumo de frutas y leche desnatada (con o sin β-Cx en su composición) y enriquecidas en EV (1,5 g EV/día), proporciona incrementos de las concentraciones séricas de β-sitosterol (49,4-64,3%) y campesterol (10,7-12,7%), sin observar cambios en estigmasterol (Garcia-Llatas et al., 2015b). Tampoco se observan cambios en los precursores de colesterol como el desmosterol (excepto para la bebida sin β-Cx) o el latosterol (Granado-Lorencio et al., 2014). Con ello, la presencia de β-Cx en bebidas a base de zumo de frutas y leche enriquecidas en EV no repercute sobre la respuesta de las concentraciones séricas de los EV, por lo que no parece existir interacción negativa entre ambos compuestos bioactivos (Garcia-Llatas et al., 2015b). Además, la presencia simultánea de β-Cx y EV en este tipo de bebidas parece mejorar el efecto hipocolesterolemiante de los EV, ya que reduce biomarcadores de riesgo de enfermedad cardiovascular y de osteoporosis (Granado-Lorencio et al., 2014).

1.5.- Funciones biológicas

1.5.1.- Efecto hipocolesterolemiante

El colesterol es una molécula de vital importancia para el ser humano, que desempeña funciones estructurales y metabólicas, y es precursor de las hormonas esteroideas, ácidos biliares y la vitamina D. Sin embargo, la acumulación excesiva de colesterol en los tejidos y altas concentraciones en sangre (hipercolesterolemia), pueden promover la aterosclerosis y representar un importante factor de riesgo cardiovascular (De Boer et al., 2018). Por lo tanto, es necesaria una adecuada regulación de la homeostasis del colesterol, la cual se logra equilibrando el aporte de colesterol (dietético y de síntesis endógena) con la excreción fecal. El colesterol excretado con las heces depende de la eficiencia de absorción intestinal del colesterol biliar y dietético, por ello, la regulación de su absorción intestinal es de gran interés para reducir la colesterolemia (Pujol, 2014).

Se ha postulado que los EV disminuyen la absorción del colesterol actuando a distintos niveles (Garcia-Llatas y Rodríguez-Estrada, 2011; Muñoz et al., 2011; De Smet et al., 2012; Jain y Bathla, 2015: Plat et al., 2015; López et al., 2016; Cabral y Klein 2017) (Figura 4):

(i) Los EV esterificados compiten con el colesterol esterificado por la acción de la enzima colesterol esterasa al actuar como sustratos de la misma, impidiendo que el colesterol esté en forma libre y pueda formar parte de la micela mixta para ser absorbido (Trautwein et al., 2003).

(ii) Por su mayor longitud y complejidad de la cadena lateral, los EV son más lipofílicos que el propio colesterol (Ikeda et al., 1988), por lo que tienen mayor afinidad para incorporarse en las micelas mixtas desplazando competitivamente al colesterol (Figura 4). El colesterol no emulsionado (desplazado de la micela), no es soluble para ser absorbido y se elimina con las heces.

(iii) Dada la similitud estructural de los EV con el colesterol, los EV compiten por el transportador activo NPC1L1, presente en el borde en cepillo de la membrana plasmática intestinal, impidiendo su paso al interior del enterocito y por lo tanto su absorción (von Bergmann et al., 2005; Davis y Altmann, 2009) (Figura 4).

(iv) Una vez en el interior del enterocito, los EV compiten por la enzima ACAT reduciendo la tasa de esterificación del colesterol en el enterocito, y en consecuencia la cantidad de colesterol exportado a la linfa en forma de quilomicrones (Figura 4). *(v)* Con ello, se acelera el eflujo de colesterol no esterificado desde las células intestinales al lumen intestinal, al verse aumentada la actividad y la expresión de los transportadores ABCG5/ABCG8 (Figura 4).

(vi) Co-cristalización de los EV y del colesterol (Figura 4) durante la lipólisis de alimentos en el tracto gastrointestinal con la consiguiente formación de cristales mixtos poco absorbibles (Rozner y Garti, 2006; Marangoni y Poli, 2010). Sin embargo, según Mel'nikov et al. (2004), este mecanismo de co-cristalización tiene un bajo impacto en la disminución del colesterol sérico, dada la alta solubilidad de los esteroles libres en los productos de hidrólisis de los lípidos.

(vii) Reducción de la síntesis de colesterol endógeno a nivel hepático inhibiendo la expresión de la hidroxi-metil-glutaril coenzima A reductasa (HMGCoA), enzima limitante en la síntesis del colesterol.

(viii) Además, hay indicios de que el colesterol se puede secretar activamente por la parte proximal del intestino delgado a través del TICE (De Boer et al., 2018). En este sentido, Brufau et al. (2011), han observado mediante ensayos con ratones, que los EV pueden incrementar la excreción del colesterol vía TICE, sin embargo, son necesarios más estudios para entender qué mecanismos moleculares están implicados.
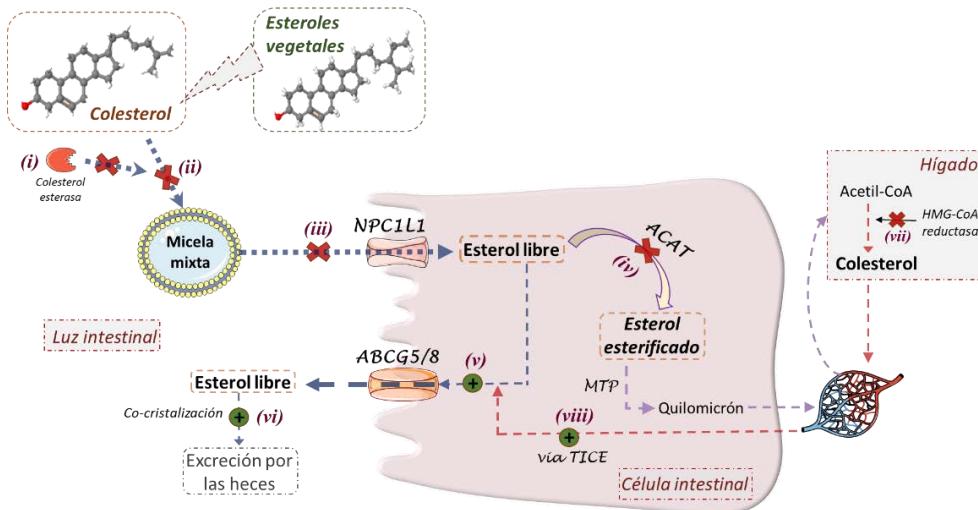
Figura 4. Principales mecanismos de acción hipocolesterolemiante de los esteroles vegetales. ABCG5/8: binding casette G5/8; ACAT: acil-coenzima A colesterol aciltransferasa; MTP: proteína microsomal de transferencia de triglicéridos; NPC1L1: Niemann-Pick C1-like protein 1; TICE: eflujo transintestinal.

1.5.1.1.- Factores implicados en el efecto hipocolesterolemiante

La habilidad de los EV para reducir el colesterol total y el c-LDL fue demostrada por primera vez en 1953 en humanos (Pollak, 1953) y ha sido posteriormente confirmada en diversos estudios clínicos. Hoy en día, se acepta que aproximadamente el 80% del colesterol sérico está determinado por el genotipo del individuo mientras que el otro 20% está determinado por la dieta y el estilo de vida (Andrade et al., 2015). La magnitud de las reducciones de colesterol total y c-LDL pueden variar según la dosis de EV, frecuencia de consumo, tipo de EV (esterificados, libres, fitoesteroles, fitoestanoles) y formulación de los alimentos (matriz) enriquecidos con EV administrados con la dieta (Andrade et al., 2015). Además, se ha constatado que dicha reducción, generalmente, se produce sin la alteración de los niveles de triglicéridos (TG) ni del colesterol ligado a lipoproteínas de alta densidad (c-HDL) (Bard et al., 2015).

♦ *Efecto de la dosis*

Un aspecto importante a considerar en la eficacia del efecto hipocolesterolemiante de los EV es la dosis de consumo. En un meta-análisis llevado a cabo por Ras et al. (2014), se indica que un consumo de EV entre 0,6-3,3 g/día

(dosis media de 2,1 g/día) reduce gradualmente las concentraciones de c-LDL, en promedio de 6-12%, alcanzándose la máxima reducción (12%) con 3 g/día. La relación dosis-respuesta ha sido también postulada previamente en otros meta-análisis/revisiones (Katan et al., 2003; AbuMweis et al., 2008; Demonty et al., 2009; Talati et al., 2010; Musa-Veloso et al., 2011). En cualquier caso, la dosis recomendada para alcanzar un efecto hipocolesterolemiante sin producir efectos adversos es de 2 g/día (Gylling et al., 2014).

♦ *Frecuencia de consumo*

En diversas revisiones, se ha indicado que no existen diferencias en la eficacia de los EV suministrados en una o en varias tomas al día, ya que los EV podrían permanecer en la luz intestinal o incluso dentro del enterocito después de su consumo; sin embargo, existen datos contradictorios sobre la importancia de consumir los EV junto con las comidas o en ayunas (Rozner y Garti, 2006; García-Llatas y Rodríguez-Estrada, 2011; De Smet et al., 2012; Moreau et al., 2018).

♦ *Esteroles vegetales libres versus esterificados*

En los alimentos enriquecidos, los EV se adicionan en forma esterificada o libre. Los fabricantes suelen añadirlos esterificados por su mayor solubilidad en los alimentos de base lipídica, sin embargo, es la forma libre la que facilita su incorporación en las micelas, ya que la esterificada necesita de una hidrólisis previa. En este sentido, la utilización de emulsiones en las formulaciones de alimentos enriquecidos con EV en forma libre es bastante común, debido a que se facilita la incorporación de los EV a las micelas mixtas y con ello la absorción (MacKay y Jones, 2011). Además, otro factor que puede afectar al metabolismo del colesterol es la distinta procedencia de las fracciones de ácidos grasos (sebo de vaca o aceites vegetales o de pescado) utilizados en la esterificación de los EV; sin embargo, se requieren más estudios para dilucidar dicha hipótesis (He et al., 2018). Por su parte, los EV en forma libre, a pesar de su alto punto de fusión, su baja solubilidad en alimentos de base lipídica e insolubilidad en agua (He et al., 2018), aportan a los alimentos menor masa añadida (30% menos sin el ácido graso) y con ello menor aporte de calorías que los EV esterificados (Clifton, 2015). En este contexto, en varios meta-análisis se ha indicado la ausencia de diferente respuesta sobre la

disminución del c-LDL entre ambas formas de adición (libre o esterificada) (AbuMweis et al., 2008; Demonty et al., 2009; Musa-Veloso et al., 2011).

♦ *Fitoesteroles versus fitoestanoles*

Dada la diferencia estructural entre los fitoesteroles y fitoestanoles, se ha estudiado la eficacia de ambos compuestos sobre los niveles del c-LDL. En recientes revisiones (He et al., 2018; Moreau et al., 2018) y diversos meta-análisis (Talati et al., 2010; Musa-Veloso et al., 2011) no se indican diferencias relevantes en las concentraciones de c-LDL tras la ingesta de fitoesteroles o fitoestanoles. Sin embargo, en el meta-análisis llevado a cabo por Ras et al. (2014), se observa que la ingesta de alimentos enriquecidos con fitoestanoles (2-2,5 g/día) presenta ligeramente mayor eficacia (2%) en la reducción del c-LDL que los fitoesteroles; sin embargo, los autores asocian esta ligera diferencia a que los estudios que contemplan fitoestanoles presentan mayor número de alimentos con formato sólido *versus* líquido, que los estudios que incluyen fitoesteroles.

♦ *Matriz alimentaria*

La consistencia de la matriz (líquida o sólida), según Musa-Veloso et al. (2011) no influye en el efecto hipocolesterolemiante de los EV (1,8-2,6 g/día). Sin embargo, en un posterior meta-análisis (Ras et al., 2014), se señala que la consistencia de la matriz tiene un rol importante en el efecto de los EV. Independientemente del tipo de EV, los alimentos líquidos muestran menor reducción del c-LDL que los alimentos sólidos (6,5% y 9,2%, respectivamente) con ingestas de EV entre 0,2-9 g/día. Este comportamiento, posiblemente podría asociarse a que los alimentos líquidos son vaciados más rápidamente del estómago que los sólidos, y en consecuencia no se estimulan las contracciones necesarias de la vesícula biliar y se inducen, en menor medida, los cambios fisiológicos que permiten que los EV tengan un efecto en la inhibición de la absorción del colesterol (Keszthelyi et al., 2013).

Por otro lado, la incorporación de los EV en las micelas mixtas es fundamental para reducir la absorción del colesterol, por lo tanto, el tipo de alimento donde se adicionan los EV influye en el efecto hipocolesterolemiante de los mismos. Si los EV suplementados permanecen cristalizados o están atrapados en su matriz, no pueden integrarse en las micelas mixtas y su capacidad hipocolesterolemiante merma

(Mackay y Jones, 2011). El primer estudio donde se demostró la influencia de la matriz de distintos alimentos sobre el efecto hipocolesterolemiante de los EV fue llevado a cabo por Clifton et al. (2004). Los autores indican que el consumo de alimentos enriquecidos con EV (1,6 g/día) como el yogur y sobre todo la leche, son más eficaces para reducir el c-LDL (15,9% y 8,6%, respectivamente) que el pan (6,5%) y cereales para el desayuno (5,4%), ya que los EV pueden ser incorporados en la membrana del glóbulo de leche y estar más disponibles para su solubilización en la micela mixta. En este sentido, Conway et al. (2013) sugieren que la membrana de glóbulo graso, MFGM, presente en el suero de leche, podría intervenir en la inhibición de la absorción de colesterol intestinal reduciendo el colesterol sérico y c-LDL (3%). Asimismo, Rosqvist et al. (2015) indican que la presencia de MFGM en productos lácteos puede contrarrestar los efectos hipercolesterolemiantes de la grasa saturada de la dieta. Aunque los mecanismos subyacentes a los efectos potenciales de MFGM sobre el colesterol plasmático no están claros, se ha sugerido que la MFGM puede estar involucrada en la supresión de los genes implicados en la absorción del colesterol (Rosqvist et al., 2015). Sin embargo, en una revisión previa llevada a cabo por Demonty et al. (2009), no se muestran diferencias significativas entre alimentos lácteos y no lácteos o grasos y no grasos, obteniéndose para una ingesta media de 1,8 g EV/día, una disminución del c-LDL, en promedio, del 28%. Por otro lado, en una reciente revisión (Ferguson et al., 2016) donde se comparan diversos tipos de matriz grasa (aceite de soja/girasol o de colza/canola y grasa animal) presentes en alimentos fortificados con EV (principalmente margarina; 1,5-4 g EV/día), se concluye que la presencia de aceite de colza/canola produce mayor reducción tanto del colesterol total como del c-LDL (6,7% y 9,7%, respectivamente) frente a la presencia de aceite de soja/girasol (5,5% y 9,5%, respectivamente). Este hecho se asocia a la combinación de ácidos grasos monoinsaturados y ácidos grasos omega-3 en cantidades adecuadas. A su vez, los autores no pueden comparar el papel de la presencia de grasa animal sobre el efecto hipocolesterolemiante, ya que el tipo de alimento y contenido graso es muy variable en este grupo; sin embargo, observan reducciones de colesterol (6,1%) y de c-LDL (8,9%) (Ferguson et al., 2016).

En un estudio llevado a cabo por nuestro grupo de investigación, se ha evaluado la influencia de la ingesta de tres bebidas a base de zumo y leche con distinta composición (β-Cx o EV o β-Cx + EV (1,5 g/día)), sobre biomarcadores de riesgo de enfermedad cardiovascular y de osteoporosis en mujeres postmenopáusicas (Granado-Lorencio et al., 2014). Los autores demuestran que la ingesta de la bebida con presencia simultánea de β-Cx y EV en la composición, disminuye los niveles séricos de colesterol total, c-LDL y c-HDL en aproximadamente 5-7%, y los marcadores de remodelación ósea en más del 5% (Granado-Lorencio et al., 2014).

En la presente Tesis Doctoral ha sido evaluado mediante un ensayo clínico (NCT 02065024), si la presencia de MFGM en la composición de la bebida a base de zumo y leche desnatada con presencia simultánea de β-Cx y EV (2 g/día), podría ser una buena opción para mejorar la funcionalidad de los EV en mujeres postmenopáusicas.

Este estudio ha dado lugar a la publicación: Alvarez-Sala, A., Blanco-Morales, V., Cilla, A., Silvestre, R. A., Hernández-Álvarez, E., Granado-Lorencio, F., Barberá, R., Garcia-Llatas, G. (2018). *Positive impact on serum lipid profile and cytokines after consumption of a plant sterol-enriched beverage with milk fat globule membrane: a clinical study* (En revisión: Food & Function).

1.5.2.- Efecto antiinflamatorio

El papel de los EV en la reducción del colesterol total y c-LDL es bien conocido; sin embargo, no hay una clara evidencia en la reducción del riesgo de enfermedades cardiovasculares, como la aterosclerosis. La aterosclerosis está asociada con elevadas concentraciones plasmáticas de colesterol total y c-LDL, y generalmente se acepta que el c-LDL es la principal fuente de depósitos de colesterol en la pared de los vasos sanguíneos (Cabral y Klein, 2017). El proceso inflamatorio crónico y la disfunción endotelial conducen a la acumulación subendotelial de c-LDL oxidado y a la formación de células espumosas, obstruyendo total o parcialmente la luz arterial. Además, se evidencia por el incremento de marcadores de inflamación sistémica como la proteína C-reactiva (CRP) y determinados tipos de citoquinas proinflamatorias, los cuales pueden utilizarse también como marcadores de enfermedad cardiovascular (Ait-Oufella et al., 2011; Nandeesha et al., 2015). Las citoquinas son proteínas de bajo peso molecular y se agrupan en distintas clases:

interleuquinas (IL), factores de necrosis tumoral (TNF), interferones, factores estimuladores de colonias, factores de crecimiento y quimiocinas (Ramji y Davies, 2015). Las citoquinas tienen propiedades proinflamatorias como: IL-1, IL-6, IL-8, IL-12-p70 y TNF-α o antiinflamatorias como IL-4, IL-10, IL-13, entre otras, aumentando o atenuando la respuesta inflamatoria, respectivamente (Ait-Oufella et al., 2011; De Oliveira et al., 2011).

1.5.2.1.- Estudios in vitro

En células endoteliales aórticas humanas (HAECs) cultivadas con células monocíticas (U937) y tratadas con β-sitosterol (0,1-200 μM), Loizou et al. (2010) indican que el β-sitosterol inhibe la adhesión de las células U937 a las HAECs (unión previamente estimulada por la acción del TNF-α), mediante la reducción de la expresión de la molécula 1 de adhesión de células vasculares (VCAM-1) y de la molécula 1 de adhesion intracelular (ICAM-1), moléculas cruciales en la patogénesis de la aterosclerosis. Además, el β-sitosterol previene la activación del factor nuclear-kB (NF-kB, un importante factor de transcripción para la liberación de citoquinas). Por consiguiente, el β-sitosterol puede ejercer una actividad antiinflamatoria mediada, al menos en parte, por la inactivación de NF-kB. Por otro lado, se ha descrito en macrófagos de ratón (RAW 264.7 y MPMs), en los cuales se induce la producción de IL-6 y TNF-α mediante lipopolisacáridos, que β-sitosterol no produce cambios en los niveles de dichas citoquinas, mientras que campesterol (160 μM) los incrementa (Kurano et al., 2011). Por su parte, en miofibroblastos cultivados, derivados de pacientes con estenosis aórtica avanzada, se ha descrito que β-sitosterol y sitostanol (1, 16 y 32 μM) producen una reducción en los niveles de expresión de ARNm de la proteína 1 quimiotáctica de monocitos (MCP-1, involucrada en la regulación de la migración e infiltración de macrófagos y monocitos) y de la citoquina IL-1β; sin embargo, no se modifican los niveles de expresión de ARNm de IL-6, IL-8, IL-10 y TNF-α (Simonen et al., 2015).

1.5.2.2.- Estudios in vivo

La mayoría de los estudios clínicos con humanos que evalúan el papel de los EV en el proceso inflamatorio, determinan concentraciones plasmáticas de CRP y en

menor medida citoquinas. En el meta-análisis llevado a cabo por Rocha et al. (2016), se concluye que la ingesta regular, durante una media de 55 días, de alimentos enriquecidos con EV (1,4-4 g/día), en su mayoría lácteos y derivados, no afecta significativamente a los niveles de CRP en sujetos hiperlipidémicos, con síndromes metabólicos o sin enfermedades coronarias establecidas, por lo que los EV no tienen un impacto sobre la inflamación sistémica de grado bajo. Recientemente, Kurano et al. (2018) tampoco encuentran correlación entre los niveles séricos de CRP y de EV (β-sitosterol o campesterol) a concentraciones fisiológicas en sujetos diabéticos (6,3 y 3,7 mg/mL, respectivamente); sin embargo, observan una correlación negativa entre el β-sitosterol y las citoquinas proinflamatorias, IL-6 y TNF-α, sugiriendo que el β-sitosterol podría tener un rol positivo en el mantenimiento de la homeostasis de la inflamación crónica relacionada con la diabetes. Estos autores asocian el distinto comportamiento de los EV sobre los niveles de CRP y citoquinas (IL-6 y TNF-α), a la distinta procedencia de estos mediadores inflamatorios, ya que la CRP proviene principalmente de los hepatocitos mientras que la IL-6 y el TNF-α se expresan de manera más ubicua, especialmente en células inflamatorias como macrófagos.

Respecto a las citoquinas, Micallef y Garg (2009) observan un descenso de IL-6 (11%) y TNF-α (10%) en sujetos hiperlipidémicos, tras el consumo de margarina enriquecida en EV (2 g/día) junto con cápsulas de aceite de pescado (ricas en ω-3). En pacientes asmáticos, Brüll et al. (2016) indican una disminución de las concentraciones de TNF-α e IL-1β (0,98 y 0,18%, respectivamente) tras el consumo de yogur enriquecido con fitoestanoles (4 g/día), sin alterar las concentraciones de IL-8, aun sí, los autores sugieren que los fitoestanoles dietéticos pueden aliviar los procesos inflamatorios. Por su parte, en sujetos sanos, Devaraj et al. (2011) muestran una reducción significativa de IL-6 (27-46%) e IL-1β (43%) tras la ingesta de un zumo de naranja enriquecido en EV (2 g/día), y de IL-6 (27%) en una bebida a base de naranja, sin modificar el resto de citoquinas estudiadas (IL-8, IL-10 y TNF-α). Recientemente, también en sujetos sanos, se indica que la ingesta de leche enriquecida en EV (1,6 g/día) induce un cambio favorable del estado inflamatorio, disminuyendo la expresión de la MCP-1 y tendiendo a aumentar la expresión del receptor de la citoquina antiinflamatoria IL-10 (Lambert et al., 2017). Por otra parte,

son diversos los estudios que no observan modificaciones en los niveles plasmáticos de las citoquinas IL-6 y/o TNF-α en sujetos con hipercolesterolemia que han ingerido entre 1,7-2,4 g EV/día mediante margarinas, leche o yogur (Hallikainen et al., 2006; Jones et al., 2007; Gagliardi et al., 2010; Turpeinen et al., 2012; Kunces et al., 2013; Heggen et al., 2015; Ho et al., 2016; Ras et al., 2016), o en pacientes con síndrome metabólico tras el consumo de un yogur bebible enriquecido con fitoestanoles (2 g/día) (Plat et al., 2009).

Por otro lado, Kunces et al. (2013) solo observan una reducción de IL-8 (11%) tras la ingesta de leche enriquecida con 2 g de fitoesteroles recristalizados con triglicéridos y no en forma libre. Aunque el mecanismo subyacente a la disminución de IL-8 no es claro, se sugiere que la disminución en la producción de esta citoquina se debe principalmente al efecto de los EV en la reducción del LDL, lo que a su vez conlleva reducción de c-LDL oxidado, con la consiguiente atenuación del reclutamiento de células inmunes hacia la pared arterial (Kunces et al., 2013). La relación entre un efecto positivo en biomarcadores inflamatorios y la reducción concomitante de aterosclerosis (60%), también ha sido observada por Nashed et al. (2005) en ratones apo E-KO alimentados con EV derivados de soja al 2%. Los autores sugieren que la reducción del colesterol plasmático supone una menor oxidación de c-LDL, así como reclutamiento de células inmunes en la íntima arterial y, en consecuencia, disminución de la secreción y producción de citoquinas proinflamatorias (IL-6 y TNF-α), especies reactivas de oxígeno (ROS) y niveles de expresión de moléculas de adhesión. Sin embargo, la EAS indica que no se debe excluir la posibilidad de acumulación de EV en las células vasculares tras su consumo, como consecuencia de un aumento de los niveles circulantes, aunque no se atribuyen efectos adversos a concentraciones fisiológicas (Gylling et al., 2014).

La bibliografía disponible muestra una gran variabilidad sobre los efectos de los EV en la producción de factores inflamatorios, lo que hace difícil llegar a una conclusión sólida sobre el efecto antiinflamatorio de los EV. En la presente Tesis Doctoral se evalúa el efecto de los EV sobre la producción de citoquinas (IL-1β, IL-6, IL-8, IL-10, IL-12p70 y TNF-α) tras el consumo, por mujeres postmenopáusicas, de una bebida a base de zumo de frutas y leche desnatada con adición de grasa de

leche y MFGM, enriquecida en EV (2 g/día), mediante un ensayo clínico aleatorizado, cruzado, doble ciego y con dosis múltiple (NCT 02065024).

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1.5.2.3.- Eriptosis

En relación al proceso inflamatorio, mediante estudios *ex vivo*, se ha descrito que los eritrocitos son particularmente sensibles a cambios sistémicos inflamatorios, siendo la CRP (Abed et al., 2017) y la citoquina IL-8 (Bester y Pretorius, 2016) desencadenantes de la eriptosis. La eriptosis es la muerte programada de los eritrocitos que se caracteriza principalmente por la contracción celular y la externalización de la fosfatidilserina (fosfolípido que usualmente se mantiene en el lado citosólico de las membranas celulares) hacia la superficie de los eritrocitos (Figura 5). En este sentido, la externalización de la fosfatidilserina en la membrana celular del eritrocito, puede conllevar a que la célula eriptótica se una mediante este fosfolípido al receptor de las células endoteliales CXCL16 (Figura 5), fomentando la unión al endotelio, causando problemas de microcirculación y contribuyendo al proceso inflamatorio del tejido endotelial que desemboca en aterosclerosis (Borst et al., 2011; Lang et al., 2014). Además, una eriptosis excesiva también se ha relacionado con varios desórdenes clínicos, que incluyen anemia, aterotrombosis, talasemia, síndrome urémico hemolítico, insuficiencia cardíaca, enfermedad de Wilson, obesidad y diabetes, entre otras. Sin embargo, la eriptosis también puede contribuir a proteger contra la malaria, atenuando la parasitemia, así como evitar que los eritrocitos sufran hemólisis, con la posterior liberación de hemoglobina al torrente sanguíneo y las consecuentes complicaciones atribuidas como insuficiencia renal. Por lo tanto, el equilibrio entre mecanismos pro- y anti-eriptóticos son vitales para mantener la homeostasis de los eritrocitos en sangre (Qadri et al., 2017; Repsold y Joubert, 2018).

La eriptosis se puede desencadenar por varios factores estresantes como choque osmótico, agotamiento de energía, exposición a compuestos xenobióticos o estrés oxidativo asociado a la producción de ROS o depleción de glutatión (GSH) (Herlax et al., 2011; Pretorius et al., 2016) (Figura 5). El proceso de eriptosis se suele iniciar con el aumento del Ca^{2+} intracelular (pudiendo ser activado por la prostaglandina E_2, que activa los canales dependientes de Ca^{2+}, facilitando la entrada del catión al interior celular). Con la entrada de Ca^{2+}, se activan los canales de K^+ sensibles a Ca^{2+}, facilitando su salida de la célula acompañada del ión Cl^-, con la consecuente liberación de agua al exterior, provocando una disminución del tamaño celular (Repsold y Joubert, 2018).
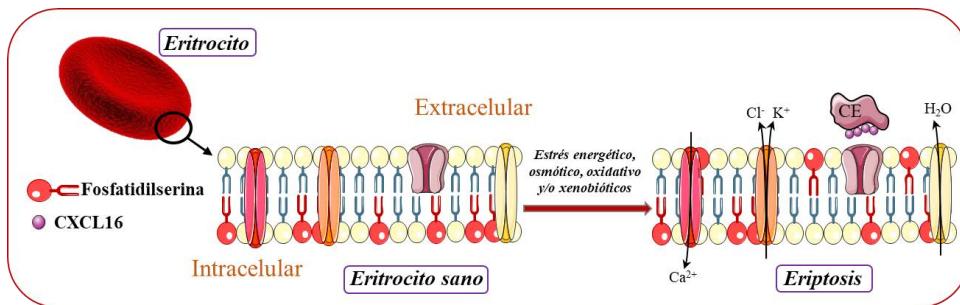
Figura 5. Principales vías desencadenantes del proceso eriptótico y adhesión del eritrocito eriptótico a la célula endotelial. CE: célula endotelial; CXCL16: receptor endotelial

Diversos estudios han evaluado el efecto osmótico de los EV sobre los eritrocitos. En ratas propensas a hipertensión, se ha descrito que la ingesta de aceite de canola conteniendo EV aumenta la fragilidad (Naito et al., 2000) y reduce la deformabilidad (Ratnayake et al., 2000) de la membrana de los eritrocitos, posiblemente debido al reemplazamiento del colesterol de la membrana por parte de los EV. Sin embargo, estos efectos no se observan en hámsters (Ebine et al., 2005). A su vez, en sujetos hiperlipidémicos no se observa alterada la deformabilidad y/o fragilidad de la membrana de los eritrocitos tras consumir margarina enriquecida con EV (Hendriks et al., 2003; De Jong et al., 2006), o tras la ingesta combinada de margarina enriquecida en EV con una dieta rica en fibra soluble y proteína de origen vegetal (Jones et al., 2005; Jenkins et al., 2007). La mayoría de estos estudios han sido

recopilados en la revisión de Bard et al. (2015), concluyendo que los efectos de los EV sobre los eritrocitos son contradictorios.

Por su parte, nuestro grupo ha participado en la investigación del efecto de productos de oxidación del colesterol (COPs, "cholesterol oxidation products") sobre diversos mecanismos implicados en la eriptosis como la externalización de la fosfatidilserina, disminución del tamaño celular, producción de ROS, aumento de Ca^{2+} intracelular y depleción de GSH, entre otros. Los COPs inducen una eriptosis dependiente del estrés oxidativo e independientes de Ca^{2+}, lo que podría contribuir al desarrollo de aterosclerosis y trombosis (Tesoriere et al., 2014). Sin embargo, no ha sido estudiado, hasta el momento, el efecto de los EV sobre estos mecanismos implicados en la eriptosis. Por todo ello, en la presente Tesis Doctoral se ha considerado de interés evaluar el efecto de los EV (β-sitosterol, campesterol y estigmasterol), a concentraciones fisiológicas halladas en suero tras la ingesta de una bebida a base de zumo de frutas y leche enriquecida en EV y conteniendo β-Cx, sobre la hemólisis y diversos mecanismos implicados en la eriptosis, para elucidar su posible papel en la inhibición de eriptosis inducida por estrés oxidativo, como un mecanismo potencial antiaterosclerótico nuevo y no descrito hasta el momento, complementario a su conocida acción hipocolesterolemiante.

Este estudio ha dado lugar a la publicación: Alvarez-Sala, A., López-García, G., Attanzio, A., Tesoriere, L., Cilla, A., Barberá, R., Alegría, A. (2018). *Effects of plant sterols and/or β-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death.* Journal of Agricultural and Food Chemistry, 66, 1157−1166.

1.5.3.- Efecto anticancerígeno

En base a la última recopilación de datos procedente de la Agencia Internacional de Investigación del Cáncer (IARC), Ferlay et al. (2015) estiman que en 2012 surgieron 14,1 millones de nuevos casos y 8,2 millones de muertes por cáncer en todo el mundo. El cáncer de pulmón es el cáncer más común en el mundo, tanto en términos de incidencia (12,9%) como de mortalidad (19,4%), seguido, en términos de incidencia, del cáncer de mama (11,9%) y cáncer colorrectal (9,3%). El cáncer es alrededor de un 25% mayor en hombres que en mujeres, predominando en éstos el

cáncer de pulmón, próstata, colorrectal y estómago y en mujeres el cáncer de mama, colorrectal, pulmón y cuello uterino (Ferlay et al., 2015).

Por su parte, ha sido constatado por la Sociedad Europea de Nutrición Clínica y Metabolismo (ESPEN) que la dieta es uno de los factores implicados en la etiología del cáncer (Arends et al., 2017). En este sentido, el papel de los EV, principalmente de β-sitosterol, en la protección a distintos tipos de cáncer ha sido ampliamente revisada (Awad y Fink, 2000; Woyengo et al., 2009; Bradford y Awad, 2010; Shahzad et al., 2017).

1.5.3.1.- Estudios in vitro

Se ha descrito el efecto antiproliferativo de los EV frente a distintos tipos de células de adenocarcinoma de colon (HT-29, HCT116, Caco-2, COLO 320 DM) (Awad et al., 1996, 1998; Choi et al., 2003; Daly et al., 2009; Jayaprakasha et al., 2007, 2010; Baskar et al., 2010; Rahman et al., 2013; Aiyelaage et al., 2015; Cilla et al., 2015; Montserrat-de la Paz et al., 2015; López-García et al., 2017), mama (MDA-MB-231, MCF-7, Hs5787) (Awad et al., 2000, 2001, 2003, 2007, 2008; Rahmat et al., 2006; Park et al, 2008; Lai et al., 2010; Rubis et al., 2010; Vundru et al., 2013; Rahman et al., 2013; Aiyelaage et al., 2015; Tor et al., 2015; Hamid et al., 2018), pulmón (A 549) (Malek et al., 2009; Vundru et al., 2013), próstata (LNCaP, PC-3, DU145) (von Holtz et al., 1998; Awad et al., 2005; Ifere et al., 2009; Tahsin et al., 2017; Yaouba et al., 2018), cuello uterino (HeLa, CasKi) (Block et al., 2004; Malek et al., 2009; Csupor-Löffler et al., 2011; Hamdan et al., 2011; Han et al., 2013; Cheng et al., 2015; Teoh et al., 2016; Karakaya et al., 2017), leucemia (U937) (Park et al., 2007) y estómago (SGC-790) (Zhao et al., 2009).

Principales mecanismos de acción anticancerígena de los EV

Se han postulado posibles mecanismos para elucidar el efecto anticarcinógeno de los EV, principalmente β-sitosterol, mayoritariamente frente al cáncer de mama, colon y próstata mediante estudios *in vitro* (Awad y Fink, 2000; Woyengo et al., 2009; Ramprasath y Awad, 2015; Shahzad et al., 2017) (Figura 6).

♦ *Alteración en la membrana y efecto sobre rutas de transducción de señales intracelulares*

Como mecanismos de acción antiproliferativa, se ha descrito en células de adenocarcinoma de colon (HT-29), que los EV pueden incorporarse a la membrana celular alterando la composición de fosfolípidos (disminución de esfingomielina e incremento de fosfatidilcolina) (Awad et al., 1996). A su vez, la incorporación de EV a la membrana conlleva a una disminución en la fluidez de la misma y como mecanismo compensatorio aumenta la actividad de enzimas desaturasas de ácidos grasos (Shahzad et al., 2017).

Dos de las principales rutas de transducción de señales estudiadas sobre los EV, son *(i)* la ruta de la proteína quinasa C, cuya principal función es mantener la integridad celular y *(ii)* el ciclo de la esfingomielina, donde se estimula la producción de ceramida, la cual promueve arresto en el ciclo celular, senescencia y apoptosis. Tras la recopilación de estudios *in vivo* e *in vitro* llevada a cabo en varias revisiones (Awad y Fink, 2000; Shahzad et al., 2017), se indica que el β-sitosterol no está implicado en la ruta de la proteína quinasa C, sin embargo, activa el ciclo de la esfingomielina, incrementando la producción de ceramida en células de cáncer de colon (HT-29) (Awad et al., 1996; Awad et al., 1998), de mama (MCF-7 y MDA-MB-231) (Awad et al., 2008) y de próstata (LNCaP) (von Holtz et al., 1998).

♦ *Efecto sobre el ciclo celular*

Los EV pueden actuar como inhibidores débiles del ciclo celular, regulando ligeramente la progresión del mismo (Bradford y Awad, 2010), principalmente en células de cáncer de colon, mama y próstata. Sin embargo, existe controversia en el comportamiento de los EV sobre las distintas fases del ciclo celular: incrementos en fase sub-G_1 en células de cáncer de colon (Choi et al., 2003; Cilla et al., 2015) y leucemia (Park et al., 2007), bloqueo de la fase G_0/G_1 en células de cáncer de colon (Montserrat-de la Paz et al., 2015; López-García et al., 2017) y mama (Vundru et al., 2013), bloqueo en la fase G_1/S en células de cáncer de próstata (Ifere et al., 2009) y pulmón (Vundru et al., 2013) o de la fase G_2/M en cáncer de mama (Awad et al., 2001) y de próstata (Awad et al., 2005). Estas divergencias podrían ser debidas a las

diferentes condiciones experimentales (tipo de células, tipo y concentración de EV y tiempo de tratamiento) llevadas a cabo en los distintos estudios.

♦ *Apoptosis*

Se ha descrito ampliamente que los EV promueven la apoptosis (muerte celular programada) de células cancerígenas (Woyengo et al., 2009; Shahzad et al., 2017). La apoptosis implica cascadas intrínsecas y extrínsecas de eventos enzimáticos, activación de caspasas, liberación del citocromo c mitocondrial y escisión del ADN genómico (Bradford y Awad, 2010). En células de cáncer de mama (MDA-MB-231 y MCF7), los EV activan la expresión de un receptor de membrana de muerte celular (FAS), y con ello la caspasa-8, produciendo apoptosis vía extrínseca (Awad et al., 2007). Además, otro estudio llevado a cabo en células MDA-MB-231, indica activación de las caspasas -3, -8 y -9, con un aumento de los niveles de las proteínas proapoptóticas Bax y disminución de las antiapoptóticas Bcl-2 (Park et al., 2008). Similares mecanismos proapoptóticos han sido descritos por Choi et al. (2003) en células de cáncer de colon (HCT116), mediante la activación de caspasa-3 y -9, junto con una menor expresión de las proteínas Bcl-2, aumento de los niveles de las proteínas Bax y liberación de citocromo c al citosol (Choi et al., 2003). Por su parte, en células de leucemia (U937) (Park et al., 2007) y de cáncer de estómago (SGC-7901) (Zhao et al., 2009), el proceso apoptótico de los EV implica la activación de la caspasa-3, así como la desregulación de las proteínas Bcl-2. Un buen marcador de la apoptosis es la externalización de la fosfatidilserina en la membrana celular.

♦ *Otros mecanismos de acción*

Otro mecanismo potencial se lleva a cabo a través del incremento de ROS producido como resultado del estrés oxidativo de las células, lo que podría conducir a daños en el ADN dando lugar a la carcinogénesis. Baskar et al. (2010) sugieren que el efecto anticancerígeno del β-sitosterol sobre el cáncer de colon (COLO 320 DM), es debido a sus propiedades antioxidantes y su capacidad para reducir la expresión de β-catenina y del antígeno nuclear de células proliferativas, PCNA, en la mucosa colónica. A su vez, el estrés oxidativo puede conllevar a activar los canales dependientes de Ca^{2+} de la membrana celular, lo que implica un aumento de Ca^{2+} intracelular (Cilla et al., 2015). Por otro lado, se ha indicado que los EV

pueden inhibir la angiogénesis (vascularización del tumor, vital en el crecimiento y multiplicación de las células cancerosas), y la metástasis (principal causa de muerte por cáncer) (Woyengo et al., 2009). En este sentido, los EV pueden ejercer efectos preventivos sobre la metástasis del cáncer a través de la produción de citoquinas, ya que ha sido indicado que el sistema inmune juega un papel vital en la etiología del cáncer y se reconoce que la inflamación crónica es un aspecto fundamental del mismo (Grattan et al., 2013). Por otro lado, se ha sugerido que los EV pueden promover la apoptosis al reducir las concentraciones de colesterol en sangre y, en consecuencia, suprimir la incorporación de colesterol en las balsas lipídicas ("lipid rafts") de las células cancerosas, disminuyendo de este modo el riesgo de sufrir cáncer (Ramprasath y Awad, 2015).
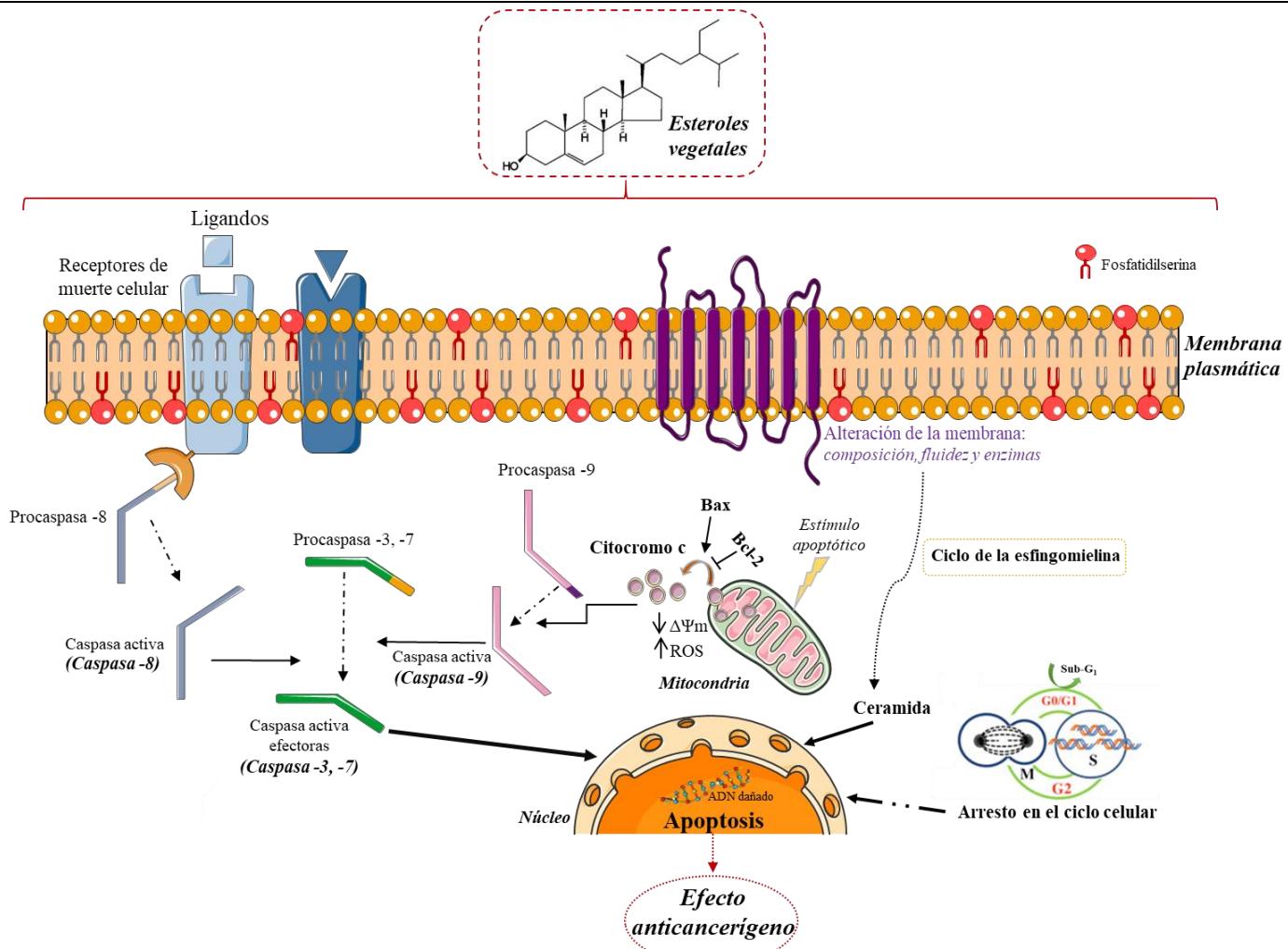
Figura 6. Efecto anticancerígeno de esteroles vegetales: principales mecanismos de acción propuestos

Parte de estos mecanismos han sido estudiados en la línea celular de adenocarcinoma de colon Caco-2, por nuestro grupo de investigación. En uno de los estudios, los EV (β-sitosterol, campesterol y estigmasterol, de forma individual o como mezcla: 12, 1, 0,25, 13,25 µM, respectivamente) a concentraciones séricas obtenidas tras la ingesta de una bebida a base de zumo y leche enriquecida en EV, muestran actividad anticancerígena vía apoptosis (aumento de la población en fase sub-G_1 y externalización de fosfatidilserina), acompañado de la activación del factor proapoptótico BAD, despolarización de la membrana mitocondrial y escisión de poli (ADP-ribosa) polimerasa dependiente de caspasa-3, junto con influjo de Ca^{2+} al citosol y aumento de los niveles de especies reactivas de oxígeno y nitrógeno (RONS) (Cilla et al., 2015). En un estudio posterior, los EV (β-sitosterol, campesterol y estigmasterol, de forma individual o como mezcla: 115, 11, 6, 132 µM, respectivamente) a concentraciones colónicas estimadas tras digerir *in vitro* una bebida a base de zumo y leche enriquecida en EV, producen actividad antiproliferativa contra las células Caco-2, en general, vía necrosis a través de un bloqueo en la fase G_0/G_1 del ciclo celular, sin despolarización de la membrana mitocondrial ni producción de ROS (López-García et al., 2017).

Sin embargo, hasta la fecha, no ha sido evaluada la posible actividad anticancerígena del ingrediente fuente de EV, *tall oil*, usado para el enriquecimiento de bebidas a base de zumo y leche (objeto de estudio a lo largo de la Tesis Doctoral). En este contexto, con la finalidad de complementar el conocido efecto hipocolesterolémico de los EV y, además, extender su uso como ingredientes funcionales en el desarrollo de alimentos enriquecidos, en la presente Tesis Doctoral se ha estudiado por primera vez, el efecto antiproliferativo del ingrediente *tall oil* y su principal EV (β-sitosterol), a concentraciones séricas obtenidas tras el consumo regular de la misma, en líneas celulares de cáncer de mama (MCF-7), colon (HCT116) y cáncer de cuello uterino (HeLa).

Este estudio, realizado en el Dipartamento Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF) de Palermo (Italia), durante una estancia de investigación de la doctoranda, ha dado lugar a la publicación: Alvarez-Sala, A., Attanzio, A., Tesoriere, L., Garcia-Llatas, G., Barberá, R., Cilla, A. (2018). *Anti-*

proliferative effect of phytosterol-ingredient and its main phytosterol (β-sitosterol) on human cancer cell lines (En revisión: International Journal of Food Science and Nutrition) y ha permitido presentar la tesis con mención internacional.

Por otro lado, los EV debido a su efecto anticancerígeno, podrían considerarse como una posible opción como adyuvantes en la quimioterapia con fármacos. A nuestro conocimiento, sólo dos estudios han evaluado el efecto de los EV, concretamente de β-sitosterol, como adyuvante para sensibilizar las células de adenocarcinoma de mama a diversos fármacos anticancerosos como el tamoxifeno (Awad et al., 2008) o el ligando inductor de la apoptosis relacionado con el TNF (TRAIL) (Park et al., 2008). Sin embargo, hasta el momento, no ha sido estudiado el papel de los EV como coadyuvantes en quimioterapia frente al cáncer de colon. Por todo ello, en la presente Tesis Doctoral se ha evaluado si los EV (β-sitosterol, campesterol y/o estigmasterol), a concentraciones colónicas estimadas tras digerir la bebida a base de zumo y leche enriquecida con EV, mejoran el efecto anticancerígeno del 5-fluorouracilo (5-FU), uno de los principales fármacos quimioterapéuticos utilizados para combatir el cáncer colorectal (Walko y Lindley, 2005). El estudio se ha llevado a cabo en dos modelos de células de cáncer de colon (Caco-2 y HT-29), evaluando la proliferación celular, efecto sobre el ciclo celular, inducción de apoptosis, activación de caspasas -3, -8 y -9, producción de ROS y efecto sobre el potencial de membrana mitocondrial.

Este estudio, realizado durante una estancia de investigación de la doctoranda en el Departamento de Ciencia y Tecnología de los Alimentos, CEBAS-CSIC (Murcia), ha dado lugar a la publicación: Alvarez-Sala, A., Ávila-Gálvez, M.A., Cilla, A., Barberá, R., Garcia-Llatas, G., Espín, J.C., González-Sarrías, A. (2018). *Physiological concentrations of phytosterols enhance the apoptotic effects of 5-fluorouracil in colon cancer cells* (En revisión: Journal of Functional Foods).

1.5.3.2.- Estudios in vivo

Los estudios *in vivo*, en comparación con los *in vitro*, no son tan abundantes (Shahzad et al., 2017). En ratas con tumores inducidos por metil nitrosourea (Raicht et al., 1980; Deschner et al., 1982) o 1,2-dimetilhidracina (Baskar et al., 2010), se ha

observado que la presencia de β-sitosterol en la dieta puede tener una acción protectora frente a la formación de tumores en el colon, disminuyendo el número de criptas aberrantes. Recientemente, en ratones con cáncer colorrectal inducido mediante sulfato de sodio de azoximetano/dextrano, se ha indicado que los EV inducen apoptosis vía extrínseca e intrínseca activando la expresión de las caspasas -3, -8 y -9 (Sadek et al., 2017). Por su parte, en ratones atímicos, a los cuales se les inyectan células de cáncer de mama, la presencia de EV en la dieta reduce el tamaño del tumor, proceso que es independiente de una respuesta estrogénica (Awad et al., 2000; Ju et al., 2004; Grattan, 2013). Sin embargo, en ratas, se ha indicado que los EV alteran el metabolismo de la testosterona al inhibir la actividad de enzimas transmembrana prostáticas (5α-reductasa y aromatasa), disminuyendo el desarrollo del cáncer de próstata (Shahzad et al., 2017).

A partir de una recopilación de estudios clínicos en humanos, se indica que el consumo de EV mejora los síntomas (índice de flujo y volumen urinario residual) de la hiperplasia prostática benigna, sin reducir el tamaño de la próstata, posiblemente debido a una alteración del metabolismo de la testosterona (Shahzad et al., 2017). En base a estudios epidemiológicos, se ha descrito que el consumo de una dieta basada en alimentos de origen vegetal, conteniendo EV, se asocia con una menor incidencia de cáncer colorectal en la población china (Huang et al., 2017), hecho que no se observa con la dieta holandesa (Normén et al., 2001). La relación entre la dieta (conteniendo EV) y una menor incidencia de cáncer de estómago, pulmón, mama y ovario, a partir de estudios epidemiológicos (Uruguay, Nueva York y Japón), ha sido revisada recientemente por Shahzad et al. (2017), estimándose una reducción del riesgo del cáncer de hasta un 20%.

1.5.4.- Otras funciones biológicas

Existen otros efectos biológicos asociados a los EV, los cuales han sido estudiados en menor profundidad. En una revisión llevada a cabo por Vanmierlo et al. (2015), principalmente basada en estudios con animales, se ha indicado que los EV pueden ejercer un papel positivo en la modulación de la neuroinflamación, neurodegeneración y la progresión de diferentes trastornos neurodegenerativos como

Alzheimer, esclerosis múltiple y el síndrome de Guam (complejo parkinsonismo-demencia-esclerosis lateral amiotrófica), ya que pueden atravesar la barrera hematoencefálica y acumularse en el sistema nervioso central; sin embargo, se requieren más estudios para dilucidar dichos efectos (Vanmierlo et al., 2015). En una posterior revisión, se sugiere que el posible mecanismo protector de los EV frente a la demencia se asocia a su acción como ligandos de receptores de transcripción nuclear como son los receptores activados por proliferadores de peroxisomas y activando la enzima sirtuina 1, los cuales están implicados en la regulación del metabolismo lipídico y la patogénesis de la demencia (Shuang et al., 2016).

Por otro lado, en ratones, se ha indicado la atenuación de la colitis ulcerosa inducida por una dieta rica en grasa, debido a la adición de EV en la dieta. Esta mejora se atribuye a diversos mecanismos: inhibición en la producción de citoquinas y enzimas proinflamatorias, disminución de las criptas aberrantes y de los niveles de hemoglobina fecal y prevención en la reducción de la longitud del colon (Kim et al., 2014; Feng et al., 2017). A su vez, se ha demostrado *in vivo* y/o *in vitro* que una dieta rica en grasa puede dar lugar a enfermedades del hígado como son la enfermedad del hígado graso no alcohólico (NAFLD) (Feng et al., 2018) o la esteatohepatitis no alcohólica (NASH) (Plat et al., 2014a). En este sentido, los EV pueden aliviar los síntomas de NAFLD principalmente a través de una disminución del colesterol, TG y ácidos grasos libres (Feng et al., 2018) o los síntomas de NASH mediante bloqueo en la expresión de marcadores hepáticos inflamatorios (Cd68, MCP-1, ICAM-1, IL-1β y TNF-α) (Plat et al., 2014a). Por otro lado, se ha indicado que la diabetes también puede ser inducida en animales tras el consumo de una dieta rica en grasa, sin embargo, no se ve atenuada con la presencia de EV (Calpe-Berdiel et al., 2008). Por el contrario, recientemente, se ha evidenciado en células L6 procedentes de músculo de rata y en un modelo animal de diabetes tipo-2 (KK-Ay), el efecto positivo de los EV sobre esta enfermedad, al verse disminuida la resistencia a la insulina a través de un aumento en la expresión y translocación del transportador de glucosa GLUT4, mejorando así la homeostasis de la glucosa (Wang et al., 2017). Además, en un ensayo clínico en mujeres embarazadas con diabetes mellitus gestacional, se ha observado que la ingesta de margarina enriquecida con EV (4 g/día) durante 10

semanas, reduce los niveles plasmáticos de glucosa en ayunas y los niveles de insulina mejorando los síntomas de este transtorno metabólico. A su vez, la incidencia de complicaciones neonatales también se reduce en términos de peso al nacer, macrosomía, glucemia y dificultad respiratoria (Gao et al., 2017).

Recientemente, en un trabajo llevado a cabo por nuestro grupo de investigación (Cuevas-Tena et al., 2018), mediante digestión gastrointestinal simulada de una bebida a base de zumo y leche enriquecida con EV y posterior fermentación colónica *in vitro*, se asocia la presencia de EV con una mayor abundancia de la especie beneficiosa *Eubacterium halii* (productora de butirato, el cual posee propiedades antiinflamatorias)*,* y menor abundancia de la familia *Erysipelotrichaceae*, relacionada con desórdenes metabólicos lipídicos. Sin embargo, en un ensayo clínico (Baumgartner et al., 2017), donde 13 sujetos sanos consumieron margarina sin o con EV (3 g/día) durante tres semanas, no se observan diferencias en la composición y diversidad de la microbiota intestinal entre los dos grupos de estudio.

Además de la funcionalidad de los EV comentados en apartados anteriores (1.5.1; 1.5.2; 1.5.3), se sugiere que estos compuestos pueden influir en otros tipos de enfermedades: inflamatoria intestinal (colitis ulcerosa), metabólicas (diabetes, NAFLD, NASH) o neurodegenerativas (Alzheimer, esclerosis múltiple y el síndrome de Guam); sin embargo, son necesarios más estudios para poder confirmar el potencial efecto beneficioso de los EV en dichas enfermedades.

2.- Productos de oxidación de los esteroles

2.1.- Formación

Los esteroles (colesterol y EV) son susceptibles de ser oxidados dando lugar a productos de oxidación conocidos como SOPs ("sterol oxidation products"). Los productos de oxidación del colesterol son denominados COPs, mientras que los derivados de los EV son conocidos como POPs ("phytosterol oxidation products") (Brzeska et al., 2016). Aunque la mayoría de los estudios acerca del mecanismo de formación se centran en los COPs y no en los POPs, la información disponible sugiere que los EV se oxidan de forma similar al colesterol. Existen dos vías principales de oxidación de los esteroles: no enzimática y enzimática (Ryan et al., 2009; Otaegui-Arrazola et al., 2010; Garcia-Llatas y Rodríguez-Estrada, 2011; Cilla et al., 2017b).

Vía no enzimática

Se lleva a cabo mediante la autooxidación (mecanismo radicalario) o por fotooxidación (mecanismo no radicalario). La autooxidación es el mecanismo de oxidación más común de los esteroles (Figura 7). Se inicia con la liberación del hidrógeno contiguo al doble enlace del carbono 7 (C-7), seguido de la reacción con oxígeno formándose radicales 7-peroxi. Seguidamente, la adición de una molécula de hidrógeno da lugar a 7-hidroperóxidos, que son moléculas más estables. A partir de los 7-hidroperóxidos mediante reacciones redox de dismutación, se forman los epímeros 7α/β-hidroxiesterol o 7-cetoesterol. La formación de este último también se puede producir a partir del 7α-/β-hidroxiesterol por deshidrogenación. Otras rutas de oxidación en las que participa el núcleo esteroideo, es la formación de 5,6α-/β-epóxidos por la acción combinada de radicales hidroperóxidos y de moléculas de esterol no oxidadas, y la formación de trioles mediante la hidratación de los epóxidos en medio ácido (Figura 7). Con todo ello, los principales SOPs producidos a partir de la oxidación de la estructura esteroidea son derivados 7α-/β-hidroxi, 7-ceto, 5,6α-/β-epoxi y triol (Hovenkamp et al., 2008; Garcia-Llatas y Rodríguez-Estrada, 2011; Brzeska et al., 2016).
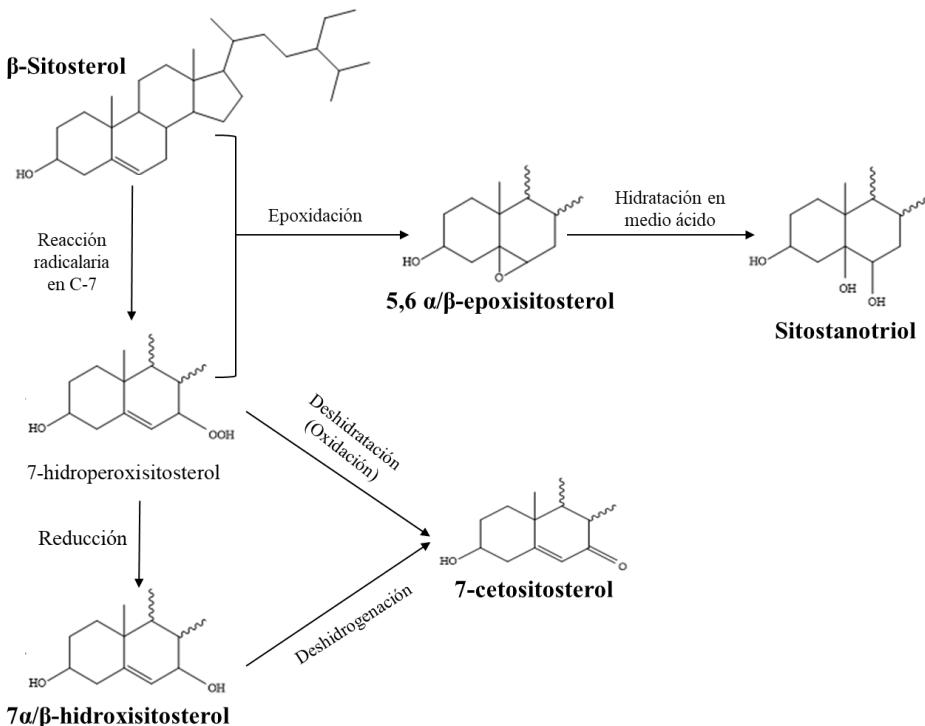
Figura 7. Vía no enzimática de formación de óxidos de β-sitosterol (adaptado de Soupas et al., 2004).

En menor medida, la autooxidación también puede producirse en la cadena lateral de la molécula esteroide, principalmente en las posiciones C-20, C-24, C-25 y C-26 generando hidroperóxidos (García-Llatas y Rodríguez-Estrada, 2011; Brzeska et al., 2016).

Por otro lado, la fotooxidación se inicia con la excitación del oxígeno, por acción de la luz sobre sustancias fotosensibles como la clorofila o riboflavina, produciéndose oxígeno singulete ($^1O_2$). El primer hidroperóxido formado a partir de la fotooxidación es 5α-hidroperóxido, el cual se reordena a 7α-/β-hidroperoxiesterol. Dado que los hidroperóxidos son moléculas altamente inestables, se convierten en productos secundarios de oxidación más estables, tales como 7-hidroxiesteroles y 7-cetoesteroles (García-Llatas y Rodríguez-Estrada, 2011).

Vía enzimática

Es la biotransformación de los esteroles en sus óxidos mediante una ruta enzimática *in vivo* y afecta principalmente a la cadena lateral de la estructura (Ryan

et al., 2009; O'Callaghan et al., 2014). Los SOPs obtenidos de la oxidación de la cadena lateral de la estructura son 24, 25 y 27-hidroxiesteroles (Figura 8). Aunque no se conoce con exactitud todas las enzimas involucradas; las citocromo P450 monooxigenasas, deshidrogenasas, epoxidasas, hidroxilasas y oxidasas que participan en el proceso de oxidación del colesterol, posiblemente, están también involucradas en el de los EV (Hovenkamp et al., 2008; Cilla et al., 2017b).
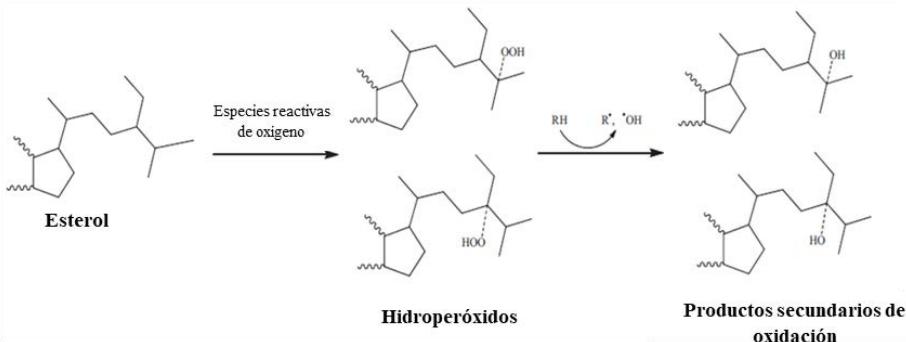
Figura 8. Vía enzimática de formación de óxidos de esteroles (adaptado de García-Llatas y Rodríguez-Estrada, 2011).

2.2.- Factores que afectan en la formación

El contenido de los SOPs en los alimentos depende de la composición de los mismos, las condiciones de almacenamiento y el procesado industrial y culinario. Todos estos procesos comparten varios factores que son elementos clave en la formación de SOPs, como matriz lipídica, temperatura, oxígeno y exposición a la luz (Barriuso et al., 2017).

Matriz alimentaria

Sus características (grado de saturación de la matriz lipídica, forma química en la que se presentan los esteroles, contenido en agua y antioxidantes) pueden influir en el proceso de oxidación de los esteroles.

♦ *Grado de saturación de la matriz y forma química de los esteroles*

El efecto protector del grado de saturación de la matriz lipídica sobre la susceptibilidad de los esteroles a la oxidación es controvertido. Se ha indicado que los lípidos pueden competir por el oxígeno con los esteroles, reduciendo la autooxidación de los mismos. De esta forma, una matriz con lípidos insaturados sería

más eficiente que una matriz con lípidos saturados en la protección de los esteroles, ya que sus dobles enlaces aumentan su probabilidad de oxidación. Sin embargo, los radicales y las especies oxigenadas derivadas de la oxidación de los lípidos pueden ejercer un efecto prooxidante frente a los esteroles, por ello se ha sugerido que la opción más probable es que exista un equilibrio entre ambos mecanismos, pero son necesarios más estudios al respecto (Barriuso et al., 2017). En este sentido, se ha constatado que el grado de susceptibilidad a la oxidación en ambos tipos de matrices (saturadas o insaturadas) podría depender de la temperatura y de la forma química en que se presenten los EV (fitoesteroles o fitoestanoles) (Soupas et al., 2006). A altas temperaturas (>140°C), los fitoesteroles son más estables en matrices insaturadas (aceite de colza) que en saturadas (tripalmitina), debido a que los lípidos insaturados se oxidan más fácilmente que los fitoesteroles protegiendo su oxidación. Por el contrario, a temperaturas más bajas (<140°C), los fitoesteroles reaccionan más rápido en una matriz ya oxidada (insaturada), por lo que son más estables en matrices saturadas. Respecto a los fitoestanoles, son más estables en matrices saturadas, independientemente de la temperatura (Soupas et al., 2006).

El efecto de la esterificación de los esteroles también podría influir en su susceptibilidad frente a la oxidación. Recientemente, Barriuso et al. (2017) indican que la esterificación de los esteroles con ácidos grasos promueve la oxidación, ya que los radicales generados están en estrecho contacto con los sitios de oxidación de los esteroles. Cuanto más insaturados son los ácidos grasos, mayor es la oxidación de los esteroles. Sin embargo, los esteroles en forma libre presentan una interacción más débil con la matriz lipídica y, por lo tanto, existe más competencia con los lípidos por el oxígeno (Barriuso et al., 2017). En este contexto, la temperatura puede ser un factor importante. A bajas temperaturas (≤ 100ºC), los EV (fitoesteroles y fitoestanoles) esterificados se oxidan en mayor proporción que los libres. Sin embargo, a temperaturas superiores (180ºC), la formación de productos secundarios de oxidación es similar para los EV (fitoesteroles y fitoestanoles) libres y esterificados, siendo siempre inferior la formación de POPs a partir de fitoestanoles (Soupas et al., 2005). Como ejemplo de una matriz alimentaria, en leche pasteurizada (127ºC, 2 seg), se observa una formación similar de POPs cuando los fitoesteroles

están presentes en forma libre o esterificada, sin embargo, es mucho inferior cuando proceden de fitoestanoles (Soupas et al., 2006).

♦ *Contenido de agua*

El contenido de agua de la matriz puede favorecer la formación de SOPs mediante dos posibles mecanismos: promoviendo la hidrólisis de los triglicéridos dando lugar a ácidos grasos libres que a su vez aceleran la oxidación de los esteroles y, por otro lado, promoviendo la oxidación lipídica al disminuir la viscosidad y favorecer el movimiento de las moléculas (Hur et al., 2007; Ryan et al., 2009; Vicente et al., 2012). Sin embargo, no se han hallado, en la bibliografía, estudios que evalúen la repercusión del contenido de agua sobre la formación de SOPs en alimentos.

♦ *Antioxidantes*

Una matriz rica en compuestos antioxidantes como son los alimentos vegetales, podría ser una buena opción para el enriquecimiento en EV, ya que protegen frente a la oxidación de los lípidos contenidos en los alimentos, entre ellos los esteroles, principalmente durante el procesado, cocción y almacenamiento (Otaegui-Arrazola et al., 2010; Vicente et al., 2012; Barriuso et al., 2017). En este sentido, en un estudio llevado a cabo por nuestro grupo de investigación (González-Larena et al., 2015), se ha demostrado que la presencia de zumo de frutas (rico en compuestos antioxidantes) en bebidas a base de zumo y leche desnatada mejora la estabilidad de los EV durante el almacenamiento (0-6 meses) a distintas temperaturas (4-37ºC).

Procesado

El calentamiento es conocido como un agente inductor en el proceso de oxidación de los lípidos dando lugar a la abstracción de hidrógenos y produciéndo radicales libres más rápidamente, por ello, la intensidad del calor y el tiempo son factores clave que afectan a la oxidación de esteroles (Barriuso et al., 2017). Se ha indicado que la degradación de los esteroles en relación a la temperatura/tiempo es muy variable, pero en general, existe una relación directa entre la temperatura y el tiempo del tratamiento térmico y la oxidación de los esteroles (Ryan et al., 2009; Vicente et al., 2012; Lin et al., 2016; Barriuso et al., 2017). Se ha indicado, bajo condiciones típicas de cocinado (160-200°C durante 5-10 min), distinta resistencia a la oxidación térmica para diversos tipos de EV (ésteres de fitoestanol > ésteres de fitoesterol >

fitoesteroles libres) en alimentos enriquecidos en EV como margarina, mantequilla y en aceites (Lin et al., 2016). Comparando distintos tipos de procesado culinario en margarina, Scholz et al. (2016) constatan que el cocinado mediante horno (200ºC, 20 min) favorece la susceptibilidad de los EV a la oxidación, frente a un calentamiento en sartén (200ºC, 9 min) o microondas (800W, 4 min). A su vez, en este estudio se pone de manifiesto que a mayor superficie del alimento en contacto con el oxígeno, mayor es la oxidación (calentamiento en cazuela *versus* en botella) y, además, se afirma que el uso de ésteres de fitoestanol para el enriquecimiento de margarina presenta mayor resistencia a la oxidación térmica que el uso de ésteres de fitoesteroles y fitoestanoles.

Almacenamiento

En una recopilación de estudios en alimentos enriquecidos con EV (leche, bebidas, margarina y chocolate), se señala que las condiciones típicas de almacenamiento (4-38ºC, 5-24 semanas) no afectan en gran medida a la formación de POPs (Lin et al., 2006). Sin embargo, diversos factores pueden contribuir a su formación durante el almacenamiento: *(i) temperatura y tiempo*; en leche en polvo (Soupas et al., 2006) y margarinas (Rudzińska et al., 2014; Panpipat et al., 2018) se ha indicado una relación positiva entre la temperatura (25-55ºC) y el tiempo (5 días-12 meses) de almacenamiento y la oxidación de los EV, sin embargo, en un trabajo llevado a cabo por nuestro grupo de investigación (González-Larena et al., 2015) con bebidas a base de leche y/o zumo indican que el tiempo de almacenamiento (2-6 meses), pero no la temperatura (4-37ºC), es un factor condicionante en este tipo de bebidas; *(ii) temperatura y forma de adición de los EV (libres o esterificados)*; en margarinas almacenadas a temperatura ambiente, la formación de POPs es superior para los EV en forma esterificada, sin embargo, cuando aumenta la temperatura (55ºC) es la forma libre la más susceptible a la oxidación (Panpipat et al., 2018); *(iii) incidencia de luz y tiempo*; la exposición a luz solar *versus* oscuridad favorece la producción de POPs durante el almacenamiento (35 días) en margarinas (Julien-David et al., 2014); *(iv) presencia de antioxidantes;* en chocolate negro almacenado a 30ºC durante 5 meses, no influye la adición de antioxidantes (α-tocoferol y ácido ascórbico) en la formación de POPs (Botelho et al., 2014); sin embargo, en un

estudio llevado a cabo por nuestro grupo de investigación (González-Larena et al., 2015), la presencia de futas (ricas en antioxidantes) disminuye la susceptibilidad de los EV a la oxidación (bebida a base de leche ≥ bebida a base de zumo de frutas > bebida a base de leche y zumo de frutas), independientemente del tiempo (0-6 meses) y temperatura (4-37ºC) de almacenamiento.

En este contexto, en esta Tesis Doctoral se han identificado y cuantificado los SOPs en bebidas a base de zumo y leche enriquecidas con EV (0,8%) y MFGM durante 6 meses de almacenamiento a temperatura ambiente.

Este estudio ha dado lugar a la publicación: Alvarez-Sala, A., Blanco-Morales, V., Cilla, A., Garcia-Llatas, G., Sánchez-Siles, L.M., Barberá, R., Lagarda, M. J. (2018). *Safe intake of a plant-sterol enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage.* Journal of Food Composition and Analysis, 68, 111–117.

2.3.- Contenidos e ingestas

Alimentos no enriquecidos en esteroles vegetales

Los esteroles (colesterol y EV) contenidos en los alimentos son susceptibles de oxidarse. Los contenidos de COPs han sido ampliamente revisados en distintos grupos de alimentos como carnes y pescados y productos derivados, leche, mantequilla, huevo y queso (Sieber, 2005; Hur et al., 2007; Otaegui-Arrazola et al., 2010; Vicente et al., 2012; Brzeska et al., 2016). En concreto, la cantidad de COPs en productos cárnicos y derivados varía desde 0,1 µg/g (carne de vacuno) a 30,9 µg/g (mortadela), mientras que en pescados y derivados se indica 0,7 µg/g en salmón y 33,6 µg/g en anchoas. A su vez, en leche se pueden detectar contenidos de COPs de entre 0,1-7,7 µg/g, siendo los contenidos, en el caso de preparados para lactantes casi inapreciables (0,23-0,28 µg/g). En mantequillas, el contenido de COPs oscila entre 1,09-27,3 µg/g y alimentos como queso rallado o huevo en polvo pueden aportar altos contenidos de COPs (4-46 y 8-311 µg/g, respectivamente), en comparación con la mayonesa (2 µg/g) o productos de bollería como el cruasán (1,1 µg/g).

Por otro lado, los estudios sobre el contenido de POPs en alimentos no enriquecidos en EV son escasos (Tabla 5). Este hecho puede ser debido a que se

hallan como componentes traza y la matriz puede interferir en su análisis. Además, pueden existir otras limitaciones, al igual que en alimentos enriquecidos en EV, como es la escasez de patrones comerciales de POPs y la existencia de un amplio número de compuestos con estructuras muy similares (García-Llatas y Rodríguez-Estrada, 2011).

Tabla 5. Productos de oxidación de los esteroles vegetales (μg/g) en alimentos no enriquecidos

Alimentos	Contenido	Alimentos	Contenido
Aceites y grasas		*Otros*	
Avellana[1]	3,2-10,6	Cacahuete[5]	49
Cacahuete[2,3]	2,6-9,6	Chocolate negro[14]	14
Colza [1,4-7]	1,9-110	Papilla de cereales y leche[15]	0,06-0,07
Girasol[2,6,8,9]	4,5-67,5	Preparados para lactantes[16]	0,7-5
Maiz[2,3,5]	4,1-60,1	Productos de repostería[17,19]	1,07-27,8
Oliva[3,10,11]	3,3-7,7	Harina[18]	0,4-2,3
Palma[2,11]	1,9-5,5	Pan[19]	0,4-8
Soja[6,17]	0-0,8		
Margarina[5,12,13]	4-74		

1: Azadmard-Damirchi y Dutta (2009); 2: Bortolomeazzi et al. (2003); 3: Johnsson y Dutta (2006); 4: Lambelet et al. (2003); 5: Rudzińska et al. (2005); 6: Zhang et al. (2006); 7: Soupas et al. (2007); 8: Dutta (1997); 9: Zhang et al. (2005); 10: D´Evoli et al. (2006); 11: Tabee et al. (2009); 12: Conchillo et al. (2005); 13: Lin et al. (2018); 14: Botelho et al. (2014); 15: García-Llatas et al. (2008); 16: Zunin et al. (1998); 17: Cercaci et al. (2006); 18: Nourooz-Zadeh y Appelqvist (1992); 19: Hu et al. (2018)

Como se muestra en la Tabla 5, las matrices con alto contenido lipídico como los aceites son las más estudiadas, siendo los aceites de colza, girasol y maíz los que mayor contenido de POPs pueden presentar. También se han detectado POPs en frutos secos, chocolate negro, productos y preparados para lactantes, harina de trigo, pan y productos de repostería.

Alimentos enriquecidos en esteroles vegetales

Dado que actualmente se están enriqueciendo alimentos con ingredientes fuentes de EV, el consumo de POPs a partir de este tipo de alimentos podría ser mucho mayor debido al aporte de POPs procedente del ingrediente utilizado. En la Tabla 6 se muestra el contenido en POPs de diferentes ingredientes fuentes de EV utilizados para el enriquecimiento de alimentos.

Tabla 6. Productos de oxidación de los esteroles vegetales presentes en ingredientes

Origen	Pureza EV	POPs	Formato	Ref.
EV[a] disuelto en: Aceite de colza Grasa láctea anhidra Aceite de coco hidrogenado Aceite de palma refinado	18% / 30%	3,25 / 10,20 2,55 / 8,38 5,42 / 4,47 5,24 / 4,15	Suspensión microcristalina	Soupas et al. (2006)[a]
Soja	Min 25%	1,70	Emulsión líquida	
Soja	68-75%	15,66	En polvo	
Tall oil	Min 25%	2,89	Emulsión líquida	
Tall oil	68-75%	7,84	En polvo	González-Larena et al. (2011)[b]
Mezcla[*¥]	43,9%	4,86	Polvo pulverizado	
Mezcla[*]	86-92%	27,12	Polvo pulverizado	
Girasol + *tall oil*	12%	2,86	Emulsión líquida	
Girasol + *tall oil*	30%	7,08	Pasta oleosa	

Expresado como mg POPs/100 g ingrediente. Contienen [a] β-sitosterol y campesterol procedentes de *tall oil*. [b]campesterol, campestanol, estigmasterol, β-sitosterol y sitostanol; EV: esteroles vegetales; *a base de aceite de soja, colza, girasol y maíz; ¥EV esterificados. Ref.: referencias

Como se observa en la Tabla 6, cuanto mayor es la pureza (% en EV del ingrediente) mayor es la presencia de POPs. En el caso de los ingredientes analizados por Soupas et al. (2006), los POPs proceden de β-sitosterol, siendo los principales 7β-hidroxisitosterol y 5,6α-/β-epoxisitosteroles y, en menores cantidades, 7α-hidroxisitosterol y 7-cetositosterol. En las suspensiones que contienen 18% de EV, existe más presencia de POPs en aceite de coco hidrogenado y aceite refinado de palma, que en aceite de colza y grasa láctea anhidra. Sin embargo, en suspensiones que contienen 30% de EV, los autores observan que el comportamiento se invierte, sin aportar justificación al respecto. En un estudio posterior llevado a cabo por nuestro grupo de investigación (González-Larena et al., 2011), los principales POPs encontrados en 8 ingredientes analizados proceden también de β-sitosterol (7α-/β-hidroxisitosterol, 7-cetositosterol y sitostanetriol), atribuyéndose al hecho de que es el EV predominante en todos estos ingredientes. Los POPs mayoritarios en los ingredientes derivados de la soja son 7α-hidroxi, 7-ceto y triol, seguidos por 7β-hidroxi. En general, los ingredientes que contienen aceite de girasol, presentan como principal POP el 7-ceto, seguido de 7α-/β-hidroxi. En cuanto a los ingredientes procedentes de *tall oil*, existe mayor variabilidad en el orden de abundancia, siendo por lo general, 7-ceto o triol los mayoritarios. Los autores sugieren que las diferencias en el contenido total y el perfil de POPs obtenido entre ingredientes, es

debido a los diversos procedimientos de obtención, presencia de otros componentes (maltodextrina, inulina, sucrosa, jarabe de glucosa, ácido cítrico, ascorbil palmitato, ascorbato sódico, conservantes, caseinato sódico, extractos ricos en tocoferoles, leche desnatada en polvo, agua, emulsificantes, estabilizantes, sorbato potásico, tocoferoles y/o antioxidantes), origen y estado físico de los ingredientes, condicionándose así la susceptibilidad de los EV a la oxidación.

En la Tabla 7, se muestra una recopilación de contenidos de POPs en distintos alimentos enriquecidos en EV, contemplándose contenidos variables debido a la influencia de los diversos factores comentados en el apartado 2.2. como son: distinta matriz alimentaria, forma química de los EV (fitoesteroles, fitoestanoles, libres, esterificados), temperatura y tiempo de almacenamiento y/o calentamiento aplicado.

Ingestas estimadas

Scholz et al. (2015) en base al consumo de 3 g/día de ésteres de fitoesterol, estiman una ingesta de POPs de 1,2-2,9 mg/día, a partir de alimentos no tratados térmicamente (mantequilla, leche y chocolate) y de 3,5-4,2 o 29,6 mg/día a partir de leche (sometida a calentamiento doméstico) y margarina líquida (calentada a 205ºC, 30 min), respectivamente. En una revisión posterior (Lin et al., 2016) donde se contemplan mayor cantidad de alimentos enriquecidos (leche, leche en polvo, bebidas, chocolate, margarina, mantequilla y aceite de canola), se indica, que en base al consumo de 0,75-3 g/día de EV, se pueden alcanzar ingestas de POPs de: *(i)* 2,9-11,4 mg/día a partir de alimentos enriquecidos con ésteres de fitoesterol sin tratar térmicamente; *(ii)* 20-78, 12-48 o 0,8-3 mg/día para alimentos enriquecidos con fitoesteroles en forma libre, esterificada o ésteres de fitoestanol, respectivamente, y tratados térmicamente (140-200ºC, 5-120 min). Además, los autores, en base a estos datos, estiman una ingesta máxima de POPs en humanos a partir de alimentos enriquecidos con fitoesteroles en forma libre o esterificada de 1,1 y 0,69 mg/kg peso corporal/día, respectivamente; cantidades 115 y 186 veces inferiores a las indicadas como NOEL ("No Observed Effect Level") en ratas (Lin et al., 2016).

Tabla 7. Formación de productos de oxidación de los esteroles vegetales en alimentos enriquecidos tras diversos tratamientos

Alimento	Tratamiento	Contenido total POPs (µg/g)	% Oxidación	POPs estudiados	Referencia
Leche FE¥/ E-FE/ E-FA (0,5%)	Pasteurización (127 ºC, 2 seg)	2,18/2,03/0,24	0,08/0,09/0,01	7α-/β-hidroxi; 5,6α-/β-epoxi; 7-ceto	Soupas et al. (2006)
Chocolate negro (7,3% E-FE)	Almacenamiento (meses): (0) 30ºC (5)	68,67 71	0,09 0,10	7α-/β-hidroxi; 6β-hidroxi; 6-ceto; 7-ceto; α-epoxi; triol	Botelho et al. (2014)
Chocolate negro (7,3% E-FE + antioxidantes)	(0) 30ºC (5)	71 82,67	0,10 0,11		
Bebida a base de leche /Bebida a base de leche y zumo/Bebida a base de frutas (0,8% EV¥)	Almacenamiento (meses): (0) 4ºC (2) 24ºC (2) 37ºC (2) 4ºC (4) 24ºC (4) 37ºC (4) 4ºC (6) 24ºC (6) 37ºC (6)	3,59/2,42/4,97 4,92/2,88/5,24 5,41/3,39/4,80 5,89/3,54/4,91 5,23/4,13/3,24 5,10/4,01/3,40 4,83/4,09/2,84 9,32/3,77/2,66 7,02 /3,36/2,97 6,51/3,72/,2,66	0,07/0,05/0,08 0,09/0,05/0,08 0,09/0,06/0,08 0,11/0,06/0,08 0,10/0,08/0,05 0,08/0,07/0,06 0,09/0,08/0,05 0,16/0,07/0,04 0,14/0,06/0,05 0,12/0,06/0,04	7α-/β-hidroxi; α-/β-epoxi; 7-ceto; triol	González-Larena et al. (2015)
Margarina esterificada con: E-EV / E-FA (8 %)	Sin tratamiento Microondas (800W, 4 min)* Fritura (200ºC, 9 min) Horno (200ºC, 20 min)* Horno (200ºC, 20 min)#	92,9/22,6 780,2/43,4 1078,8/137,0 104,6/2902,6 225,5/5109,3	0,12/0,03 1,04/0,04 1,44/0,08 3,87/0,07 6,81/0,11	7α-/β-hidroxi; 5,6α-/β-epoxi; 7-ceto	Scholz et al. (2016)
Margarina (1,7% FE) / (1,7% E-FE)	25ºC (5-20 días) 55ºC (5-20 días)	2,5-25,5/2,5-42,5 20-160/10-60	-	7-ceto	Panpipat et al. (2018)

EV: fitoesteroles y fitoestanoles; E-EV: ésteres de EV; E-FA: ésteres de fitoestanoles; E-FE: ésteres de fitoesteroles; FE: fitoesteroles; ¥ en forma libre.; POPs: productos de oxidación de los EV; M: meses; Tratamiento * en botella, # en cazuela. % Oxidación: (contenido total POPs / contenido total EV) x 100.

2.4.- *Biodisponibilidad (absorción y metabolismo)*

La BA de SOPs ha sido evaluada por nuestro grupo de investigación (Alemany et al., 2013a), a partir de una digestión gastrointestinal simulada en bebidas lácteas y/o zumo de frutas enriquecidas en EV (0,8%). En todas las bebidas los únicos POPs detectados son los óxidos de β-sitosterol (EV más abundante en las mismas), como son los derivados triol, 7α-/β-hidroxi, α-/β-epoxi y 7-ceto, además de COPs como 7α-hidroxi, 7β-hidroxi y 7-ceto. En general, el 7-ceto derivado del β-sitosterol y del colesterol es el más abundante en todas las bebidas. Por su parte, en las FB no se detecta α-/β-epoxisitosterol ni 7-cetocolesterol. La ausencia de derivados epoxi de β-sitosterol podría justificarse por su conversión a trioles (hecho que se ve favorecido por el medio ácido en la etapa gástrica de la digestión). Además, los autores evidencian que la BA de los POPs es superior respecto a la correspondiente de los EV no oxidados (19-48% *versus* 2-7%), sin embargo, la BA de los COPs es menor en relación con la del colesterol (2-17% *versus* 53-99%), siendo sitostanotriol y 7β-hidroxicolesterol los que mayor BA presentan.

Por otro lado, se ha indicado que los COPs presentan menor absorción que el colesterol *in vivo*, debido posiblemente a su menor solubilidad en las micelas, la menor susceptibilidad a la esterificación en el interior de los enterocitos e incluso a los efectos citotóxicos en las células de la mucosa (Otaegui-Arrazola et al., 2010). En este sentido, en condiciones fisiológicas normales, se ha indicado que la concentración sanguínea de COPs es menor que la de colesterol (aproximadamente 0,01-0,1 µM *versus* 5000 µM), aumentándose a 20 µM cuando existe hipercolesterolemia (Cilla et al., 2017b). Por su parte, se estima que la absorción de POPs es mayor, en general, que la de los EV no oxidados, aunque existe controversia al respecto (Otaegui-Arrazola et al., 2010; O'Callaghan et al., 2014). Se ha indicado que la absorción de los derivados 7α-/β-hidroxi suele ser mayor que la de epoxi y 7-ceto derivados y que, además, como ocurre con los EV no oxidados, la estructura de la cadena lateral influye en el grado de absorción de los POPs. En este sentido, se ha señalado que la absorción de óxidos derivados de campesterol es mayor que la de los derivados de β-sitosterol tanto a nivel tisular como plasmático o linfático, sugiriendo que el menor número de átomos de carbono en la cadena lateral del campesterol

favorece su absorción (Hovenkamp et al., 2008; Otaegui-Arrazola et al., 2010; O'Callaghan et al., 2014).

El origen de los POPs en plasma puede deberse a: *(i)* la absorción de una pequeña cantidad de POPs presentes en los alimentos, *(ii)* transformación *in vivo* a partir de sus formas no oxidadas y *(ii)* la oxidación catalizada por radiaciones UV de EV, previamente absorbidos vía cutánea procedentes de productos cosméticos (Ryan et al., 2009; Brzeska et al., 2016). En este sentido, las concentraciones de POPs plasmáticas en sujetos sanos que no consumen alimentos enriquecidos en EV oscilan entre 4,18-109 µg/L (Scholz et al., 2015), siendo del orden de 1000 veces inferior a las determinadas en sujetos con sitosterolemia, donde los óxidos derivados del β-sitosterol alcanzan valores de 4,10-4,49 µg/mL (Plat et al., 2001). En cambio, en sujetos sanos que consumen margarina enriquecida con EV los datos son variables: Husche et al. (2011) observan un incremento sérico de 7β-hidroxisitosterol (86,7%) tras la ingesta de 3 g/día de fitoesteroles durante 28 días, sin embargo, el contenido total de POPs no se modifica. Baumgartner et al. (2013) no muestran incremento en el contenido plasmático de los POPs tras el consumo (4 semanas) de margarina enriquecida en fitoesteroles (3 g/día), mientras que observan una reducción de 7β-hidroxicampesterol (14%) tras la ingesta de margarina enriquecida en fitoestanoles (3 g/día). Posteriormente, este mismo grupo (Baumgartner et al., 2017), muestran reducciones plasmáticas de 7β-hidroxicampesterol (24%), 7β-hidroxisitosterol (17%), 7-cetositosterol (13%) y de POPs totales (15%), tras la ingesta durante 3 semanas de margarina enriquecida en fitoestanoles (3 g/día).

Existen pocos estudios donde se evalúe la distribución de los POPs en el organismo; a modo de ejemplo, en hamsters a los que se les suplementa con aceite de soja con POPs (100-2500 ppm), se cuantifican de forma dosis dependiente, distintas concentraciones (ng/mg) en los tejidos estudiados (aorta (10,4-75,7), hígado (0,7-90,5), riñón (19,5-275,4) y corazón (3,3-326,6)), principalmente con prevalencia de triol derivados de campesterol y β-sitosterol (Grandgirard et al., 2004).

2.5.- Funciones biológicas

Los COPs están involucrados no solo en procesos fisiológicos como la modulación del metabolismo de colesterol/lípidos o la estructura de la membrana, sino también en la iniciación y progresión de enfermedades crónicas tales como aterosclerosis, procesos inflamatorios, fallo renal, cáncer, diabetes, procesos neurodegenerativos y enfermedades visuales (Otaegui-Arrazola et al., 2010; Vicente et al., 2012; Poli et al., 2013; Kulig et al., 2016; Cilla et al., 2017b; Olkkonen et al., 2017). Cabe esperar, dada la similitud estructural, que el efecto sobre el organismo de los POPs sea comparable, habiéndose publicado diversas revisiones al respecto (Hovenkamp et al., 2008; Ryan et al., 2009; García-Llatas y Rodríguez-Estrada, 2011; Vanmierlo et al., 2013; Alemany et al., 2014; O'Callaghan et al., 2014).

Modulación del metabolismo del colesterol

Los posibles mecanismos mediante los cuales los COPs podrían influir en el metabolismo del colesterol han sido ampliamente revisados: bloqueo de la proteína de unión de esteroles (SREBP), degradación de la HMGCoA reductasa (implicada en la síntesis de colesterol) y estimulación del receptor X del hígado (LXR), receptor implicado en la transcripción de genes involucrados en el metabolismo lipídico, como la familia de las proteínas ABC (Otaegui-Arrazola et al., 2010). Sin embargo, existe controversia en el efecto de los POPs sobre la alteración de la expresión de proteínas y enzimas implicadas en el metabolismo del colesterol (O'Callaghan et al., 2014). En este sentido, en hámsters, se ha descrito que los óxidos derivados de estigmasterol y β-sitosterol (mezcla de 7α-/β-hidroxi, α-/β-epoxi y 7-ceto; 0,1%) no reducen el contenido de colesterol plasmático y no alteran la expresión de la enzima ACAT o de las proteínas MTP, NPC1L1 y ABCG8, sin embargo, dichos óxidos disminuyen la expresión de la proteína transportadora ABCG5, y solo los óxidos derivados de estigmasterol reducen la expresión de los genes implicados en la síntesis del receptor de c-LDL, además de la actividad de la enzima HMGCoA reductasa (Liang et al., 2011). Por su parte, en un trabajo llevado a cabo por nuestro grupo de investigación en células de epitelio intestinal (Caco-2) con 7-cetoestigmasterol y 7-cetocolesterol (60 μM), se confirma la ausencia de efecto sobre

la expresión de NPC1L1, sin embargo, se ve disminuida la expresión de las proteínas transportadoras ABCG5/8 y aumentada la actividad de la enzima HMGCoA reductasa (Alemany et al., 2013b). Además, se ha especulado, mediante ensayos *in vitro*, que los POPs procedentes de una oxidación enzimática de la cadena lateral (derivados sintéticos del campesterol (22(E)-campesta-5,7,22-trieno-3β,25-diol), estigmasterol (estigma-5-en-25-ol-3-ona) y ergosterol (22(E)-ergost-22-en-1α,3β-diol)) pueden estar más involucrados en la modulación del metabolismo del colesterol mediante activación del receptor LXR, que los procedentes de una oxidación enzimática del núcleo esteroideo (7α/β-hidroxi, 7-ceto y 5,6α/β-epoxi derivados de β-sitosterol, campesterol y estigmasterol) (Hovenkamp et al., 2008).

Enfermedad aterosclerótica

La implicación de los COPs en el proceso de aterosclerosis ha sido ampliamente estudiada y parece intervenir a distintos niveles: *(i)* disfunción de las células endoteliales aumentando su permeabilidad; *(ii)* adhesión y transmigración de monocitos (expresión de moléculas de adhesión celular, quimiocinas y citoquinas); *(iii)* generación de células espumosas; *(iv)* inflamación y formación de una capa fibrótica; *(v)* apoptosis vascular y degradación de la matriz extracelular (Otaegui-Arrazola et al., 2010). Por otro lado, se ha indicado que los COPs causan eriptosis (Tesoriere et al., 2014), proceso que contribuye a complicaciones tromboembólicas, y corrobora el potencial efecto de los COPs en la aterogénesis (Cilla et al., 2017b). Sin embargo, el papel de los POPs sobre el efecto aterogénico no ha sido estudiado en profundidad. En este sentido, se ha observado, mediante estudios *in vivo* (ratones y ratas), que el consumo de POPs no supone un incremento en el tamaño de la lesión aterosclerótica (Tomoyori et al., 2004; Plat et al., 2014b), aunque otras evidencias como el hecho de que dificulten la vasorelajación, pudiendo perjudicar la funcionalidad de la aorta, sugieren que existe un potencial efecto aterogénico en dichos compuestos (Yang et al., 2013). Dado que los datos sobre el papel aterosclerótico de los POPs son escasos, no es posible afirmar su participación en dicha enfermedad (O'Callaghan et al., 2014).

Proceso inflamatorio

Los COPs son considerados reguladores de la expresión del factor de transcripción NF-κB a través de la activación de la proteína quinasa C, lo que implica un aumento de las reacciones inflamatorias mediante la expresión y síntesis de biomarcadores inflamatorios como citoquinas, quimiocinas y moléculas de adhesión. Además, se ha indicado que 7α-/β-hidroxicolesterol y 7-cetocolesterol causan fenotipo inflamatorio en células endoteliales y, a su vez, 7-cetocolesterol induce la creación de células espumosas (Poli et al., 2013; Brzeska et al., 2016). Respecto a los POPs, Vejux et al. (2012), en células monocíticas (U937), observan que 7β-hidroxisitosterol no influye en la secreción de la proteína quimiotáctica MCP-1 o la citoquina proinflamatoria IL-8. En este sentido, en ratones, Plat et al. (2014b) tampoco observan cambios en las concentraciones séricas de MCP-1 ni en TNF-α, en respuesta al consumo de una dieta enriquecida con POPs (0,025%). Recientemente, se ha indicado que los POPs (7β-hidroxi o 7ceto derivados de campesterol/β-sitosterol) no presentan una clara capacidad para mediar respuestas inflamatorias en macrófagos procedentes de ratas (Oligschlaeger et al., 2018). Por el contrario, en un estudio llevado a cabo por nuestro grupo de investigación en células Caco-2, se indica que 7-cetoestigmasterol presenta mayor efecto proinflamatorio que 7-cetocolesterol, asociado a un mayor aumento en la producción de citoquinas proinflamatorias (IL-8 y TNF-α). Los autores sugieren que el aumento en TNF-α puede tener consecuencias importantes sobre la integridad del epitelio intestinal (Alemany et al., 2013b).

Citotoxicidad

Los efectos citotóxicos de los SOPs se asocian con la inducción de muerte celular (apoptosis), actividades oxidativas e inflamatorias, fosfolípidosis, variaciones de los niveles citoplasmáticos de Ca^{2+}, alteraciones de la membrana mitocondrial y microsomal y metabolismo de las poliaminas, con la consiguiente sobreproducción intracelular de ROS (Cilla et al., 2017b). Diversas recopilaciones de estudios con líneas celulares, muestran que los POPs presentan efectos citotóxicos similares a los de los COPs, pero son necesarias mayores concentraciones para ello (> 60 µM), siendo en general, los óxidos derivados de β-sitosterol y campesterol, seguidos por

los óxidos de estigmasterol (principalmente triol, 7β-hidroxi y 7-ceto), los que mayor efecto citotóxico presentan (Hovenkamp et al., 2008; Ryan et al., 2009; O'Callaghan et al., 2014; Scholz et al., 2015). En un estudio realizado por nuestro grupo en células de epitelio intestinal (Caco-2) se indica que, a diferencia de 7-cetocolesterol, 7-cetoestigmasterol (30-120 µM) no presenta efectos citotóxicos e incluso reduce su toxicidad disminuyendo los efectos metabólicos perjudiciales sobre la funcionalidad e integridad mitocondrial y restaurando la distribución del contenido de ARN en las fases G_1 y G_2 del ciclo celular (Alemany et al., 2012). Asimismo, recientemente se ha indicado que diversos fitoquímicos dietéticos como polifenoles, carotenoides, vitaminas C y E podrían proteger frente la citoxicidad asociada a los SOPs (Cilla et al., 2017b).

Actividad estrogénica/androgénica

En general, se ha señalado una ausencia de actividad agonista de los POPs sobre los receptores andrógenicos y estrógenicos. Sin embargo, determinados POPs poseen cierta afinidad estructural con ligandos de receptores de activación de estrógenos, limitando la unión de éstos a dichos receptores (Vanmierlo et al., 2013). Este hecho se ha observado en células de cáncer de cuello uterino (HeLa) para POPs específicos (sitostanotriol, campestanotriol y β-epoxisitosterol), al inducir, aunque en menor medida que los correspondientes COPs, actividad luciferasa (marcador de la activación de los receptores estrogénicos) (Sato et al., 2004). Además, se ha indicado que los óxidos de estigmasterol modulan la acción del estradiol en células de adenocarcinoma de mama (MCF-7) y de endometrio humano (Ishikawa) (Newill et al., 2007). Por otro lado, en ensayos *in vivo* existe controversia, ya que en pez mosquito (mosquitofish), los óxidos de β-sitosterol y estigmasterol no producen efecto androgénico (van den Heuvel et al., 2006), mientras que en pez cebra, los óxidos de β-sitosterol se han asociado con dicha acción (Christianson-Heiska et al., 2007).

Diseño experimental y difusión de resultados
Experimental design and results dissemination

En la Figura 9 y 10 se detallan el esquema del diseño experimental realizado en la presente Tesis Doctoral y la difusión de resultados en artículos, respectivamente.

Figure 9. Experimental design

Section 1. Sterol determination in PS-enriched skimmed milk-based fruit beverages

Impact of lipid components and emulsifiers on plant sterols bioaccessibility from milk-based fruit beverages. Journal of Agricultural and Food Chemistry, 2016, 64, 5686−5691. (Annex 1)

Safe intake of a plant-sterol enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage. Journal of Food Composition and Analysis, 2018, 68, 111−117. (Annex 1)

Section 2. Biological effects

Positive impact on serum lipid profile and cytokines after consumption of a plant sterol-enriched beverage with milk fat globule membrane: a clinical study (Food & Function) (under review). (Annex 2)

Effects of plant sterols and/or β-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death. Journal of Agricultural and Food Chemistry, 2018, 66, 1157−1166. (Annex 1)

Anti-proliferative effect of phytosterol-ingredient and its main phytosterol (β-sitosterol) on human cancer cell lines (International Journal of Food Sciences and Nutrition) (under review). (Annex 2)

Physiological concentrations of phytosterols enhance the apoptotic effects of 5-fluorouracil in colon cancer cells (Journal of Functional Foods) (under review). (Annex 2)

Articles

Figure 10. Results dissemination
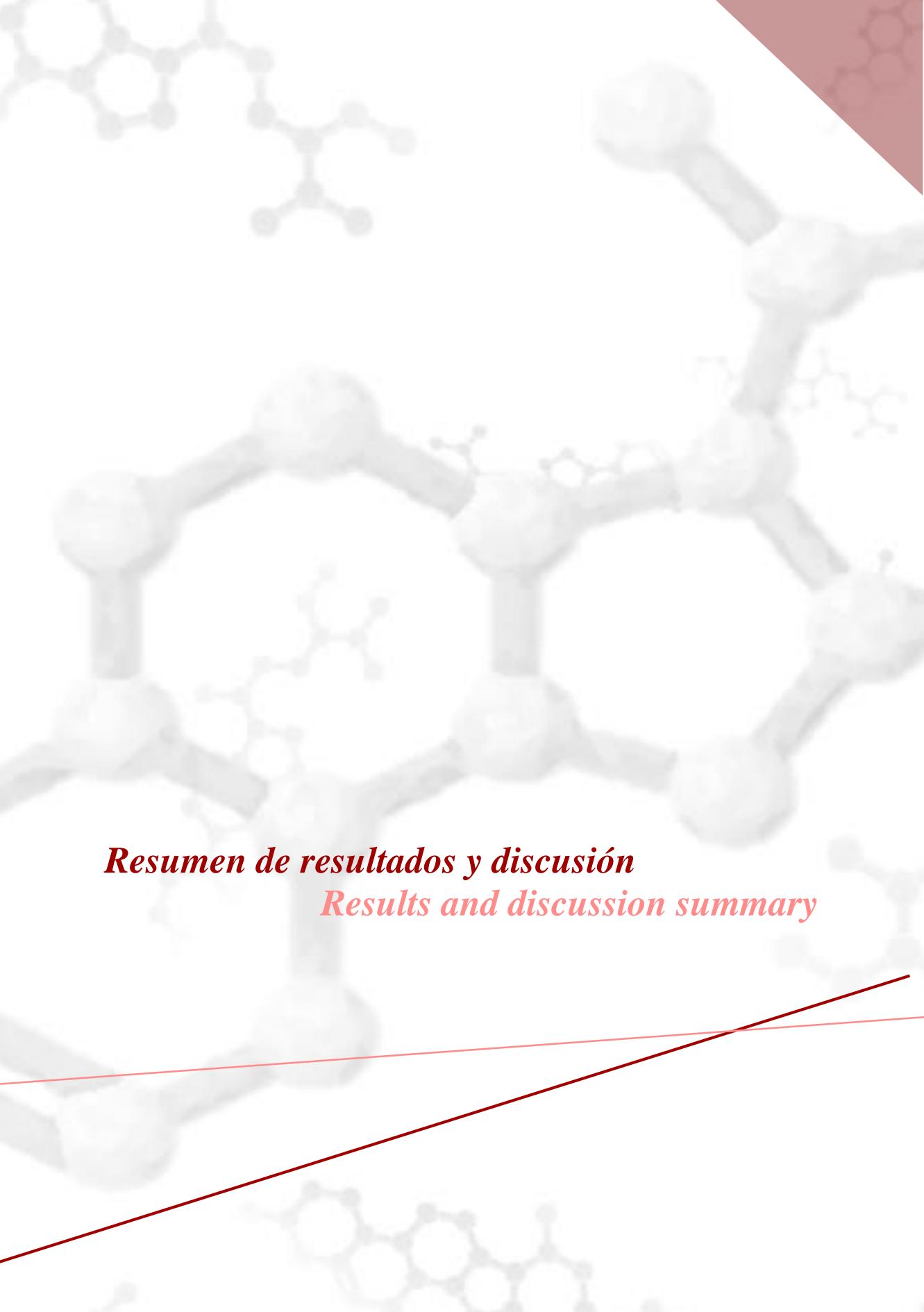
Resumen de resultados y discusión
Results and discussion summary

According to the objectives above (see pages 9-12), the summary of results of the present Doctoral Thesis are shown below. The methodology applied in each of the studies is described in detail in the corresponding articles (see annexes).

1.- Sterol determination in plant sterol (PS)-enriched skimmed milk-based fruit beverages

♦ *Sterol bioaccessibility (BA) of three PS-enriched skimmed milk-based fruit beverages with different type and/or content of fat and emulsifiers*

A prerequisite to improve the knowledge of functional efficiency associated with PS consumption is to know not only the amount of PS but also their bioavailability. The measurement of the BA is a good option as an *in vitro* tool to estimate bioavailability. The presence of certain substances within a food matrix could influence PS bioavailability. Thus, different factors were evaluated due to their potential interaction with the BA of PS. The factors evaluated were type of fat (milk fat or extra virgin olive oil), quantity of fat (1.1-2.4%), and emulsifier type (milk fat globule membrane (MFGM) or lecithin) in the context of three PS-enriched skimmed milk-based fruit beverages; *(i)* without fat addition (MFb), *(ii)* with addition of milk fat and whey protein concentrate enriched with MFGM (MFbM), and *(iii)* with extra virgin olive oil and soy lecithin addition (MFbO). All the beverages contained food stabiliser (pectin), skimmed milk, mandarin juice as source of β-cryptoxanthin (β-Cx), banana puree, grape juice, and free microcrystalline PS from *tall oil* in powder form (0.8%).

In the three beverages, cholesterol as well as PS (β-sitosterol, sitostanol, campesterol, campestanol, and stigmasterol) were identified and quantified. Cholesterol content in the beverages ranged from 2 to 8 mg/100 g beverage, being higher in MFbM, because of the addition of milk fat and MFGM. Total PS contents ranged from 613 to 856 mg/100 g of beverage, and the relative PS abundance was headed by β-sitosterol, followed by sitostanol and campesterol, with lower amounts of campestanol and stigmasterol – a profile that corresponds to the PS-ingredient used (*tall oil*) for enrichment of the beverage (González-Larena et al., 2011). Relative percentages of each PS obtained in these beverages, comply with the PS

profiles specified by the European Commission for milk based fruit drinks (Decision 2004/336/EC). Similar PS profiles were reported by our research group in other milk-based fruit beverages using the same PS source (González-Larena et al., 2012; Alemany et al., 2013a; Blanco-Morales et al., 2018). As far as we are aware, only our research group carries out these kind of studies with PS-enriched milk-based fruit beverages.

The BA was tested through simulated micellar gastrointestinal digestion in three phases (salivary, gastric and intestinal). The BA of cholesterol (53.5-64.2%) was significantly ($p<0.05$) higher than that of total PS (8.7-31.4%). In model systems using different bile salts to assess sterol solubility, it has been found that cholesterol presented the highest solubility, possibly due to its lower hydrophobicity compared with PS (Armstrong & Carey, 1987; Matsuoka et al., 2008, 2010, 2015). Higher BA of cholesterol than PS has also been observed in similar PS-enriched skimmed milk-based fruit beverages (Alemany et al., 2013a; Blanco-Morales et al., 2018).

The BA of total PS from the beverages ranked as follows: MFbM (31.4%) = MFbO (28.2%) > MFb (8.7%). The individual PS in the same beverage showed similar BA, except in MFb, where the greatest BA corresponded to campestanol, and in MFbO, where a higher variability between individual PS was observed; being stigmasterol, although not statistically significant ($p>0.05$), the PS with the lowest BA. The structure of PS seems to play an important role in its solubility. Solubility appears to be favoured when the sterol side chain does not have double bonds and has fewer carbon atoms (Armstrong & Carey, 1987; Matsuoka et al., 2008, 2010, 2015); this fact could explain why campestanol (one of the PS with fewer carbon atoms) shows higher BA, and stigmasterol (which has an unsaturated double bond on the side chain) lesser BA. The greater contribution of campestanol BA found in MFb could explain the higher (1.3- to 2-fold) BA of total PS compared with a similar skimmed milk-based fruit beverage, although manufacturing conditions were different (laboratory or industrial scale) (Alemany et al., 2013a). Regarding the MFbM beverage, Blanco-Morales et al. (2018), recently showed similar total BA of PS to our study (37.2% *versus* 31.4%) after the same micellar gastrointestinal digestion with the same beverage formulation but with higher PS enrichment (1%

versus 0.8%). The BA was lower (14.4%) when the beverage was subjected to Infogest gastrointestinal digestion method according to Minekus et al. (2014). The need to adapt the Infogest method for its application to food matrices containing lipophilic bioactive compounds such as sterols has been suggested since this method is mainly a consensus established for food protein matrices.

The higher BA of MFbM and MFbO compared with MFb beverage could be due to the higher fat content (2.4% *versus* 1.1%) and the presence of emulsifiers (MFGM or lecithin), which could promote the formation of mixed micelles, making the PS incorporation easier into the micelles during gastrointestinal digestion. Generally, the favourable influence of lecithin upon PS solubility has been shown in model systems (Ikeda et al., 1989; Ostlund et al., 1999; Matsuoka et al., 2012), and recently, He et al. (2017) reported that soy lecithin has a positive emulsifying effect on PS in milk. In the case of MFGM, its influence upon PS solubility has not been directly studied yet, but it is considered to be an efficient natural surface-active emulsifier, highly effective in lowering the interfacial tension (Contarini & Povolo, 2013). Moreover, the PS may be incorporated into the milk globule membrane and be readily available for transfer into the micellar membrane (Clifton et al., 2004).

The results obtained in this study have been published in: Alvarez-Sala, A., Garcia-Llatas, G., Cilla, A., Barberá, R., Sánchez-Siles, L. M., Lagarda, M. J. (2016). *Impact of lipid components and emulsifiers on plant sterols bioaccessibility from milk-based fruit beverages.* Journal of Agricultural and Food Chemistry, 64, 5686−5691.

♦ *Sterol stability and oxidation during shelf-life of the selected beverage*

Taking into account that sterols can be oxidised (see section 2. of the literature review), and since the MFbM beverage will be used for a clinical study, the sterol stability, the formation of sterol oxidation products (SOPs), and their BA during storage (0-6 months) at room temperature (20-25ºC) have been evaluated. As mentioned above, this beverage (containing MFGM) showed the highest BA of PS (although not statistically different ($p > 0.05$) with respect to MFbO). This fact, added to the several health benefits attributed to the compounds of MFGM (including the

lowering of blood cholesterol levels and inhibition of cancer cell growth, among others (Bernard et al., 2017)), makes the selection of the MFbM beverage a good option to carry out a subsequent human intervention trial in order to study its possible health benefits.

At all time points (0, 3 and 6 months), there were no statistically significant differences ($p > 0.05$) in total PS and individual sterols (PS and cholesterol). Total PS and cholesterol contents ranged from 824 to 856 and 7.6 to 8.2 mg/100 g of beverage, respectively. The PS stability observed in the MFbM beverage during the storage period, as previously suggested for similar milk-based fruit beverage, could be due to the presence of natural antioxidants from fruit juices (polyphenols and carotenoids), as well as an increase in total antioxidant capacity (probably due to later Maillard reaction products formed during storage), and to the antioxidant activity of casein and whey proteins (González-Larena et al., 2012). This is in agreement with previous studies carried out with these kind of beverages (but without milk fat and MFGM added), which showed no loss in initial PS (β-sitosterol, campesterol, stigmasterol, sitostanol and campestanol) content over time. Samples were analysed after 0, 2 and 4 months of storage at room temperature (Garcia-Llatas et al., 2015b) or after 0, 2, 4 and 6 months of storage at 4, 24 and 37ºC (González-Larena et al., 2012).

In the MFbM beverage, the BA of individual as well as total PS (29-32%) remained stable during the storage period, while BA of cholesterol ranged from 64-75%, being slightly higher at 6 months of storage. The higher BA of cholesterol *versus* PS could be ascribed to greater micellar solubility of cholesterol (Matsuoka et al., 2012), as it has been previously discussed for this kind of beverage.

In spite of the fact that PS appear to remain stable during storage, a possible formation of SOPs cannot be ruled out, because the sterols present in food (cholesterol and PS) are susceptible to oxidation, that in turn, could have important consequences for consumer health (Alemany et al., 2014). The oxidised products formed are respectively known as cholesterol oxidation products (COPs) and plant sterol oxidation products (POPs). Overall, COPs and POPs are referred to SOPs (Brzeska et al., 2016).

In the course of the storage period (0-6 months) at room temperature, the same oxides of cholesterol and β-sitosterol were detected (7α-/β-hydroxy, α-/β-epoxy, triol and 7-keto). Only POPs corresponding to β-sitosterol were identified, which would agree with the fact that it is the most abundant PS in the beverage. This fact was in agreement with previous studies in similar PS-enriched beverages, where β-sitosterol oxides were the only POPs detected (Alemany-Costa et al., 2012; Alemany et al., 2013a).

The total content of POPs ranged from 187 to 204 µg/100 g of beverage, with no statistically significant differences ($p > 0.05$) during storage (0-6 months). Regarding the individual POP contents from the beverage analysed just after elaboration, the most abundant oxide was β-epoxysitosterol followed by α-epoxysitosterol, 7β-hydroxysitosterol and 7-ketositosterol, being sitostanetriol and 7α-hydroxysitosterol the least abundant. Only a slight significant ($p < 0.05$) increase was recorded for sitostanetriol after 6 months of storage. In similar skimmed milk-based fruit beverages (without milk fat and MFGM added), just after manufacture and after 6 months of storage at 24ºC, González-Larena et al. (2015), in agreement with our own study, β-epoxysitosterol was found to be the most abundant POP. However, in previous studies on these kind of beverages, 7β-hydroxy (Alemany-Costa et al., 2012) or 7-ketositosterol (Alemany et al., 2013a) were the most abundant POPs just after elaboration of the beverages. The differences observed could be attributed to several factors such as the POP profile present in the ingredient source of PS, the beverage matrix (without milk fat and MFGM added) and the manufacturing conditions involved (laboratory or industrial scale).

Regarding COPs, the total amounts ranged between 99-103 µg/100 g of beverage, with no changes during 6 months of storage, being cholestanetriol and 7-ketocholesterol the main COPs, and 7α/β-hydroxycholesterol the lowest. A slight significant ($p < 0.05$) rise in 7α/β-hydroxycholesterol was observed from three months onwards during the storage period. Only one study (Alemany et al., 2013a) reported contents of COPs in skimmed milk-based fruit beverages; while the order of abundance of individual COPs was in agreement with our study, the authors found higher total COPs amounts (201 µg/100 g of beverage), despite the lower cholesterol

content in those beverages compared with our own study (1.4 *versus* 8.2 mg/100 g beverage).

When comparing the formation of COPs and POPs respect to the content of its non-oxidised analogues in the MFbM beverage, cholesterol presented higher rates of oxidation than β-sitosterol (1.2-1.3% *versus* 0.027-0.029%). This behaviour was in agreement with the study of Alemany et al. (2013a). Further, it is suggested that the incorporation of milk fat and MFGM in the elaboration of the MFbM beverage, do not imply greater PS oxidation, since the oxidation percentages of β-sitosterol were lower than reported by González-Larena et al. (2015) in PS-enriched milk-based fruit beverages during storage at room temperature (0.03-0.06%) (0-6 months).

As with the analogous non-oxidised sterols, the BA of total POPs were lower (45-49%) than COPs (58-80%), with no statistically significant (p>0.05) differences through 6 months of storage. The oxides with the highest BA values were triol, α-epoxy and 7β-hydroxysitosterol for POPs, and 7α-/β-hydroxycholesterol for COPs.

Taking into account the percentage of PS or cholesterol oxidation ratios and the PS (2.14 g) or cholesterol (20.4 mg) intake from the consumption of 250 g (daily portion) of beverage, the estimated contribution of the MFbM beverage to daily SOPs intake was 0.5 and 0.25 mg/day of POPs and COPs, respectively. The SOPs value intake are below that indicated by others authors: 1.2-11.4 mg POPs/day from PS-enriched foods as milk, chocolate and margarine (Scholz et al., 2015; Lin et al., 2016) and 1.8-3.1 mg COPs/day from a diet including raw food or baked/fried/grilled food with or without fruit and vegetables (van de Bovenkamp et al., 1988). Moreover, the bioaccessible amount of SOPs (1.5-2.2 µM), obtained after simulated gastrointestinal digestion of the beverage, was far from cytotoxic concentrations (30-120 µM) reported in different cell cultures (O'Callaghan et al., 2014; Laparra et al., 2015).

Considering that no loss in initial sterol content occurred during 6 months of storage of the beverage, and that the estimated intake of SOPs is low, the MFbM beverage can be considered suitable as a PS-enriched food matrix during its shelf-

life, and its consumption appears to be safe for consumers during the intervention period of the *in vivo* study.

The study of PS stability has been included in the research article (under review): Alvarez-Sala, A., Blanco-Morales, V., Cilla, A., Silvestre, R. A., Hernández-Álvarez, E., Granado-Lorencio, F., Barberá, R., Garcia-Llatas, G. (2018). *Positive impact on serum lipid profile and cytokines after consumption of a plant sterol-enriched beverage with milk fat globule membrane: a clinical study* (Food & Function).

The results obtained from sterol oxidation formation during shelf-life of the beverage have been published in: Alvarez-Sala, A., Blanco-Morales, V., Cilla, A., Garcia-Llatas, G., Sánchez-Siles, L.M., Barberá, R., Lagarda, M. J. (2018). *Safe intake of a plant-sterol enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage.* Journal of Food Composition and Analysis, 68, 111–117.

2.- Biological effects

♦ *Clinical study: influence of the consumption of a PS-enriched milk-based fruit beverage (MFbM) on serum lipid profile and inflammatory markers*

Plant sterols have many beneficial functions, mainly a cholesterol-lowering effect at intakes of 1.5-3 g/day (Gylling et al., 2014), and it has been suggested that MFGM provided by dairy products is also associated with cholesterolemia-lowering (Bernard et al., 2018). Therefore, in the present Doctoral Thesis, continuing with previous studies of our research group, it was evaluated whether the presence of milk fat and MFGM in the PS-enriched MFbM beverage, increases the PS (β-sitosterol, campesterol and stigmasterol) and cholesterol-precursors (desmosterol and lathosterol) bioavailability besides the expected hypocholesterolemic effect upon consumption. To achieve this purpose, a randomized, double-blind, crossover, multiple-dose bioavailability study (NCT 02065024) with postmenopausal women (n=38, 49-66 years and BMI of 24.6±4.7 kg/m^2) presenting untreated mild hypercholesterolemia was performed. The habitual diet of the volunteers was

supplemented with 1 x 250 mL beverage/day for 6 weeks in random order by using a computer-based table of pseudo-random numbers with a washout period of 4 weeks between the interventions. Postmenopausal women are considered to be at higher risk of bone demineralisation, and hypercholesterolemia is a risk factor for osteoporosis in this group. In a previous study carried out by our research group, a positive effect has been demonstrated on cardiovascular risk and bone turnover markers after the consumption of a milk-based fruit beverage containing β-Cx plus PS but without milk fat and MFGM added in its formulation (Granado-Lorencio et al., 2014).

The intake of the MFbM beverage induced statistically significant reductions ($p<0.05$) (post-treatment *versus* pre-treatments values) in serum total cholesterol (212.9±25.8 *versus* 220±27.8 mg/dL) and LDL-cholesterol (121.7±24.4 *versus* 129.4±28.5 mg/dL), which meant changes of -2.9% and -5.1%, respectively. HDL-cholesterol levels after consumption of this beverage were comparable to levels prior the intervention (71.4±20 *versus* 71.7±16.9 mg/dL). In addition, the consumption of the MFbM beverage significantly ($p<0.05$) increased serum campesterol (43.1%), β-sitosterol (32.5%) and lathosterol (11.2%) concentrations after the intervention, while the stigmasterol (minor PS in the beverage) and desmosterol concentrations showed no variations. The increase in serum lathosterol, a marker of cholesterol synthesis, can be related to a compensatory homeostatic mechanism of cholesterol absorption inhibition by PS (Santosa et al., 2007). In turn, the lack of changes in stigmasterol (with unsaturated double bond on the side chain) level could be due to lower solubility, as discussed in section 1.1.

Upon comparing the results obtained in the present study with those previously published by our research group (Granado-Lorencio et al., 2014; Garcia-Llatas et al., 2015b), involving a clinical trial with postmenopausal women who ingested a similar PS-enriched milk-based fruit beverage (without milk fat and MFGM added in their formulation), it was observed that the presence of milk fat and MFGM in the MFbM beverage did not improve the hypocholesterolemic effect, since a slight lower reduction in total and LDL-cholesterol (3-5% *versus* 5-7% (Granado-Lorencio et al., 2014)) was detected. Furthermore, different sterol level modifications were reported

in these previous studies (1.4-fold higher increase in β-sitosterol and 2.2-fold lower in campesterol serum levels (Garcia-Llatas et al., 2015b) and no modifications of lathosterol concentrations (Granado-Lorencio et al., 2014)). The discrepancies observed after comparing with the present study, could be due to the slight differences in the formulation of the beverages, with lower PS dose consumed (1.5 *versus* 2 g/day), lesser percentage of campesterol (5.5% *versus* 7%), lower fat content (1.3% *versus* 1.9% (with addition of milk fat and MFGM)), as well as shorter duration of the study intervention (4 *versus* 6 weeks).

As described in the literature review, the magnitude of serum total cholesterol and LDL-cholesterol reductions and the increase in sterol (cholesterol-precursors and PS) levels, may vary depending on daily amount of PS consumed, PS type, food matrix and study design, among other factors (Ras et al., 2013, 2014; Ferguson et al., 2016; He et al., 2018; Moreau et al., 2018).

The postmenopausal period in women has also been associated with increments of proinflammatory cytokines (Camilleri et al., 2012). Although PS are well known for their cholesterol-lowering effect, their impact on inflammation is unclear (Rocha et al., 2016). Therefore, the impact of MFbM consumption on serum proinflammatory (IL-1β, IL-6, IL-8, IL-12p70, and TNF-α) and antiinflammatory (IL-10) cytokines, was also evaluated for the first time. A statistically significant ($p < 0.05$) decrease in proinflammatory IL-1β (6.7%) and a concomitant increase in antiinflammatory IL-10 cytokine levels (22.5%) was recorded, and no variations in other cytokines were detected during the intervention.

Few studies have evaluated the relationship between serum/plasma cytokine levels and the consumption of beverages and dairy products enriched with PS. These in turn have shown variable results; while some authors showed reductions in proinflammatory cytokines (IL-1β, IL-6, IL-8 and/or TNF-α) (Devaraj et al., 2011; Kunces et al., 2013; Brüll et al. 2016; Kurano et al., 2018), other authors found no changes (Plat el al., 2009; Kunces et al., 2013; Ho et al., 2016). Thus, regarding our results, the MFbM beverage could be an adequate matrix which may contribute to decrease cardiovascular disease risk through cholesterol-lowering and improvement of inflammatory biomarkers, although more studies are required.

These studies have allowed to submit a research article (under review): Alvarez-Sala, A., Blanco-Morales, V., Cilla, A., Silvestre, R. A., Hernández-Álvarez, E., Granado-Lorencio, F., Barberá, R., Garcia-Llatas, G. (2018). *Positive impact on serum lipid profile and cytokines after consumption of a plant sterol-enriched beverage with milk fat globule membrane: a clinical study* (Food & Function).

♦ *Ex vivo study: impact of PS and/or β-Cx at physiological serum concentrations on suicidal erythrocyte death*

As aforementioned, the simultaneous presence of β-Cx and PS in milk-based fruit beverages has a positive effect on cholesterol-lowering and bone turnover markers (Granado-Lorencio et al., 2014). Moreover, the antiproliferative and antioxidant effects of β-Cx and/or PS have been demonstrated (Baskar et al., 2010; Cilla et al., 2015) on human colon adenocarcinoma cells. The oxidative stress, among others, is a powerful trigger of eryptosis in red blood cells (Pretorius et al., 2016), and it is assumed that the use of antioxidant compounds may prevent this phenomenon. Eryptosis is the principal form of erythrocyte death, hemolysis being the other death process (Herlax et al., 2011). Thus, the eryptotic and hemolytic effects of a PS mixture [22 μM: β-sitosterol (13 μM), campesterol (8 μM), and stigmasterol (1 μM)] and/or β-Cx (1 μM), at physiological serum concentrations obtained after the intake of PS-enriched milk-based fruit beverages containing β-Cx (Granado-Lorencio et al., 2014; Garcia-Llatas et al., 2015b), and their effect against oxidative stress induced by *tert*-butyl hydroperoxide (tBOOH) (75 and 300 μM) were evaluated in the present Doctoral Thesis. Blood was drawn from healthy human volunteers (n = 8, aged 24-59 years), with informed consent.

Regarding treatments with isolated bioactive compounds (PS mixture or β-Cx), it was observed that PS had no eryptotic nor hemolytic effect. This neutral effect may be due to the fact that PS incorporation into the erythrocyte membrane does not alter membrane properties such as rigidity, osmotic fragility and deformability (Hendriks et al., 2003; de Jong et al., 2006; Jones et al., 2005; Jenkins et al., 2007). Conversely, β-Cx induced a pro-eryptotic effect through an increase of eryptotic cells and depletion of intracellular glutathione (GSH) content (56 and 10% *versus*

control, respectively), without a concomitant reactive oxygen species (ROS) overproduction and Ca^{2+} entry. These results suggested that the eryptotic action of β-Cx was triggered through a Ca^{2+}-independent mechanism, and thus, more molecular pathways could be involved; such as the activation of protein kinase C (De Jong et al., 2002) or caspase-3 (Mandal et al., 2002). The antioxidant character of these bioactive compounds (PS or β-Cx) may justify the lack of ROS overproduction. Moreover, the absence of increased ROS production with a concomitant GSH depletion provided by β-Cx treatment, could be due to the fact that ROS generation is a late event in stressed erythrocytes and that eryptosis can occur via early GSH depletion independently of ROS generation (Officioso et al., 2016).

Simultaneously, two differentiated erythrocyte populations were observed: forward-scatter (FSC) <20 (cell size associated with eryptosis) and FSC >80 (associated with a preliminary step of hemolysis). In turn, β-Cx decreased the percentage of cells with low FSC (53%), with a concomitant increase in high FSC cells (143%) *versus* control cells, probably associated to their hemolytic effect (52% *versus* control). In agreement with our study, Briglia et al. (2015) has also observed a hemolytic effect with fucoxanthin (a compound structurally similar to β-Cx). According to the authors, β-Cx may have a positive effect since it could allow the clearance of erythrocytes if these were infected with the malaria pathogen *Plasmodium*.

Co-incubation of both bioactive compounds (PS mixture + β-Cx) protected partially against β-Cx damage; it seems that PS had a cytoprotective effect when both bioactive compounds were coincubated, because the increase of erythrocytes with high FSC was blunted (37%), as well as the associated hemolysis (55%) induced by β-Cx alone. Moreover, the GSH depletion was restored, showing a cellular response against oxidative stress.

To induce oxidative stress, the oxidant agent tBOOH (75 and 300 µM) was used as an effective inductor of eryptosis, as previously reported (Officioso et al., 2016). The tBOOH treatment showed an increase of eryptotic cells (1.3- and 2-fold *versus* control, respectively), triggered through the activation of entry Ca^{2+} (26% and 78%) with a concomitant ROS overproduction (1.6- and 12-fold), and an intracellular GSH

depletion (15% and 22%) at 75 and 300 μM *versus* control, respectively. However, only with tBOOH at 300 μM produced a limited hemolytic effect (18% higher *versus* control). Therefore, considering all results, it was concluded that tBOOH at low concentrations (75 μM) induces erythrocyte death, mainly via eryptosis, while at high concentrations (300 μM) both eryptosis and hemolysis ocurred.

With mild oxidative stress (tBOOH 75 μM), the pre-incubation with PS had a preventive effect upon Ca^{2+} entry and, moreover, protected the erythrocytes against ROS overproduction and GSH depletion, while at higher oxidative stress (tBOOH 300 μM) it only prevented hemolysis. Conversely, β-Cx exacerbated the hemolytic effect with both tBOOH concentrations, and eryptosis only with the highest concentration − through a marked GSH depletion (1.4 fold) with a concomitant increase of eryptotic cells (3-fold) associated to a raise of cells with FSC<20 (45%). The co-incubation with both bioactive compounds (PS mixture plus β-Cx), indicated that the presence of PS prevented hemolysis completely and partially prevented eryptosis (phosphatidylserine externalization and GSH depletion) induced by the β-Cx and/or tBOOH. Therefore, PS could provide protection against diseases associated with eryptosis, such as anemia, atherosclerosis, malignancy, obesity, diabetes, and chronic inflammatory diseases, among other disorders (Qadri et al., 2017; Repsold & Joubert, 2018).

The results obtained in this study have been published in: Alvarez-Sala, A., López-García, G., Attanzio, A., Tesoriere, L., Cilla, A., Barberá, R., Alegría, A. (2018). *Effects of plant sterols and/or β-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death.* Journal of Agricultural and Food Chemistry, 66, 1157−1166.

♦ *In vitro study: antiproliferative effect of PS-ingredient and its main PS (β-sitosterol) in human cancer cell lines*

The global burden of cancer has gained great attention, as it is among the leading causes of morbidity and mortality worldwide. Dietary interventions may effectively control cancer development, with PS being a class of cancer chemopreventive dietary phytochemicals (Bradford & Awad, 2010; Shahzad et al., 2017). The selected

beverage (MFbM) in this Doctoral Thesis was enriched in PS, using *tall oil* as the source of this bioactive compound. Thus, the antiproliferative effect of this PS-ingredient and its main PS (β-sitosterol) was evaluated in the most prevalent cancer cells in women; breast (MCF-7), colon (HCT116) and cervical (HeLa) cells (Ferlay et al., 2015). The compounds (PS-ingredient and β-sitosterol) were provided at physiological serum levels (13, 26 and 52 µM) drawn from values reported in the literature. The lowest PS concentration assayed in the present work (13 µM), is similar to the serum β-sitosterol concentration (15 µM) obtained after the consumption of milk-based fruit beverages (Garcia-Llatas et al., 2015b).

Regarding MCF-7 cells, only the PS-ingredient at 13 µM (17% or 53%) and the β-sitosterol standards (19-22% or 50-67%) showed a significant ($p<0.05$) decrease in cell viability at 24 and 48 h, respectively. The growth inhibition percentages observed for these compounds at 48 h are within levels recorded in previous studies (29-81%) with β-sitosterol (16 µM) tested between 1-5 days of incubation (Awad et al., 2007, 2008; Rubis et al., 2010). The percentage of cells in the sub-G_1 phase (considered a marker of DNA fragmentation) increased in all treated *versus* control cells, following the order: β-sitosterol 13 µM (7.1 fold) > β-sitosterol 26 µM (6.3 fold) > PS-ingredient 13 µM (5.4 fold) > β-sitosterol 52 µM (3.5 fold). All treatments also induced an increase in early apoptosis (16.8-24.1%), with the same order behaviour as evaluated in the cell cycle assay. These results suggest that the PS-ingredient and β-sitosterol could exert an antiproliferative effect through apoptosis involving the modulation of cell cycle progression – with no statistically significant ($p>0.05$) dose-response effect among all the concentrations tested. The β-sitosterol action via the apoptosis pathway was also reported previously at 16 µM upon MCF-7 (Awad et al., 2007, 2008), without changes in the cell cycle at lower concentrations (1 and 5 µM) (Rubis et al., 2010) than the ones used in our study.

Concerning HCT116 cells, the greatest viability reductions were observed with the PS-ingredient at 13 µM (36% or 46% at 24 and 48 h, respectively) and β-sitosterol at 13, 26 and 52 µM (27-42% or 44-49% at 24 and 48 h, respectively), though with no clear time- and dose-response effect. After 48 h of exposure, the percentage of cells in the sub-G_1 phase also increased, showing a higher value for

PS-ingredient at 13 µM and for β-sitosterol at 13 and 26 µM (6.7-8.8 fold) followed by β-sitosterol at 52 µM (3.3-fold) *versus* control cells. At the same time-point, early apoptosis was induced (12.9-20.9%), following the order: PS-ingredient 13 µM ≥ β-sitosterol 13 µM ≥ β-sitosterol 26 µM > β-sitosterol 52 µM. These results again indicated the absence of a dose-response effect in HCT116 cells. In this sense and as suggested previously by López-García et al. (2017) in Caco-2 cells, a possible biphasic effect could be involved, since a greater apoptosis response was observed with lower concentrations (13 and 26 µM) than with higher concentration (52 µM) of β-sitosterol. In agreement with our own findings, previous studies in Caco-2 cells carried out by our research group with β-sitosterol or PS mix standards, at serum concentrations obtained after the intake of PS-enriched milk-based fruit beverages (12 and 13.25 µM) (Cilla et al., 2015) or at estimated colonic concentrations obtained after gastrointestinal digestion of such beverage (115 and 132 µM) (López-García et al., 2017), showed no evident additive or synergistic effect with a PS mixture. As far as we are aware, only one other study (Choi et al., 2003) has been carried out with β-sitosterol standard (7.5-20 µM) in the HCT116 cell line, inducing slightly greater growth inhibition in a dose-dependent manner (50-75%) with a concomitant apoptosis induction by increasing the sub-G_1 cell population, as occurred in our own study.

In HeLa cells a time-dependent effect was observed, since PS-ingredient at 26 and 52 µM and β-sitosterol (13 µM) produced a statistically significant ($p < 0.05$) greater decrease (1.3-1.4 fold) at 48 h *versus* 24 h of exposure. To the best of our knowledge, only one other study (Cheng et al., 2015) reported an antiproliferative effect (40%) after exposure of β-sitosterol (20 µM) in HeLa cells at 24 h; being this effect similar to our own results (44.8%) only for β-sitosterol (13 µM) at 48 h.

In contrast with the results obtained for MCF-7 and HCT116 cells, a dose-dependent effect for β-sitosterol was observed in the cell cycle, because the greatest increase in the percentage of cells in the sub-G_1 phase corresponded to PS-ingredient 13 µM (3.4-fold), followed by β-sitosterol 52 µM (1.9-fold) ≥ 26 µM (1.5-fold) ≥ 13 µM (1.3-fold) *versus* control cells. The same trend was observed for the early apoptosis assay, where PS-ingredient at 13 µM showed the highest proportion of

early apoptosis (24.5%), and a dose-dependent effect of β-sitosterol standards was observed (14.3-20.4%), with a greater apoptotic effect induced by β-sitosterol 52 µM.

The antiproliferative activity of the *tall oil* PS-ingredient has not been evaluated so far; however, different studies have evaluated the antiproliferative effect of PS (mainly β-sitosterol or PS mixtures) isolated from plant extracts in MCF-7, HCT116 and HeLa cells, mainly through the determination of IC_{50}. Although there is controversy as to when a plant extract can be considered to have an active cytotoxic effect based on IC_{50} (active with $IC_{50} \leq 20$ µg/mL (Malek et al., 2009) or with $IC_{50} < 500$ µg/mL (Tahsin et al., 2017)), generally no antiproliferative effect in these kind of cell lines are reported. Thus, concerning the above results, the effect of PS-ingredient upon the cell lines may be due to the apparent predominant action of its main PS (β-sitosterol), and the use of PS as functional ingredients in the development of PS-enriched foods could exert a potential preventive effect against human breast, colon and cervical cancer; although further *in vivo* studies are required.

This study, carried out in the Dipartamento Scienze and Technologie Biologiche Chimiche e Farmaceutiche (STEBICEF), Palermo (Italy), where the PhD student performed a research stay that allowed to present this Doctoral Thesis with international mention, has allowed to submit a research article (under review): Alvarez-Sala, A., Attanzio, A., Tesoriere, L., Garcia-Llatas, G., Barberá, R., Cilla, A. (2018). *Anti-proliferative effect of phytosterol-ingredient and its main phytosterol (β-sitosterol) on human cancer cell lines* (International Journal of Food Sciences and Nutrition).

♦ *In vitro study: PS enhances the apoptotic effect of 5-fluorouracil (5-FU) in colon cancer cells*

Plant sterols, due to their anticancer effect, could be considered as a possible option as co-adjuvants in chemotherapy with conventional drugs. A widely used drug against colorectal cancer is 5-FU; however, the therapy frequently becomes ineffective due to resistance and the cytotoxic side effects to this drug (Walko & Lindley, 2005). Since PS undergo less absorption (0.5-2%) (Gylling et al., 2014),

these compounds may reach the colon and exert local actions. Thus, it was evaluated if the PS alone: β-sitosterol, 115 μM; campesterol, 11 μM; stigmasterol 6 μM, or as mixture (132 μM), at concentrations achievable in the human colon obtained after simulated gastrointestinal digestion of MFbM beverage (López-García et al., 2017), might enhance the chemotherapeutic effectiveness of 5-FU (50 μM) in two different lines of human colon cancer cells (HT-29 and Caco-2).

All PS treatments induced (p<0.05) an antiproliferative effect compared to control cells (15-31% cell growth inhibition), higher in Caco-2 than HT-29 cells, mediated by apoptosis induction mainly via both extrinsic (caspase-8) and intrinsic (caspase-9) pathways. Based on the antiproliferative data, the combination treatment with all individual PS and their mixture and 5-FU had an additive anticancer effect in both cell lines *versus* 5-FU alone. Despite of the treatment with PS mixture showed a trend to have the greatest additive effect with the combination of 5-FU, no synergistic or additive effect was observed compared to single PS treatments. In a previous study (Cilla et al., 2015), neither additive nor synergistic effect were observed for a PS mixture (7.125, 13.25 or 26.5 μM) compared to individual PS.

The 5-FU alone induced statistically significant (p<0.05) cell cycle arrest at S phase and apoptosis induction, with a concomitant activation of caspases-3, -8 and -9 as well as ROS overproduction and loss of mitochondrial membrane potential upon both cell lines (Caco-2 and HT-29). These 5-FU results were in agreement with previous studies (González-Sarrías et al., 2015; Pariente et al., 2018). Regarding cell cycle, co-treatment of PS plus 5-FU showed a chemosensitisation effect in the S phase arrest in both cell lines compared to 5-FU alone, although it was only statistically significant (p<0.05) in HT-29 cells at 48 h. Moreover, this combination (PS + 5-FU) increased efficacy of apoptosis induction with an increase in both caspases activation *versus* to 5-FU alone. In Caco-2 and HT-29 cells, the combination of 5-FU plus PS mixture showed a tendency to have the greatest apoptotic effect. It should be noted that HT-29 cells were more resistant to all PS treatments and also to 5-FU treatment with respect to Caco-2 cells. No statistically significant (p>0.05) effects were observed on ROS levels (except for stigmasterol and PS mixture in Caco-2 cells) and mitochondrial membrane potential (except for

PS mixture in HT-29 cells). This behaviour was in agreement with the results reported by Lopez-García et al. (2017), who observed that Caco-2 cells treated with colonic concentrations of PS did not show dissipation of the mitochondrial membrane potential or changes in intracellular ROS content (except for stigmasterol and PS mix) at 24 h, linking this fact possibly to their antioxidant properties.

To the best of our knowledge, only β-sitosterol among the PS studied, has been investigated as a potential co-adjuvant of other chemotherapy drugs to sensitize cancer cells. In a previous study, sub-toxic concentrations of β-sitosterol (8 µM) in combination with TNF-related apoptosis-inducing ligand (TRAIL) sensitised MDA-MB-231 breast cancer cells by a TRAIL-mediated apoptosis. In agreement with our data, β-sitosterol exerted the activation of caspases-3, -8 and -9 (Park et al., 2008). In addition, another study reported that β-sitosterol (4 and 16 µM) potentiated the inhibition of breast cancer MCF-7 and MDA-MB-231 cells exerted by the antiestrogen drug tamoxifen (Awad et al., 2008).

The present study conducted during a research stay in the Department of Food Science and Technology, CEBAS-CSIC (Murcia), provides for the first time, new findings regarding PS sensitising colon cancer cells to 5-FU chemotherapy in a preclinical context; although further *in vivo* studies are required. This study has allowed to submit a research article (under review): Alvarez-Sala, A., Ávila-Gálvez, M.A., Cilla, A., Barberá, R., Garcia-Llatas, G., Espín, J.C., González-Sarrías, A. (2018). *Physiological concentrations of phytosterols enhance the apoptotic effects of 5-fluorouracil in colon cancer cells* (Journal of Functional Foods).
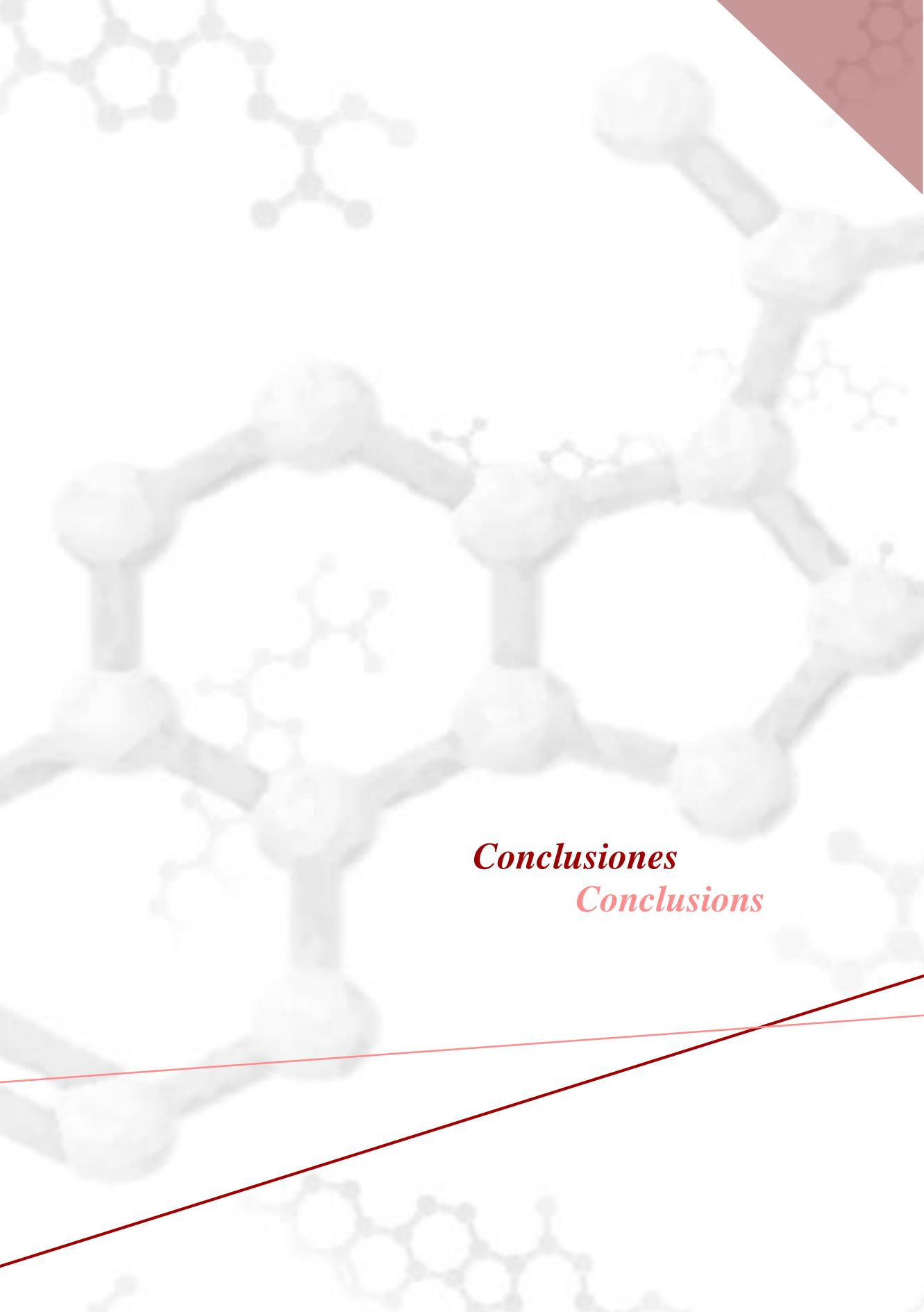
Conclusiones
Conclusions

Los estudios llevados a cabo para la determinación de esteroles y sus productos de oxidación (contenido y BA) en bebidas a base de zumo de frutas y leche desnatada enriquecidas en EV y conteniendo β-Cx, han dado lugar a las siguientes conclusiones:

1ª Las tres bebidas analizadas (MFb: sin adición de grasa; MFbM: con adición de grasa láctea y concentrado de proteínas de suero enriquecidas con MFGM; MFbO: con adición de aceite de oliva virgen extra y lecitina de soja), presentan el siguiente perfil de EV: β-sitosterol > sitostanol > campesterol y, en menores cantidades, campestanol y estigmasterol, acorde a la legislación vigente y al perfil del ingrediente fuente de EV (*tall oil*) utilizado para el enriquecimiento de las mismas.

2ª En la BA de los EV influyen el tipo y cantidad de grasa y el emulsionante empleado en la formulación de las bebidas. La BA de los EV totales en las tres bebidas analizadas sigue el orden: MFbM (31,4%) = MFbO (28,2%) > MFb (8,7%). La mayor BA de EV en MFbM y MFbO *versus* MFb se relaciona con el mayor contenido de grasa (2,4% *versus* 1,1%) y la presencia de emulsionantes (MFGM o lecitina).

3ª Se selecciona la bebida MFbM para llevar a cabo el ensayo clínico, en base a la BA de los EV y posibles efectos beneficiosos atribuidos al MFGM. Esta bebida es estable en las condiciones de almacenamiento (20-25ºC, 6 meses) ensayadas, ya que no se modifica ni el contenido ni la BA de los EV y su grado de oxidación es bajo (0,022-0,023%).

Los estudios *in vivo*, *ex vivo* e *in vitro* sobre los efectos biológicos de los EV permiten concluir que:

4ª La ingesta durante 6 semanas de 250 mL de MFbM/día, conteniendo 2 g EV, por mujeres postmenopáusicas: *(i)* reduce el colesterol total (2,9%) y el c-LDL (5,1%) sérico, sin modificar los niveles de c-HDL; *(ii)* incrementa las concentraciones séricas de campesterol (43,1%), β-sitosterol (32,5%) y latosterol

(11,2%); *(iii)* disminuye los niveles de la citoquina IL-1β proinflamatoria (6,7%) y aumenta los de la citoquina IL-10 antiinflamatoria (22,5%).

5ª Una mezcla de EV (22 μM) conteniendo β-sitosterol (13 μM), campesterol (8 μM) y estigmasterol (1 μM), compatibles con concentraciones séricas fisiológicas, previene los efectos hemolíticos y parcialmente los pro-eriptóticos inducidos por β-Cx, sin y con estrés oxidativo.

6ª El ingrediente fuente de EV (*tall oil*) utilizado para el enriquecimiento de la bebida MFbM y su principal EV (β-sitosterol), a las concentraciones ensayadas (13, 26 y 52 μM) compatibles con concentraciones séricas fisiológicas, muestran un efecto antiproliferativo en células de cáncer de mama (MCF-7), colon (HTC116) y cuello uterino (HeLa)). El efecto se produce mediante inducción de la fragmentación del ADN y apoptosis, sin un claro efecto dosis- tiempo-dependiente.

7ª En células de cáncer de colon (Caco-2 y HT-29) se observa un efecto antiproliferativo aditivo de los EV [132 μM: β-sitosterol (115 μM), campesterol (11 μM) y estigmasterol (6 μM)], a concentraciones colónicas, como coadyuvantes del agente quimioterapéutico 5-FU. Los EV mejoran la eficacia del 5-FU sobre el arresto del ciclo celular, inducción de apoptosis y aumento en la activación de caspasas.

De manera global, se puede indicar que la bebida MFbM es una matriz adecuada para el enriquecimiento en EV y segura para su consumo. Su ingesta por mujeres postmenopáusicas proporciona, de forma complementaria al conocido efecto hipocolesterolemiante, un efecto antiinflamatorio. Por otro lado, a las concentraciones fisiológicas estudiadas, los EV previenen de efectos hemolíticos y eriptóticos inducidos por otros compuestos como la β-Cx y, con ello, podrían mejorar los desórdenes clínicos asociados a los mismos. A su vez, los EV pueden considerarse compuestos anticarcinogénicos contra el cáncer de mama, colon y cuello uterino, y actuar como coadyuvantes del agente quimioterapéutico 5-FU en el tratamiento del cáncer de colon, aunque son necesarios ensayos *in vivo* para confirmar estos hallazgos preclínicos.

The studies for the determination of sterols and their oxides (content and BA) in milk-based fruit beverages enriched with PS and containing β-Cx, allow the following conclusions to be drawn:

1st From the three beverages analysed (MFb: without fat addition; MFbM: with addition of milk fat and whey proteins enriched with MFGM; MFbO: with extra virgin olive oil and soy lecithin addition), the PS profile exhibited the following order: β-sitosterol > sitostanol > campesterol, with lower amounts of campestanol and stigmasterol – profile in accordance with current legislation and with the PS-ingredient (*tall oil*) used for the enrichment of the beverages.

2nd The BA of PS is influenced by the type and quantity of fat and the emulsifier added to the beverages. The BA of total PS from the beverages followed the order: MFbM (31.4%) = MFbO (28.2%) > MFb (8.7%). The higher BA of MFbM and MFbO compared with MFb beverage could due to the highest fat content (2.4% *versus* 1.1%) and the presence of emulsifiers (MFGM or lecithin).

3rd The MFbM beverage was selected to carry out the clinical trial, based on the BA of the PS and the possible health benefits attributed to the MFGM compounds. This beverage is stable under storage conditions assayed (20-25ºC, 6 months), since no modifications were observed in the content and BA of PS and their oxidation rate is low (0.022-0.023%).

From the *in vivo*, *ex vivo* and *in vitro* studies of the PS biological effects, the following can be concluded:

4th The consumption of 250 mL of MFbM/day containing 2 g PS during 6 weeks by postmenopausal women: *(i)* reduces serum total (2.9%) and LDL-cholesterol (5.1%), without modifying HDL-cholesterol levels; *(ii)* increases serum campesterol (43.1%), β-sitosterol (32.5%) and lathosterol (11.2%) concentrations; *(iii)* decreases the levels of the proinflammatory cytokine IL-1β (6.7%) with a concomitant increase in antiinflammatory IL-10 cytokine levels (22.5%).

5th The mixture of PS (22 μM) containing β-sitosterol (13 μM), campesterol (8 μM) and stigmasterol (1 μM), at physiological serum concentrations, prevents the hemolytic effect and partially the pro-eryptotic effect induced by β-Cx with or without oxidative stress.

6th The PS-ingredient (*tall oil*) used for enrichment of the MFbM beverage and its main PS (β-sitosterol), at the concentrations tested (13, 26 and 52 μM) compatible with physiological serum concentrations, shows an antiproliferative effect in breast (MCF-7), colon (HTC116) and cervical (HeLa) cancer cells. The effect is produced by induction of DNA fragmentation and apoptosis, without a clear dose- and time-dependent effect.

7th In colon cancer cells (Caco-2 and HT-29) an additive antiproliferative effect of PS [132 μM: β-sitosterol (115 μM), campesterol (11 μM) and stigmasterol (6 μM)], at colonic concentrations, as coadjuvants of the chemotherapeutic agent 5-FU was observed. The PS improved efficacy of 5-FU on cell cycle arrest at S phase, apoptosis induction and caspase activation increase.

Overall, the MFbM beverage is a suitable matrix for PS enrichment and its consumption is safe; its intake by postmenopausal women provides, besides the well-known hypocholesterolemic effect, an antiinflammatory action as well. Moreover, at physiological concentrations studied, the PS prevents the pro-eryptotic and hemolytic effect induced by other compounds as β-Cx, and with it, alleviates the clinical disorders associated with them. In turn, PS may be regarded as natural anticarcinogenic compounds against breast, colon and cervical cancer cells, as well as acting as coadjuvants of the chemotherapeutic 5-FU agent in the treatment of colon cancer, although further *in vivo* studies are required to confirm these preclinical findings.
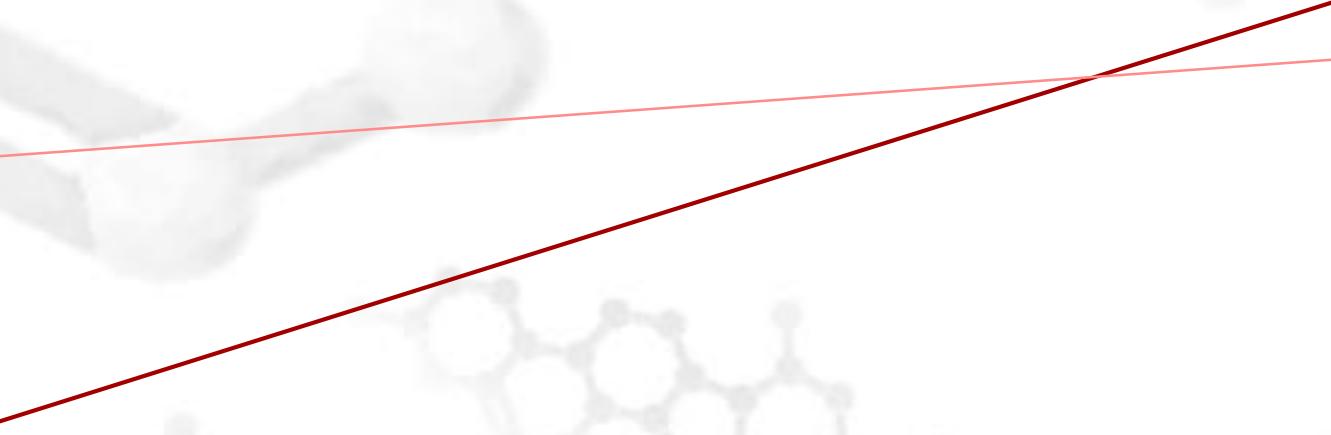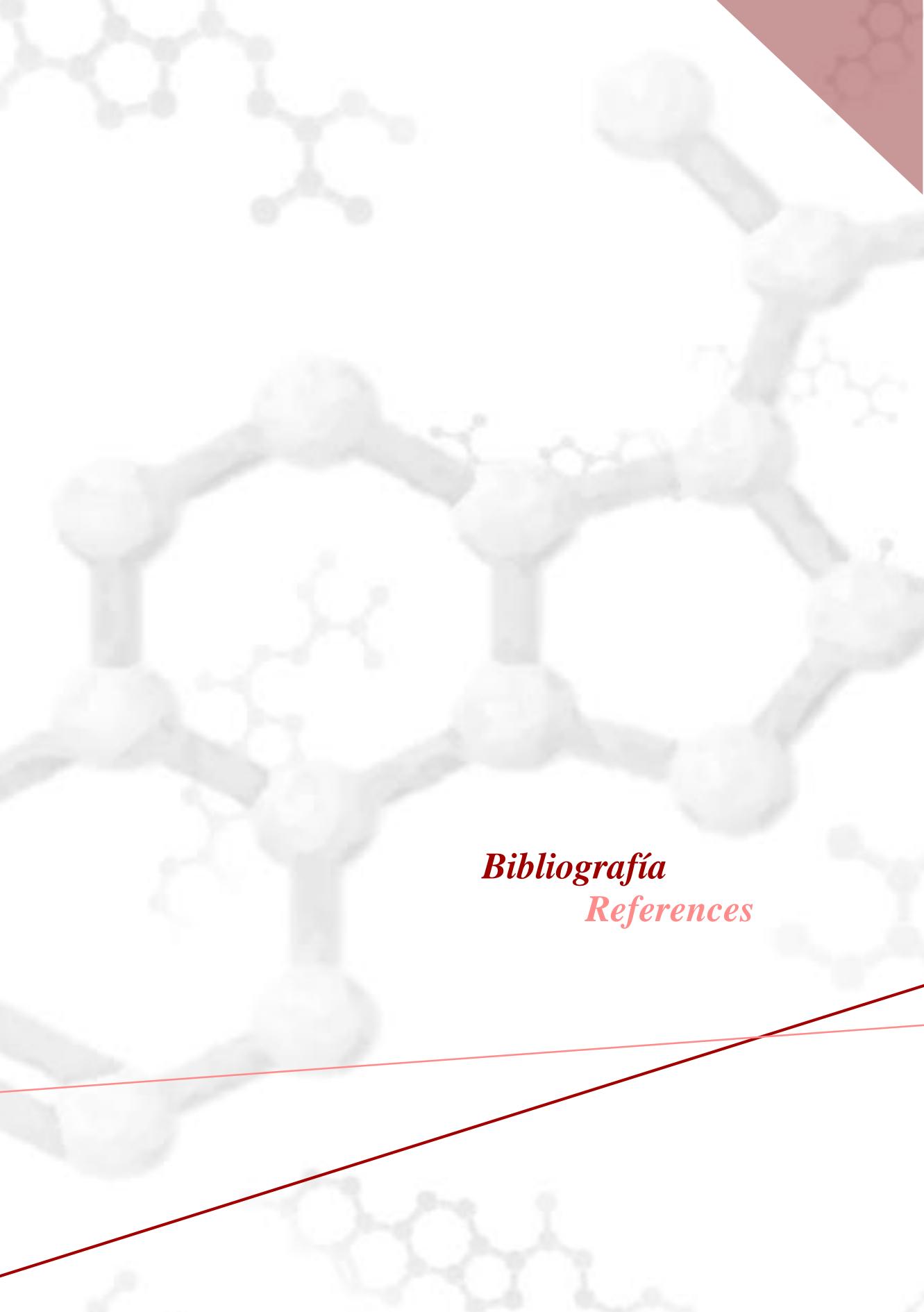
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Anexo 1. Artículos publicados
Annex 1. Published articles

Impact of lipid components and emulsifiers on plant sterols bioaccessibility from milk-based fruit beverages

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Impact of Lipid Components and Emulsifiers on Plant Sterols Bioaccessibility from Milk-Based Fruit Beverages

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ABSTRACT: Sterol bioaccessibility (BA) of three plant sterol (PS)-enriched milk-based fruit beverages (MFb) with different fat contents (1.1–2.4%), lipid sources (animal or vegetable), and without or with emulsifiers (whey proteins enriched with milk fat globule membrane (MFGM) or soy lecithin) was evaluated after simulated gastrointestinal digestion. The BA of total PS followed the order 31.4% (MFbM containing milk fat and whey proteins enriched with MFGM) = 28.2% (MFbO containing extra virgin olive oil and soy lecithin) > 8.7% (MFb without fat addition). Total and individual PS content in the bioaccessible fractions followed the order MFbM > MFbO > MFb. Consequently, formulation with MFGM is proposed in beverages of this kind to ensure optimum bioavailability of PS. Our results suggest that the BA of PS is influenced by the type and quantity of fat and the emulsifier type involved.

KEYWORDS: *functional beverages, cholesterol, MFGM, mixed micelles, phytosterols, simulated gastrointestinal digestion, food matrix*

■ INTRODUCTION

The European Atherosclerosis Society Consensus Panel specified that a plant sterols/stanols (PS) intake of 2 g/day produces a 10% reduction in serum low-density lipoprotein-cholesterol in the context of the prevention of cardiovascular disease with the absence of adverse signs.[1] Other functions such as immune, antiinflammatory, and anticarcinogenic effects have also been attributed to PS.[2,3] The Western diet provides a maximum of 440 mg PS/day, but the value can reach 1 g PS/day in vegans.[4] This amount alone is unable to offer the effective dose for securing a cholesterol-lowering effect. Consequently, a broad range of products enriched with free or esterified PS have been authorized by the European Union (EU). Moreover, health claims have been approved by the European Commission, such as Decision 2014/686, which indicates that "plant sterols have been shown to lower/reduce blood cholesterol. High cholesterol is a risk factor in the development of coronary heart disease".[5]

Because PS are susceptible to oxidation,[6−8] the inclusion of fruits (natural sources of antioxidants) is a good option for preventing the oxidation of these bioactive compounds. Different dairy products or fruit beverages have been enriched with PS, and their profiles have been analyzed: orange juice,[9] milk,[10−12] or different fermented milks.[11−15] Currently, incorporating PS into low-fat foods such as milk-based fruit beverages (where the addition of PS is allowed in the EU)[16] is a convenient way for consumers to obtain the daily recommended amount of PS in individuals with moderate hypercholesterolemia. To the best of our knowledge, the only studies that have evaluated PS content (total and individual) in fruit milk-based beverages enriched with PS are those conducted by our research group.[6,7,17]

To improve our knowledge of functional efficiency associated with PS consumption, it is interesting to know not only the quantities of PS but also their bioavailability. In this regard, in vitro digestion models can supply relative results in a short time and could serve as a tool for screening food ingredients.[18] These procedures estimate the bioaccessibility (BA) of PS, evaluating their transfer from food matrix to mixed micelles in the intestinal chyme during simulated digestion of lipids. The presence of certain substances in the emulsion-based delivery system could influence PS bioavailability.[19] In this sense, Baldi and Pinotti[20] report that milk fat fraction are an efficient delivery system for highly lipophilic microconstituents such as PS. In addition, the presence of lecithin[21] or unsaturated fatty acids (oleic, linoleic, and α-linolenic acid)[22] could enhance the incorporation of cholesterol and/or PS in model bile mixed micelles.

Different authors[23−30] have evaluated the solubility (transfer to mixed micelles) of sterol/stanol from standard solutions and/or their impact on cholesterol solubility mimicking in vitro micellar systems. Moreover, there are few data on the transference of PS from food model systems to the aqueous micellar phase,[31] or on their possible interaction with cholesterol in micellar incorporation,[32] through in vitro digestion. As far as we aware, only three studies have addressed sterol solubility after simulated digestion in food products from a commercial PS-enriched orange juice or multivitamin/multimineral tablet dissolved in orange juice,[33] commercial fermented milk beverages enriched with PS,[15] and in enriched PS skimmed milk and/or fruit beverages.[17] These studies showed an important effect on the part of the matrix and the PS-ingredient used in the food formulation upon solubility and

BA of PS. In this context, the aim of the present study was to develop and improve a functional beverage, modifying lipid components/emulsifiers to obtain better BA of PS for further use in a human clinical study. Accordingly, we carried out a modification of the food matrix from previous beverages assayed by our research group.[17] This same approach has recently been used to evaluate the BA of β-cryptoxanthin.[34]

■ MATERIALS AND METHODS

Reagents. The internal standard (IS) used was 5β-cholestan-3α-ol (epicoprostanol) (purity 95%). Other standards used were 5-cholesten-3β-ol (cholesterol) (purity 99%), 24α-ethyl-5α-cholestan-3β-ol (stigmastanol) (purity 97.4%), (24S)-ethylcholest-5,22-dien-3β-ol (stigmasterol) (purity 97%), and (24R)-ethylcholest-5-en-3β-ol (β-sitosterol) (purity 97.3%) from Sigma Chemical Co. (St. Louis. MO, United States). (24R)-Methylcholest-5-en-3β-ol (campesterol) (purity 98.6%) was purchased from Steraloids (Newport, RI, United States).

For sterol determination we used trimethylchlorosilane (TMCS), purchased from Fluka (Buchs, Switzerland). Ammonium chloride, anhydrous sodium sulfate, chloroform, ethanol, hydrochloric acid (purity 37%), methanol, potassium chloride (KCl), potassium dihydrogen phosphate, sodium chloride, sodium bicarbonate, and urea were supplied by Merck (Whitehouse Station, NJ, United States). Sodium hydroxide was from Panreac (Barcelona, Spain). Uric acid was purchased from Prolabo (Sacramento, CA, United States). Diethyl ether, n-hexane, potassium hydroxide (KOH), and 2-propanol were from Scharlau (Barcelona, Spain). Anhydrous pyridine, α-amylase from human saliva, bovine bile, bovine serum albumin (BSA), butylhydroxytoluene (BHT), calcium chloride dehydrate, cholesterol esterase from bovine pancreas, colipase from porcine pancreas, glucose, glucosamine hydrochloride, glucuronic acid, hexamethyldisilazane (HMDS), lipase from human pancreas, magnesium chloride, mucin from porcine stomach type II, pancreatin from porcine pancreas, pepsin from porcine stomach, phospholipase A2 from porcine pancreas, potassium thiocyanate, sodium dihydrogen phosphate, sodium taurocholate, and tris(hydroxymethyl)amino-methane were all from Sigma Chemical Co. (St. Louis, MO, United States). All reagents were of analytical grade. A Millipore Q water purification system (Milford, MA, United States) was used to obtain ultrapure water.

Samples. Three PS-enriched milk-based fruit beverages were developed: MFb, without fat addition; MFbM, with addition of milk fat and whey proteins enriched with milk fat globule membrane (MFGM); and MFbO, with extra virgin olive oil and soy lecithin addition. All the beverages contained skimmed milk (50%), mandarin juice as the source of β-cryptoxanthin (48%), banana puree (1%), grape juice (1%) and free microcrystalline PS from tall oil in powder form[35] (2 g PS/250 mL beverage) and were prepared by the Hero Technology Center (Alcantarilla, Murcia, Spain). Briefly, dairy powder ingredients (skimmed milk, whey enriched with MFGM) were dissolved in water. After complete ingredient hydration, fat-rich ingredients (cream or olive oil/soy lecithin) were added and mixed in a high shear mixer. In a separate tank, the microencapsulated water-dispersible PS ingredient was mixed with the reconstituted fruit juices using a high-speed mixer device. The milk phase was acidified by the addition of the PS-enriched fruit juice base and, to prevent protein destabilization, pectin was added. The resulting mixed phases were heated to 70 °C and homogenized at 150 bar in two steps (100 + 50 bar). After homogenization, the product was pasteurized at 90 °C for 30 s by indirect heat exchanger, cooled to 20 °C, and filled aseptically in 250 mL tetra bricks. The process and conditions were identical for all the beverages. Energy and nutritional information on the beverages is provided in Table 1.

Simulated Gastrointestinal Digestion. This process was performed according to Vaghini et al.,[15] in three phases: salivary, gastric, and intestinal, including the formation of mixed micelles. Briefly, 20 g of beverage was placed in an Erlenmeyer flask, and a saliva solution was added (9 mL, pH 6.5 ± 0.2). This saliva solution contained organic and inorganic components, mucin, and α-amylase

Table 1. Plant Sterols-Enriched Milk-Based Fruit Beverages: Energy and Nutritional Information per 100 mL[a]

	MFb	MFbM	MFbO
energy (kJ/kcal)	197.4/49.5	263.0/65.3	244.7/61.0
protein (g)	2.2	3.1	2.2
carbohydrates (g)	8.7	8.9	8.7
fat without plant sterols (g)	0.3	1.6	1.6
fiber (g)	1.5	1.5	1.5
plant sterols (g)	0.8	0.8	0.8

[a]MFb: without fat addition. MFbM: with addition of milk fat and whey proteins enriched with milk fat globule membrane. MFbO: with olive oil and soy lecithin addition.

(0.19 mg, 20 U). The mixture was incubated in a shaking water bath (SBS30 Stuart Scientific) for 5 min at 37 °C and 95 orbitations per minute (opm). Then, 13.5 mL of gastric juice (pH 1.07 ± 0.07) containing organic and inorganic solutions, mucin, BSA, and pepsin from porcine stomach was added, and the mixture was incubated (1 h, 37 °C, 95 opm). Subsequently, for the gastrointestinal digestion phase, we used 25 mL of duodenal juice (pH 7.8 ± 0.2) and 9 mL of bile solution (pH 8.0 ± 0.2). After neutralization of the digest pH value (6.8−7.2), cholesterol esterase (5 U), colipase (12.5 μg), human pancreatic lipase (1 U), phospholipase A2 (502 U), and sodium taurocholate (0.02 mg) were added. The flasks were incubated (2 h, 37 °C, 95 opm) and centrifuged (90 min, 4 °C, 3100 g) to obtain the aqueous-micellar fraction (supernatants) considered the bioaccessible fraction (BF) (soluble fraction that is released in the gastrointestinal tract available for absorption) of the digested beverages. BA of sterol (percentage soluble available for absorption with respect to sterol content in beverage before its digestion) of sterols was calculated from the following equation: [BF content (mg sterol/100 g beverage)/total content in undigested beverage (mg sterol/100 g beverage)] × 100.

Extraction of the Lipid Fraction. A modification of the method of Folch et al.[36] was used for lipid extraction. According to Alemany-Costa et al.[6] and Vaghini et al.,[15] 5 g of beverage providing approximately 40 mg of PS were taken. To the beverage, 25 mL of chloroform:methanol (1:1, v/v) mixture with 0.05% BHT was added, followed by homogenization (Polytron PT 2000, Kinematica AC, Switzerland) for 3 min. Then, 12.5 mL of chloroform was added, and mixing was again performed with the Polytron. The sample was filtered (Whatman no. 1.90 mm) through a Buchner funnel, and 20 mL of KCl 1 M solution was added to the filtrate and kept at 4 °C overnight. After separation of the organic fraction, the chloroform phase was concentrated in a rotary evaporator and taken to dryness under a nitrogen stream.

Determination of Sterols. *Beverages.* The lipid fraction obtained was dissolved in 10 mL of hexane:isopropanol (4:1). To a fraction (1/20) of the extracted fat we added 200 μg of IS. Hot saponification of the lipid fraction was performed according to Vaghini et al.,[15] with 2 mL of a KOH 1N in ethanol/Milli Q-water (9:1) solution in the water bath (1 h, 65 °C). Then, the unsaponifiable fraction was extracted with diethyl ether and derivatized with HMDS:TMCS in anhydrous pyridine (2:1:5, v/v/v) (25 min, 40 °C). The trimethylsilyl ether (TMSE) derivatives were solubilized in n-hexane, filtered (syringe driven Millex FH with filter 1 mL, 0.45 μm, Millipore, Milford, MA, United States) and evaporated under a nitrogen stream. Afterward, the TMSE derivatives were dissolved in 100 μL of n-hexane and analyzed (1 μL) by gas chromatography−flame ionization detection (GC-FID) under the conditions described by Alemany-Costa et al.[6]

The sterols were identified by comparing their retention times with those of the standards derivatized by the same procedure as the samples. Sterols quantification was performed with calibration curves containing 200 μg of IS and the corresponding commercial standards (stigmastanol was used for stanols quantification, because this is the only phytostanol that is commercialized). The calibration curves employed were as follows: cholesterol (4.95−99.00 μg; $y = 0.0085x - 0.0343$, $r = 0.996$; retention time (RT), 20.49 min), campesterol (49.30−394.40 μg; $y = 0.0082x + 0.0712$, $r = 0.998$; RT, 24.90 min);

β-sitosterol (194.60−3892.00 μg; $y = 0.0051x - 0.0995$, $r = 0.998$; RT, 29.18 min); stigmasterol (4.85−194.00 μg; $y = 0.0055x + 0.0059$, $r = 0.999$; RT, 26.28 min); stigmastanol (9.70−954.50 μg; $y = 0.005x + 0.039$, $r = 0.995$; RT, 29.50 min), where y is Area$_{sterol}$/Area$_{IS}$ and x is micrograms of sterol. The RT for campestanol was 25.8 min, and the RT for the IS was 18.01 min.

Bioaccessible Fraction. To 5 g of the obtained BF, was added 200 μg of IS, and the mixture was saponified directly (with 10 mL of a KOH 2N solution in 90% ethanol) in the water bath (1 h, 65 °C).[15] Then, the unsaponifiable material was extracted with diethyl ether, and all of it was used for sterols quantification using the same derivatization and determination conditions described for sterols determination in the beverages. The TMSE derivatives were dissolved in 100 μL for cholesterol, campesterol, campestanol, and stigmasterol determination and in 250 μL for β-sitosterol and sitostanol determination of n-hexane for the GC-FID analysis.

Statistical Analysis. The analysis of all samples was performed in triplicate. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was applied to determine statistically significant differences in the same compound (individual or total sterol content) and in the same kind of sample (beverage, BF, or BA) (within lines) or in BF or BA of the same sample (MFb, MFbM, MFbO) (within columns). A significance level of $p < 0.05$ was adopted for all comparisons, and the Statgraphics Centurion XV statistical package (Statpoint Technologies Inc., VA, United States) was used throughout.

■ RESULTS AND DISCUSSION

Sterol Contents in the Beverages. All beverages were identified, and cholesterol, campesterol, campestanol, stigmasterol, β-sitosterol, and sitostanol were quantified. Table 2 summarizes the sterol contents (milligrams of sterol/100 g of beverage) of the beverages.

Total PS contents ranged from 613 to 856 mg/100 g of beverage (Table 2). Cholesterol content ranged from 2 to 8 mg/100 g beverage, being higher in MFbM, because of addition of milk fat. Cholesterol content in MFb (Table 2) was in agreement with results published by Alemany et al.[17] in similar samples (1.4−2 mg/100 g beverage). Because the same PS source (tall oil-derived PS ingredient) has been used in all beverages, the relative PS abundance was headed by β-sitosterol, followed by sitostanol and campesterol, with lower amounts of campestanol and stigmasterol, a profile previously characterized by our group in an ingredient from tall oil.[35] Similar relative percentages of each PS with respect to total content were obtained in all beverages (β-sitosterol ≈ 79%, sitostanol ≈ 12%, campesterol ≈ 7%, campestanol ≈ 1%, stigmasterol ≈ 0.8%). These values comply with the PS profiles specified by the European Commission for milk products and milk-based fruit beverages.[16] The observed PS profiles were in agreement with those reported by González-Larena et al.[7] and Alemany et al.[17] in beverages using the same PS source and contents in enrichment of the beverages (tall oil-derived PS ingredient).

Bioaccessibility of Sterols. The sterol contents in the BF of the beverages, expressed as milligrams of sterol/100 g of beverage, and their corresponding BA (%) are shown in Table 2.

The total PS content in the BF ranged from 53 to 267 mg/100 g of beverage, being highest for MFbM, followed by MFbO and MFb. The total PS content in the BF with respect to that of the corresponding beverage was 12-fold lower in MFb and 3- or 4-fold lower in MFbM or MFbO, respectively. In all beverages, the profile of individual PS in the BF was headed by β-sitosterol, followed by sitostanol and campesterol, with lesser amounts of campestanol and stigmasterol. Cholesterol content

Table 2. Sterol Contents in Plant Sterols-Enriched Milk-Based Fruit Beverages and in Bioaccessible Fraction (mg sterol/100 g beverage), and their Bioaccessibility[a]

sterol	MFb			MFbM			MFbO		
	beverage	BF	BA	beverage	BF	BA	beverage	BF	BA
cholesterol	2.19 ± 0.09a	1.26 ± 0.05a,y	57.60 ± 3.37a,y	8.15 ± 0.41b	5.32 ± 0.33b,y	64.18 ± 6.64a,y	2.30 ± 0.20a	1.23 ± 0.01a,v	53.50 ± 4.23a,v
campesterol	23.94 ± 2.51a	2.15 ± 0.13a,y	9.03 ± 0.59a,w	34.70 ± 4.11b	11.63 ± 0.18b,w	33.86 ± 3.83b,w	25.27 ± 2.40a	6.60 ± 0.41c,v	26.37 ± 3.94c,wx
campestanol	3.19 ± 0.34a	0.88 ± 0.04a,y	27.75 ± 2.76a,x	9.46 ± 0.82b	3.55 ± 0.41b,y,w	36.92 ± 3.84b,w	3.91 ± 0.35a	1.20 ± 0.04a,v	31.41 ± 4.74a,b,w
stigmasterol	3.75 ± 0.23a	0.22 ± 0.03a,y	5.72 ± 0.60a,w	5.19 ± 0.38b	1.69 ± 0.05b,x	32.64 ± 3.28b,w	4.03 ± 0.20a	0.78 ± 0.02c,v	19.37 ± 0.98c,x
β-sitosterol	506.00 ± 58.69a	42.44 ± 1.76a,w	8.45 ± 0.81a,w	704.72 ± 70.60b	215.12 ± 3.75b,y	30.72 ± 2.61b,w	513.08 ± 36.34a	143.73 ± 11.66c,w	28.14 ± 3.53b,w,x
sitostanol	77.47 ± 10.37a	7.52 ± 0.35a,x	9.79 ± 1.02a,w	102.77 ± 9.10b	35.13 ± 0.46b,z	34.37 ± 2.90b,w	75.44 ± 3.32a	22.52 ± 1.74c,x	29.94 ± 3.45b,w
total PS	613.10 ± 70.28a	53.13 ± 2.16a	8.73 ± 0.81a	856.29 ± 84.41b	267.11 ± 3.85b	31.39 ± 2.70b	621.74 ± 42.41a	174.43 ± 13.80c	28.19 ± 3.58b

[a]Values are expressed as mean ± standard deviation of three replicates. BF: Bioaccessible fraction content. BA: Bioaccessibility, calculated as [BF content (mg sterol/100 g beverage)/total content in undigested beverage (mg sterol/100 g beverage)] × 100. MFb: without fat addition. MFbM: with addition of milk fat and whey proteins enriched with milk fat globule membrane. MFbO: with olive oil and soy lecithin addition. PS: plant sterols. Different letters denote significant differences ($p < 0.05$) in the same compound and in the same kind of sample (beverage, BF, or BA) (a−c) or in the BF or BA of the same sample (MFb, MFbM, or MFbO, within columns) (v−z).

in BF ranged from 1.23 to 5.32 mg/100 g of beverage, being higher in MFbM because of the presence of milk fat and MFGM, with contents of the same order in MFbO and MFb. However, the cholesterol content in BF was about one-half of that present in the corresponding beverages, with no differences in cholesterol BA between the beverages.

The BA of total PS from the beverages followed the order: MFbM = MFbO > MFb. The individual PS in the same beverage showed similar BA, except in MFb, where the greatest BA corresponded to campestanol, and in MFbO, where lesser homogeneity was observed; stigmasterol was the PS with the lowest BA (Table 2). In all three beverages, the BA of total PS was significantly lower than that of cholesterol because the PS exhibited less solubility and higher hydrophobicity compared with cholesterol. Accordingly, cholesterol would have the highest solubility in the mixed micellar phase.[23,27,28]

Few studies involving simulated gastrointestinal digestion of sterols in different food products have been published, and the existing studies mainly focus on the effect of PS upon cholesterol micellarization. Bohn et al.[33] evaluated the effect of test meals, including orange juice with 190 mg of PS (β-sitosterol + campesterol + stigmasterol) and 27 mg of cholesterol dissolved in olive oil, upon cholesterol micellarization through simulated gastrointestinal digestion similar to that used in the present study. The percentage of cholesterol micellarization (bioaccessibility) of the test meal including PS-enriched orange juice (around 55%) was similar to that recorded in our beverages (see Table 2), and the test meal with PS-containing multivitamin/multimineral tablet dissolved in orange juice decreased cholesterol micellarization approximately 3-fold. The authors suggested that the different effects on cholesterol micellarization may be due to the fact that the PS formulations present differences in production techniques, especially coating formulations, and the minerals and vitamins present in the tablet formulation could increase micelle stability and/or formation during digestion, possibly implying increased micellar access of PS versus cholesterol in these products.

To date, the only studies assessing the BA of PS in milk fruit-based beverages have been carried out by our group. In this sense, when the BA of total PS in MFb versus beverages with similar formulations are compared,[17] the BA of total PS in MFb was found to be 1.3- to 2-fold higher, possibly due to the greater contribution of campestanol to BA in the present study. Vaghini et al.,[15] in a fermented milk with orange juice beverage, found BA of total PS (8.9%) to be similar to that of MFb, with generally no significant differences in BA of the individual PS, as in our study.

On the other hand, PS standard solutions and/or their possible interaction with cholesterol have been studied through one-step simulated intestinal digestion models.[31,32] Malaki Nik et al.[31] found that the BA (transference to the aqueous micellar phase) of β-sitosterol, campesterol, and stigmasterol ranged from 72 to 93% at 5−120 min of simulated duodenal digestion. These values are higher than in our study, probably because of the less complex food matrix involved (emulsion made of soy bean oil and soy protein isolate), the lower PS content (0.055 g PS/100 g versus 0.8 g PS/100 g in our beverages), and the one-step digestion model used. Moran-Valero et al.[32] reported that the greater hydrophobicity exhibited by PS with saturated lateral chains (campesterol, campestanol, β-sitosterol, sitostanol) allows greater adherence/transference to the micelles, displacing cholesterol. Furthermore, if the micelles become saturated because of the presence of sterols, these may

coprecipitate, thereby also eliminating cholesterol. The authors found that with higher levels of total PS presenting saturated lateral chains (93.5% versus 73.5%), the incorporation of cholesterol to the micelle decreases up to 60% versus 85%, respectively. These results are in agreement with our cholesterol BA (∼60%), because in our beverages total PS with saturated lateral chains reached 99.4%.

Models using different bile salts to assess sterol solubility have found that cholesterol always shows higher solubility, followed by β-sitosterol.[23,25,27,28,30] These results are in agreement with our own study regarding cholesterol.

To the best of our knowledge, no studies have evaluated the effect of different fat contents from foods upon sterol BA based on simulated gastrointestinal digestion. The beverages with the highest fat content (2.4%), i.e., MFbM and MFbO, were those showing the greatest BA for total PS (31.4% and 28.2%, respectively), compared with MFb (8.7%), which presented a lesser fat content (1.1%). Therefore, the fat content of the matrix could promote the formation of mixed micelles in the gastrointestinal digestion. These results are in agreement with the study published by Biehler et al.,[37] where the BA of a β-carotene (a lipophilic compound like PS) (around 18% versus 12% and 14%) was found to increase with the fat content of the matrix (4% versus 1.5% and 3.5%). On the other hand, a greater fat content from canola oil and cream (18% fat) increased the BA of β-carotene around 3−4.5-fold, without significant differences between them versus milk alone (4% fat).[38] Thus, in the same way as in our own beverages, the BA of lipidic compounds is influenced more by the amount of fat than by the lipid source, because the BA values of PS do not differ significantly between MFbM (animal origin) and MFbO (vegetable origin), where the fat contents are the same and higher than in MFb. Furthermore, these authors observed that the presence of emulsifiers had a clear positive impact on the BA of β-carotene. Accordingly, the higher BA of total PS (Table 2) obtained in MFbM and MFbO versus MFb (without emulsifier) could be explained by the presence of different emulsifiers in their composition.

The presence of lecithin and unsaturated fatty acids in MFbO could have a positive effect upon its BA, and in the case of MFbM the BA could be influenced by the use of MFGM and whey protein in the formulation. It has been reported[21] that the addition of lecithin to sitostanol or cholesterol solutions increases solubility around 17- or 37-fold, respectively. The use of lecithin to solubilize sitostanol might recreate a more natural situation in which PS are associated with phospholipids in plant cell membranes. It has been suggested that the presence of sitostanol requires only a small amount of phospholipid and that the micelles thus formed are compatible with nonfat foods. In this sense, Ikeda et al.[24] studied micellar solubility from micellar solutions containing sterols (cholesterol alone or combined with β-sitosterol or sitostanol) and composed of bile salt-monoolein-oleic acid with or without lecithin. The authors found the micellar solubility of cholesterol increase approximately 1.3-fold in the presence of lecithin; however, in binary mixtures of cholesterol and either β-sitosterol or sitostanol, the presence of lecithin did not affect the micellar solubility of cholesterol or of β-sitosterol and barely increased that of sitostanol. In contrast, Matsuoka et al.[29] reported that the presence or absence of lecithin in a micellar intestinal model solution containing bile salt-oleic acid-monoolein does not influence cholesterol solubility. However, the combination of cholesterol and β-sitosterol in the presence of lecithin resulted

in a slight decrease in the solubility of both sterols. The authors also found that variations in lecithin concentration (2−10 mM) in the micellar solutions strongly affected the solubility of both sterols when combined, exhibiting a decrease in the case of cholesterol and an increase in the case of β-sitosterol. The observed differences in the influence of lecithin upon sterols solubility in our work versus the previous studies may be due to the use of model solutions versus a real food; the lecithin concentration involved (0.6 mM,[24] 1−10 mM,[29] versus 7.9 mM in our study) or differences in the proportion cholesterol:PS [1:1,[24,29] versus 1:300 (MFb and MFbO) or 1:100 (MFbM) in our beverages]. In the beverage of our study containing oleic acid and lecithin (MFbO), a 3-fold increase was recorded in the solubility of β-sitosterol and sitostanol in comparison with MFb (without emulsifiers). However, cholesterol solubility was of the same order (see Table 2).

The effect of different fatty acids on micellar sterol content has also been evaluated in model bile mixed micelles by Brown et al.,[22] who indicated that the presence of saturated fatty acids (palmitic and stearic) does not modify the incorporation of cholesterol or PS (campesterol, β-sitosterol, stigmasterol, stigmastanol). However, a slight yet significant increase was noted with the presence of unsaturated fatty acids (linoleic, α-linolenic, and oleic). In our study both the beverage with the highest saturated fatty acids content (MFbM) and the beverage with the highest unsaturated fatty acids content (MFbO) showed an increase in the BA of the individual and total PS, with no effects upon the BA of cholesterol versus MFb. However, the higher individual and total PS contents in the BF of MFbM versus MFbO could be due to the greater interaction between β-lactoglobulin-pectin in MFbM. In this sense, it has been reported that this kind of interaction is a good way to improve emulsion stability because of the negatively charged pectin adsorbed onto the surface of the positively charged β-lactoglobulin stabilized emulsion droplets.[39,40] This situation is consistent with the study published by Vaghini et al.,[15] in which the highest values of BF of total and individual PS were found in the fermented milk beverages with whey protein and pectin in their formulation. Similarly, it has been seen that the presence of whey proteins in oil−water emulsions containing β-carotene favors the BA of this compound.[41] Moreover, the presence of MFGM in MFbM has been shown to act as a natural emulsifier, allowing fat globules to remain dispersed in the water phase and reducing interfacial tension emulsion, thereby facilitating formation of the emulsion.[42] Thus, the addition of MFGM-enriched whey and pectin in MFbM could explain its highest BF content in individual and total PS (Table 2). In addition, several health benefits have been attributed to the compounds of MFGM, including the lowering of blood cholesterol levels and the prevention of coronary heart disease and other related comorbidities.[43] A recent study[44] has shown that the presence of MFGM contained in milk fat may counteract the hypercholesterolemic effects of saturated fat provided by dairy products.

In conclusion, the BA of PS is influenced by the lipid components and emulsifiers present in PS-enriched milk-based fruit beverages. The fat content of 2.4% (MFbM and MFbO) versus 1.1% (MFb) increased the BA of PS. Moreover, the addition of milk fat and whey proteins enriched with MFGM affords higher BF contents of individual and total PS and higher BA for campestanol and stigmasterol. Consequently, the beverage MFbM, containing milk fat and MFGM, seems to

be a good choice for achieving optimum BA and potential health effects of PS.

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Notes
The authors declare no competing financial interest.

■ **ABBREVIATIONS USED**

ANOVA, analysis of variance; BA, bioaccessibility; BF, bioaccessible fraction; BSA, bovine serum albumin; BHT, butylhydroxytoluene; GC-FID, gas chromatography−flame ionization detection; HMDS, hexamethyldisilazane; IS, internal standard; KCl, potassium chloride; KOH, potassium hydroxide; MFb, plant sterol-enriched milk-based fruit beverage without fat addition; MFbM, plant sterol-enriched milk-based fruit beverage with addition of milk fat and whey proteins enriched with milk fat globule membrane; MFbO, plant sterol-enriched milk-based fruit beverage with olive oil and soy lecithin addition; MFGM, milk fat globule membrane; opm, orbitations per minute; PS, plant sterols; TMCS, trimethylchlorosilane; TMSE, trimethylsilyl ether

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Safe intake of a plant sterol-enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage

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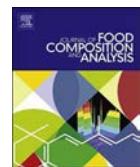
Original research article

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A B S T R A C T

Sterols in foods are susceptible to oxidation to form oxysterols. It is interesting for consumer health to know real intake and the possible associated adverse effects associated to oxysterols. This study measured oxysterol formation and bioaccessibility (BA) in a plant sterol (PS)-enriched milk-based fruit beverage with milk fat globule membrane (MFGM) added at 0, 3 and 6 months of storage at room temperature. The same cholesterol (COPs) and phytosterol oxidation products (POPs) (exclusively from β-sitosterol) (7α/β-hydroxy, α/β-epoxy, triol and 7-keto) were detected in the beverage and its bioaccessible fraction. Total COPs and POPs contents were maintained during storage, and their BA ranged between 58 and 80% and 45–49%, respectively, without significant differences throughout storage. β-Sitosterol showed a lower mean oxidation percentage (0.028%) than cholesterol (1.24%), but the estimated POPs intake (0.5 mg/day) was two-fold higher than that of COPs (0.25 mg/day) from 250 g of beverage. These results show that the presence of milk fat and MFGM in the formulation of this beverage did not imply an increase in the contents of oxysterols and their BA. Thus, the beverage is suitable as a PS-enriched food matrix for the length of its shelf-life, and its consumption appears to be safe for consumers.

1. Introduction

Plant sterols (PS) are well known for their ability to reduce low-density lipoprotein (LDL)-cholesterol concentrations. A recent meta-analysis (Ras et al., 2014) has shown that an average intake of 2.1 g PS/day (3 g PS/day being the maximum allowed dose) gradually reduces LDL-cholesterol by an average of 6–12%. In addition, antiinflammatory and anticarcinogenic properties (against cancer of the breast, prostate, lung, stomach and ovary) have also been proposed as further beneficial effects of the consumption of PS (Othman and Moghadasian, 2011; Bin Sayeed and Ameen, 2015; Ramprasath and Awad, 2015; Shahzad et al., 2017).

In this regard, several foods currently can be enriched with PS due to their cholesterol-lowering effect, since the estimated daily intake of PS from the Western diet usually does not exceed 440 mg PS. Only in the case of vegans can an intake of 1 g PS/day be reached (García-Llatas and Rodríguez-Estrada, 2011; Klingberg et al., 2012; Ras et al., 2015). Such levels fall short of the effective PS doses.

Sterols present in food (cholesterol and PS) are susceptible to oxidation. The oxidized products formed are respectively known as cholesterol oxidation products (COPs) and plant sterol oxidation products (POPs). Overall, COPs and POPs are referred to as sterol oxidation products (SOPs) or oxysterols (García-Llatas and Rodríguez-Estrada, 2011; Brzeska et al., 2016). Milk-based fruit beverages, where the addition of PS has been approved (Commission Decision, 2004), are a good option for obtaining the recommended daily amount of PS in subjects with moderate hypercholesterolemia, and are postulated as a good vehicle for preventing the possible formation of SOPs, thanks to the presence of fruits (natural sources of antioxidants) (González-Larena et al., 2015).

The effects upon the body of consuming COPs have been intensively studied, though less information is available in the case of POPs (Hovenkamp et al., 2008; Otaegui-Arrazola et al., 2010; García-Llatas and Rodríguez-Estrada, 2011; Olkkonen et al., 2015; Brzeska et al., 2016). Recently, Kulig et al. (2016) have reviewed the biological importance of COPs in the human organism and their association to chronic diseases such as atherosclerosis, neurodegenerative disorders or cancer. Given the structural similarity between PS and cholesterol, it

can be assumed that their oxidation products have similar effects in the human body, though unclear and contradictory information has been published in this regard. The biological actions associated to the consumption of POPs include effects on cholesterol metabolism, atherosclerosis and inflammation processes and cytotoxicity (García-Llatas and Rodríguez-Estrada, 2011; Vanmierlo et al., 2013; O'Callaghan et al., 2014).

Regarding oxysterol occurrence in foods, it has been found that the intensity and time of heat treatment employed are key factors affecting the oxidation of sterols, as well as food composition.

The effect of the food lipid matrix, temperature, free or esterified PS (with different degree of unsaturated fatty acids) upon sterol oxidation remains subject to controversy, and has been addressed by a number of recent reviews (Otaegui-Arrazola et al., 2010; Barriuso et al., 2016a). Soupas et al. (2004) indicated that for temperatures above 140 °C, unsaturated lipid matrices result in a lesser PS oxidation rate, since unsaturated lipid matrices are more readily oxidized, thus protecting sterols, while PS in saturated lipid matrices at higher temperatures facilitate sterol reactivity. Similar observations have been made by Soupas et al. (2007) and Barriuso et al. (2016b). However, for temperatures under 140 °C, PS oxidation is highest in unsaturated matrices. Since the mechanism underlying this effect is not clear, sterols could react more rapidly in lipid matrices where oxidation occurs more easily (Soupas et al., 2004; Otaegui-Arrazola et al., 2010). Moreover, the interaction between PS and lipids depends on whether or not PS are esterified: if PS are esterified with fatty acids, the vicinity of the radicals generated is close to the oxidation points of PS, while if the fatty acids are unsaturated, further oxidation of PS may result (Barriuso et al., 2016a). On the other hand, free PS undergo less interaction with the lipid matrix, and the more unsaturated the fatty acids surrounding PS happen to be, the more protected the sterols can be against oxidation. However, highly unsaturated lipids (such as DHA) might not exert this protective effect, due to rapid degradation of the fatty acid and the generation of a high proportion of oxidizing species (Barriuso et al., 2016a). Xu et al. (2011) also reported that polyunsaturated fatty acids possibly may compete for oxygen with sterols – the latter oxidizing first – though the effect of fatty acids upon sterol oxidation is time-dependent and is most unlikely related to their degree of unsaturation. In this regard, Soupas et al. (2007) indicate that at 160 °C, a saturated lipid matrix (butter oil) increases free PS oxidation *versus* an unsaturated lipid matrix (liquid margarine or rapeseed oil) to a greater extent (2- to 3-fold higher) than in the case of esterified PS. Furthermore, sterol oxidation is favored in oil-water emulsions, since they allow more interactions with the aqueous phase and promote the presence of free fatty acids, which accelerate oxidation of the sterols (Cercaci et al., 2007; Pignoli et al., 2009). In designing PS-enriched beverages, it could be of interest to evaluate sterol bioaccessibility (BA), defined as the maximum sterol content available for absorption, as a previous step for *in vivo* studies (Garcia-Llatas et al., 2015). In this sense, we have observed that the presence of milk fat globule membrane (MFGM), a natural emulsifier, and a fat content of 2.4% provided by milk fat, improve sterol BA in milk-based fruit beverages enriched with PS (Alvarez-Sala et al., 2016). Accordingly, the same behavior could be expected referred to the BA of SOPs, which could also be favored in this kind of beverage. To the best of our knowledge, only one study to date has assessed the BA of SOPs in a similar PS-enriched milk-based fruit beverage, though not containing MFGM in its formulation (Alemany et al., 2013). Several beneficial effects from MFGM compounds (phospholipids and gangliosides) present in bovine milk, such as improved blood lipid profiles (Vesper et al., 1999), the lowering of blood cholesterol, and the prevention of coronary heart disease (Rueda, 2014), could counteract the possible atherosclerotic effect of SOPs. Therefore, the aim of the present study was to evaluate the formation of SOPs and their BA during storage in milk-based fruit beverages enriched with PS and containing MFGM.

2. Material and methods

2.1. Chemicals and reagents

The internal standard (IS) used was 5α-cholest-5-en-3β,19-diol (19-hydroxycholesterol) (purity 98%). Other standards of COPs were cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) (purity 98%), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol) (purity 97%), 5β,6β-epoxycholestan-3β-ol (β-epoxycholesterol) (purity 90%) and cholestane-3β,5α,6β-triol (cholestanetriol) (purity 95%), all acquired from Steraloids (Newport, RI, USA). 5α,6α-Epoxycholestan-3β-ol (α-epoxycholesterol) (purity 80%), and 5-cholesten-3β-ol-7-one (7-ketocholesterol) (purity 90%) were from Sigma Chemical Co. (St. Louis, MO, USA).

Trimethylchlorosilane (TMCS) was purchased from Fluka (Buchs, Switzerland). Ammonium chloride, anhydrous sodium sulfate, chloroform, ethanol, hydrochloric acid (purity 37%), methanol, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium bicarbonate and urea were supplied by Merck (Whitehouse Station, NJ, USA). Sodium hydroxide was from Panreac (Barcelona, Spain). Uric acid was purchased from Prolabo (Sacramento, CA, USA). Diethyl ether, *n*-hexane, potassium hydroxide and 2-propanol were from Scharlau (Barcelona, Spain). Anhydrous pyridine, α-amylase from human saliva, bovine bile, bovine serum albumin (BSA), butylhydroxytoluene (BHT), calcium chloride dehydrate, cholesterol esterase from bovine pancreas, colipase from porcine pancreas, glucose, glucosamine hydrochloride, glucuronic acid, hexamethyldisilazane (HMDS), lipase from human pancreas, magnesium chloride, mucin from porcine stomach type II, pancreatin from porcine pancreas, pepsin from porcine stomach, phospholipase A2 from porcine pancreas, potassium thiocyanate, sodium dihydrogen phosphate, sodium taurocholate, and tris(hydroxymethyl)aminomethane were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents were of analytical grade. Silica solid-phase extraction (Si-SPE) cartridges (Supelclean LC-Si, 500 mg/3 mL) were purchased from Supelco (Bellefonte, PA, USA). The syringe-driven Millex-FH filters (1 mL, 0.45 μm) were purchased from Millipore, and ultrapure water was obtained by means of a Millipore Q water purification system (Milford, MA, USA).

2.2. Sample

A beverage containing skimmed milk, milk fat, whey protein concentrate enriched with MFGM (Lacprodan® MFGM-10 from Arla Foods Ingredients) (50%), mandarin juice (48%), banana puree (1%) and grape juice (1%) with the addition of microencapsulated free microcrystalline PS (Lipohytol® ME Dispersible from Lipofoods) (2 g PS/250 mL beverage) from tall oil in powder was elaborated. The beverage was prepared by the Hero Global Technology Center (Alcantarilla, Murcia, Spain) specifically for this study (product not commercially available). This sample is one of those used in the study of Alvarez-Sala et al. (2016). Energy and nutritional information per 100 mL of beverage was: energy (kJ/kcal) 263/65.3; protein (g) 3.1; carbohydrates (g) 8.9; fat without considering PS (g) 1.6; fiber (g) 1.5; PS (g) 0.8. The mean sterol contents (mg/100 g of beverage) were: β-sitosterol 704, sitostanol 102, campesterol 34.7, campestanol 9.46, cholesterol 8.15, and stigmasterol 5.19 (Alvarez-Sala et al., 2016).

The beverage was analyzed just after manufacture (time 0) and after 3 and 6 months of storage at room temperature (20–25 °C). The storage time of up to 6 months is the common and usual turnover period for products of this kind at sales points.

2.3. Determination of sterol oxidation products

2.3.1. Beverage

Lipids were extracted according to the procedure described by Alvarez-Sala et al. (2016) To 5 g of beverage (providing approximately

40 mg of PS), 25 mL of a chloroform:methanol (1:1, v/v) mixture with 0.05% BHT (as antioxidant to avoid SOPs artifact formation during saponification) was added and subsequently homogenized (Polytron PT 2000, Kinematica AC, Switzerland) for 3 min at 19,800 rpm. After adding 12.5 mL of chloroform and mixing again with the Polytron, the sample was filtered (Whatman no. 1.90 mm) through a Buchner funnel, and 20 mL of potassium chloride 1 M solution was added to the filtrate and kept at 4 °C overnight. After the organic fraction was separated, the chloroform phase was concentrated in a rotary evaporator and taken to dryness under a nitrogen stream.

For saponification, the procedure described by González-Larena et al. (2015) was used. The lipid fraction was dissolved in 10 mL of hexane:isopropanol (4:1), and 5 mL was taken, with the addition of 10 µg of 19-hydroxycholesterol as IS. Cold saponification was performed at room temperature with 10 mL of 1 N methanolic potassium hydroxide, in darkness and under continuous agitation in a shaker at 150 rpm during 18–20 h. Afterwards, the unsaponifiable material was extracted with diethyl ether, dried under nitrogen and dissolved in 10 mL of hexane:isopropanol (4:1). Five mL of the unsaponifiable material was purified by Si-SPE. The SOPs were finally eluted with acetone (10 mL) and then subjected to derivatization with HMDS:TMCS in anhydrous pyridine (2:1:5, v/v/v) (25 min, 40 °C). The trimethylsilyl ether (TMSE) derivatives were solubilized in *n*-hexane, filtered and evaporated under a nitrogen stream. Afterwards, the TMSE derivatives obtained were dissolved in 40 µL of hexane, and 1 µL was analyzed by gas chromatography-tandem mass spectrometry (GC–MS/MS) for identification purposes, and by gas chromatography-flame ionization detection (GC-FID) for quantification purposes (González-Larena et al., 2011, 2015).

2.3.2. Bioaccessible fraction

Simulated gastrointestinal digestion was applied to 20 g of beverage according to Vaghini et al. (2016) and Alvarez-Sala et al. (2016). This process includes three phases – salivary, gastric and intestinal – with the formation of mixed micelles to obtain the aqueous-micellar fraction (supernatants) regarded as the bioaccessible fraction (BF) (*i.e.*, the soluble fraction that is released in the gastrointestinal tract and is available for absorption) of the digested beverages.

To 10 mL of BF, 5 µg of 19-hydroxycholesterol was added as IS, and the mixture was subjected to cold saponification (based on Alemany et al., 2013) with 20 mL of 2 N methanolic potassium hydroxide (0.05% BHT) in darkness and under continuous agitation in an orbital shaker at room temperature (150 rpm, 18–20 h). Afterwards, the unsaponifiable material was extracted with diethyl ether, and the totality of this fraction was purified by Si-SPE. Derivatization and SOPs determination were carried out as described for the beverages.

With the aim of eliminating any possible presence of cholesterol and its oxides (since it has been reported that crude bile extract used in the simulated digestion process can contain traces of these compounds) (Alemany et al., 2013), digestions of blanks (composed of 20 g of water, in triplicate) were performed to subtract these compounds from the BF.

The BA of SOPs (*i.e.*, the percentage of SOPs available for absorption with respect to the total SOPs content in beverage before digestion) was calculated as: [SOPs content in BF (µg SOPs/100 g beverage)/total SOP content in undigested beverage (µg SOPs/100 g beverage)] × 100.

2.3.3. Identification and quantification

Identification of COPs was performed based on the resulting fragmentation patterns and chromatographic retention times of commercial standards obtained by GC–MS/MS, while the identification of POPs was established based on the fragmentation patterns described in previous studies of the group (González-Larena et al., 2011, 2015). Due to the structural similarity between COPs and POPs, and since POP standards are not commercially available, the quantification of SOPs contained in the beverage and BF using GC-FID was performed by employing calibration curves obtained with COP standards. In this

Table 1
Calibration curves for COP standards obtained by CG-FID.

COP standards	Linearity range (µg)	Calibration equation	Correlation coefficient (r)
7α-Hydroxy	0.008–0.122	y = 1.2794x − 0.0007	0.9999
7β-Hydroxy	0.025–0.245	y = 1.3735x − 0.0067	0.9998
α-Epoxy	0.099–0.694	y = 0.9219x − 0.0027	0.9971
β-Epoxy	0.102–0.713	y = 0.7082x + 0.0016	0.9990
Triol	0.051–0.303	y = 2.9507x − 0.1519	0.9928
7-Keto	0.008–0.281	y = 1.084x − 0.0047	0.9965

$y = \text{Area}_{COP}/\text{Area}_{IS}$ and $x = \mu g$ of COP/µg of IS. COP: Cholesterol oxidation products.

sense, calibration curves were prepared with 7α- and 7β-hydroxy, α- and β-epoxy, triol and 7-ketocholesterol at increasing concentrations, containing 5 µg of IS (19-hydroxycholesterol), with derivatization and analysis by GC-FID under the same conditions as for the beverages and BF. The calibration equations obtained are shown in Table 1.

2.3.4. Statistical analysis

For the study of the formation of SOPs during storage, one-way analysis of variance (ANOVA), followed by Tukeýs *post hoc* test, was applied to determine statistically significant differences in the same compound and in the same kind of sample (beverage, BF, or BA) as a function of storage time (0, 3 or 6 months).

The analysis of all samples was performed in triplicate. A significance level of $p < 0.05$ was adopted for all comparisons, and the Statgraphics® Centurion XVI.I statistical package (Statpoint Technologies Inc., VA, USA) was used throughout.

3. Results and discussion

3.1. Beverage

A GC–FID chromatogram of the SOPs present in the beverage analyzed after 6 months of storage is shown in Fig. 1. In the course of the storage period (0–6 months), the same oxides of cholesterol and β-sitosterol were detected (7α- and 7β-hydroxy, α- and β-epoxy, triol and 7-keto). Only POPs corresponding to β-sitosterol were identified, since β-sitosterol was the most abundant PS in the beverage, in agreement with previous studies in similar PS-enriched beverages (Alemany-Costa et al., 2012; Alemany et al., 2013) where β-sitosterol oxides were the only oxysterols detected. In addition, in another stability study involving similar functional beverages, β-sitosterol and campesterol oxides were identified – the latter being less abundant (González-Larena et al., 2015).

Table 2 reports the contents of SOPs in the beverage. The total mean contents of POPs ranged from 187 (t = 0) to 204 µg/100 g of beverage (t = 6 months), with no statistically significant differences during storage. The contents of POPs just after elaboration were lower than those found by Alemany-Costa et al. (2012) (580–830 µg/100 g) and González-Larena et al. (2015) (243 µg/100 g), but higher than those reported by Alemany et al. (2013) (74 µg/100 g) in PS-enriched milk-based fruit beverages. In the present study, formulation of the beverage comprised the addition of milk fat and whey proteins concentrate enriched with MFGM, yielding a 4-fold higher saturated fat content compared with the abovementioned previous studies (1.6 *vs.* 0.4 g/100 mL). Therefore, fat type and content do not seem to influence the POP content in these types of beverages.

Regarding the individual POP contents of the beverage analyzed just after elaboration, the prevalent oxide was β-epoxysitosterol followed by α-epoxysitosterol and 7β-hydroxysitosterol, with 7α-hydroxysitosterol being the least prevalent. In milk-based fruit beverages stored at 24 °C, González-Larena et al. (2015), in agreement with our own study, found α/β-epoxysitosterols to be the most abundant oxysterols. However, in previous studies on milk based fruit beverages, 7β-hydroxy followed by
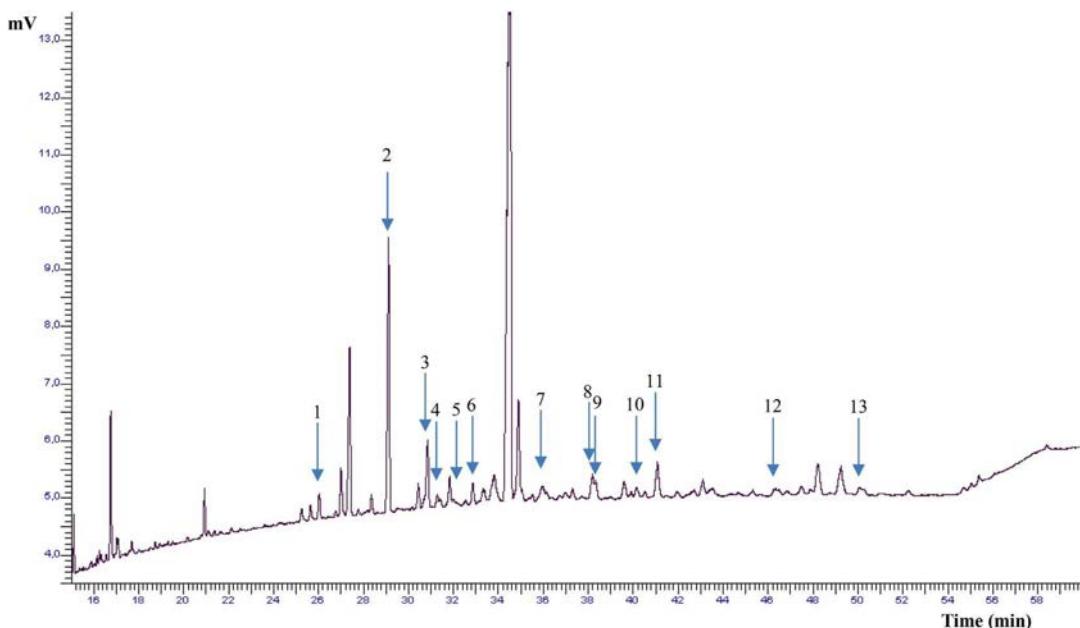
Fig. 1. Sterol oxidation products in beverage obtained by GC–FID.1: 7α-Hydroxycholesterol; 2: 19-hydroxycholesterol (IS); 3: 7β-hydroxycholesterol; 4: α-epoxycholesterol; 5: β-epoxycholesterol; 6: 7α-hydroxysitosterol; 7: cholestanetriol; 8: 7β-hydroxysitosterol; 9: 7-ketocholesterol; 10: α-epoxysitosterol; 11: β-epoxysitosterol; 12: sitostanetriol; 13: 7-ketositosterol.

β-epoxy and 7-ketositosterol (Alemany-Costa et al., 2012) or 7-ketositosterol (Alemany et al., 2013) were the most abundant POPs. The differences observed could be attributed to several factors such as the POPs profile present in the ingredient source of PS, and the manufacturing conditions involved (laboratory or industrial scale). Only a slight

significant increase was recorded for sitostanetriol after 6 months of storage.

Throughout the 6 months of storage, the relative percentages of each β-sitosterol oxide with respect to the total POPs content reflected a tendency to maintain constant proportions for all the oxides: β-epoxy

Table 2
Sterol oxidation products contents in beverage and bioaccessible fraction (μg/100 g beverage) after manufacture and during storage.

	Month	β-Sitosterol oxides (POPs)			Cholesterol oxides (COPs)		
		Beverage	BF	BA	Beverage	BF	BA
7α-Hydroxy	0	11.85 ± 3.26[a]	2.78 ± 0.44[a]	23.48 ± 3.69[a]	5.57 ± 0.24[a]	4.92 ± 0.41[a]	88.30 ± 7.32[a]
	3	12.21 ± 4.29[a]	3.90 ± 0.11[b]	31.94 ± 0.88[a]	7.47 ± 0.62[b]	6.01 ± 0.30[a]	80.50 ± 4.04[a]
	6	11.18 ± 1.52[a]	3.39 ± 0.17[a,b]	30.32 ± 1.52[a]	7.48 ± 0.37[b]	9.06 ± 0.97[b]	121.04 ± 12.97[b]
7β-Hydroxy	0	33.96 ± 2.06[a]	19.65 ± 0.78[a]	57.86 ± 2.30[a]	8.22 ± 0.65[a]	7.67 ± 0.62[a]	93.32 ± 7.52[a,b]
	3	34.59 ± 3.29[a]	19.45 ± 2.45[a]	56.24 ± 7.08[a]	10.35 ± 0.51[b]	8.49 ± 0.65[a,b]	81.99 ± 6.31[a]
	6	38.86 ± 0.33[a]	22.92 ± 3.92[a]	58.98 ± 10.10[a]	9.84 ± 0.30[b]	9.74 ± 0.52[b]	99.00 ± 5.27[b]
α-Epoxi	0	32.11 ± 4.49[a]	15.82 ± 2.26[a]	49.27 ± 7.05[a]	13.28 ± 2.63[a]	8.21 ± 1.24[a]	61.80 ± 9.31[a]
	3	34.11 ± 4.70[a]	20.66 ± 1.71[a]	60.55 ± 5.01[a]	15.07 ± 2.71[a]	5.65 ± 0.95[a]	37.49 ± 6.33[a]
	6	30.00 ± 1.72[a]	18.58 ± 4.46[a]	58.02 ± 12.51[a]	11.21 ± 1.37[a]	5.89 ± 1.96[a]	52.54 ± 17.45[a]
β-Epoxi	0	62.15 ± 5.90[a]	27.85 ± 0.03[a]	44.82 ± 0.05[a]	12.44 ± 4.72[a]	12.20 ± 2.97[a]	98.11 ± 23.88[a]
	3	72.05 ± 1.11[a]	28.77 ± 0.21[a]	39.94 ± 0.29[b]	15.72 ± 0.79[a]	11.05 ± 0.49[a]	70.30 ± 3.09[a]
	6	72.50 ± 6.19[a]	29.72 ± 1.04[a]	41.00 ± 1.43[b]	15.06 ± 3.57[a]	11.93 ± 2.14[a]	79.22 ± 14.18[a]
Triol	0	18.80 ± 1.58[a]	11.65 ± 0.43[a]	61.97 ± 2.30[a]	27.58 ± 1.69[a]	19.09 ± 0.17[a]	69.21 ± 0.62[a]
	3	18.98 ± 0.88[a]	11.59 ± 0.21[a]	61.05 ± 1.09[a]	27.04 ± 0.80[a]	18.58 ± 2.50[a]	68.71 ± 9.24[a]
	6	21.96 ± 0.71[b]	12.38 ± 0.81[a]	56.34 ± 3.68[a]	29.91 ± 1.93[a]	19.53 ± 2.72[a]	65.29 ± 9.10[a]
7-Keto	0	28.30 ± 8.60[a]	7.76 ± 1.82[a]	27.44 ± 6.44[a]	27.15 ± 6.15[a]	10.11 ± 1.92[a]	37.23 ± 7.09[a]
	3	24.86 ± 3.08[a]	8.06 ± 2.13[a]	32.44 ± 8.59[a]	27.43 ± 4.85[a]	10.12 ± 1.85[a]	36.90 ± 6.73[a]
	6	26.32 ± 0.83[a]	7.32 ± 0.98[a]	27.81 ± 3.71[a]	25.61 ± 3.91[a]	18.37 ± 5.75[a]	71.71 ± 22.45[a]
Total	0	187.16 ± 12.92[a]	85.52 ± 2.06[a]	45.69 ± 1.10[a]	99.84 ± 2.96[a]	62.66 ± 5.32[a,b]	62.77 ± 5.33[a,b]
	3	192.53 ± 3.86[a]	94.35 ± 3.20[a]	49.00 ± 1.66[a]	103.09 ± 4.56[a]	59.90 ± 4.69[a]	58.11 ± 4.55[a]
	6	204.07 ± 4.44[a]	92.54 ± 12.43[a]	45.35 ± 6.09[a]	99.12 ± 5.96[a]	79.31 ± 5.93[b]	80.02 ± 5.98[b]

Values are expressed as mean ± standard deviation of three replicates. BF: Bioaccessible fraction content. BA: Bioaccessibility, calculated as [oxides content in BF (μg/100 g beverage)/oxides content in undigested beverage (μg/100 g beverage)] × 100. Different superscript letters indicated significant differences ($p < 0.05$) in the same compound and in the same kind of sample (beverage or BF or BA) in the different months of storage (within columns, a–b).

(33–36%), 7β-hydroxy (18–19%), α-epoxy (15–17%), 7-keto (13–15%), triol (10–11%), and 7α-hydroxy (5–6%). In contrast, González-Larena et al. (2015) after 6 months of beverage storage, recorded no changes in sitostanetriol, with an increase in α/β-epoxysitosterols and a decrease in 7β-hydroxysitosterol and 7-ketositosterol.

Total amounts of COPs ranged between 99.1–103 μg/100 g of beverage, with no statistically significant changes during the storage period (Table 2). Only one study (Alemany et al., 2013) reported contents of COPs in milk-based fruit beverages, obtaining higher amounts (201 μg/100 g of beverage) than in our study, despite the lower cholesterol content in these beverages compared to the present study (1.4 *versus* 8.2 mg/100 g beverage).

Cholestanetriol and 7-ketocholesterol were the main COPs (each representing 25–27% of total COPs), while the lowest contents corresponded to 7α/β hydroxycholesterol (5–8%). This order of abundance is in agreement with the study reported by Alemany et al. (2013), where 7-ketocholesterol was the main COP and 7α/β-hydroxycholesterol were present in minor amounts. In that study, no cholestanetriol or α/β-epoxycholesterol was found. During the storage period of our beverage, only a slight significant rise in 7α/β-hydroxycholesterol was observed from three months onwards, increasing to 7–10% of total COPs.

The sterol oxidation percentages in the beverage analyzed are shown in Table 3. The oxidation percentages of β-sitosterol ranged from 0.027–0.029%, which are higher than those previously reported by Alemany et al. (2013) (0.013%) at time zero, but lower than or similar to those reported for PS-enriched milk-based fruit beverages at time zero (0.07%) (Alemany-Costa et al., 2012) or during storage at room temperature (0.03–0.06%) (0–6 months) (González-Larena et al., 2015). Thus, it can be affirmed that formulation and elaboration of the beverages of the present study, including milk fat and MFGM, do not imply greater PS oxidation.

Cholesterol, in agreement with the study of Alemany et al. (2013), presented higher rates of oxidation than β-sitosterol (Table 3). However, the percentages in our beverage after elaboration (t = 0) were lower than those reported by the aforementioned authors (1.2% *versus* 14.9%), despite the fact that our beverage had a greater cholesterol content (8.2 *versus* 1.4 mg/100 g beverage). This is in agreement with the study by Nielsen et al. (1996), where higher saturated fat and cholesterol contents implied lower cholesterol oxidation in dairy spread and butter stored at 20 °C. In contrast, at higher temperatures (180 °C), Ansorena et al. (2013) reported the opposite effect in food lipid models in the presence of triacylglycerols. In our study, the higher oxidation percentage of cholesterol *versus* PS (β-sitosterol) (see Table 3) can be ascribed to the distinct surface activity presented by the sterols, which can differently affect their susceptibility to oxidation (Cercaci et al., 2007). In this regard, cholesterol has a higher degree of surface activity than β-sitosterol – a fact that is linked to its greater capacity to reduce interfacial tension than this phytosterol, and thus cholesterol is more

concentrated at oil-water interfaces in the emulsions where oxidative stress is high. Moreover, the PS ingredient employed in the formulation of our beverage was in a microencapsulated form that can afford protection against oxidation.

3.2. Contribution of the plant sterol-enriched beverage to daily sterol oxidation product intake

Regarding the daily intake of SOPs from the diet, it is important to underscore that: i) the existing data are scarce, due to the fact that most studies consider a specific meal and not the total diet; ii) the diets or meals in these studies normally do not include PS-enriched foods; iii) the analytical methodology used to determine COPs and mainly POPs is complex and difficult, thereby making proper determination of these compounds in foods difficult; and iv) in some cases the levels of dietary SOPs are calculated based on theoretical oxidation percentages. Therefore, our calculated contribution to daily SOPs intake in the context of the abovementioned diets should be regarded as an approximation to the real situation.

Daily POPs intake has been estimated by Lin et al. (2016) based on PS intake from PS-enriched food (0.75–3 g PS/day) multiplied by the oxidation rate of phytosterols in these foods. For non-heated and stored free PS-enriched foods, the estimated daily POPs intakes are in the range of 2.9–11.4 mg/day. For pan-fried foods the estimated daily POPs intakes are 3.0, 47.7 or 78.3 mg/day, depending on the phytosterol forms added (free PS, plant sterol esters or plant stanol esters, respectively) and daily PS intakes. Lower POPs intakes from PS-enriched foods have been reported by Scholz et al. (2015) using two approaches: i) from POPs contents in these foods and the assumption that the upper daily PS intake is of 3 g from these foods (POP intake from non-heated and heated foods ranging between 1.2–2.9 and 3.5–29.6 mg/day, respectively); and ii) based on data referred to the dietary intake of PS from different surveys and the assumption in foods of a minimum (0.1%) and maximum oxidation rate (1%) (mean intakes of POPs were 0.35–2.45 and 3.5–24.5 mg/day, respectively).

For the contents of β-sitosterol oxides (POPs) in the PS-enriched beverage of this study (see Table 2), and given that the daily serving size/portion corresponds to 250 g of beverage, the daily POP intake provided was 0.47 mg just after elaboration and 0.51 mg after 6 months of storage. These values fall within the indicated interval reported by Scholz et al. (2015) for pasteurized milks (0.1–3.5 mg POPs/day). Although it has been indicated that the absolute POP contents of foods are positively associated to their phytosterol contents (Lin et al., 2016), greater PS enrichment in the beverage analyzed in the present study (0.8%) *versus* (0.3–0.5%) in the abovementioned review does not imply higher POP contents.

On the other hand, taking into account the percentage PS oxidation ratios (see Table 3) and the PS intake from the consumption of 250 g of beverage (2.14 g PS), the estimated POP intake is 0.5 mg/day from the beverage through storage. These POPs intakes are lower than those indicated by Lin et al. (2016) for non-heated foods, and are included in the range indicated by Scholz et al. (2015) for minimum oxidation rates (0.1%). It must be pointed out that the oxidation percentage of the PS-enriched beverage analyzed is comprised between 0.022–0.023% as a function of time storage (see Table 3).

Regarding COPs, intakes between 1.8–3.05 mg/day from three diets (consumed in The Netherlands and including all raw food, fried/baked or grilled food, or fried/baked or grilled food plus fruits and vegetables) have been reported by van de Bovenkamp et al. (1988). Subsequently, COPs intakes of 11.5 mg from a test meal (scrambled eggs containing egg powder, butter and whole milk) (Emanuel et al., 1991) and 2.5 mg from a typical fish and chips meal (Lake and Scholes, 1997) have been reported. In our case, the COPs intake provided by 250 g of beverage calculated similarly to POPs intake according to the COPs content during storage (see Table 2) or taking into account the cholesterol oxidation percentage and content (20.4 mg/250 g beverage) (see

Table 3
Oxidation percentage of plant sterols and cholesterol in beverage.

	Sterol content (mg/100 g beverage)[a]	Month	Oxidation (%)
Plant sterols		0	0.022
	856.29 ± 84.41	3	0.022
		6	0.023
β–Sitosterol		0	0.027
	704.72 ± 70.60	3	0.027
		6	0.029
Cholesterol		0	1.225
	8.15 ± 0.41	3	1.265
		6	1.216

[a] Values from Alvarez-Sala et al. (2016). Oxidation (%) calculated as: [(total oxides content/100 g beverage)/(total sterol content/100 g beverage)] × 100.

Table 3), was 0.25 mg/day. These values are far lower than in the previously mentioned studies, since they were calculated from a single food, whereas the other studies considered a diet or meal including cholesterol-rich foods and food deep-fried in beef fat.

3.3. Bioaccessible fraction and bioaccessibility

The SOPs contents in BF, expressed as µg/100 g of beverage, and their corresponding BA (%) are shown in Table 2. The total β-sitosterol and cholesterol oxidation product contents in the BF were 85.5–94.4 and 59.9–79.3 µg/100 g of beverage, respectively, without significant changes during the storage period. However, for individual SOPs a slight increase during storage was observed for 7α-hydroxy derivatives of β-sitosterol and cholesterol, and 7β-hydroxycholesterol.

In the only study that has assessed the effect of simulated gastro-intestinal digestion upon the SOPs contents in PS-enriched milk fruit-based beverages (Alemany et al., 2013), the POPs (32.7 µg/100 g of beverage) or COPs (5.6 µg/100 g of beverage) contents in the BF were lower than in the present study as expected, due to the lower oxysterol contents reported for the non-digested beverages (38.6 µg/100 g of beverage) in the study by Alemany et al. (2013) compared to our own study (148 µg/100 g of beverage).

Regarding the BA values, the BA of total POPs ranged between 45.4–49% during the 6 months of storage, being higher for COPs (58.1–80%), with no statistically significant differences through storage. This difference in BA could be attributed to the greater micellar solubility of cholesterol compared to β-sitosterol described in several studies using model systems or food products under different micellar-ization conditions, namely three kinds of micellar solutions including bile salts (Ikeda et al., 1989), in sodium taurocholate and sodium glycodeoxycholate solutions (Armstrong and Carey, 1987), in bile salt micelles (Matsuoka et al., 2010), from a simplified system (bile salt solutions) compared to a more complex system (model intestinal solution including bile salts, phospholipids and fatty acids) (Matsuoka et al., 2012), and from *in vitro* gastrointestinal digestion of functional beverages (Alvarez-Sala et al., 2016). By analogy, the same behavior can be expected for the corresponding oxides of cholesterol and β-sitosterol.

Regarding the individual BA of the oxides, triol, α-epoxy and 7β-hydroxysitosterol were the oxides with the highest values for POPs, and 7α- and 7β-hydroxycholesterol for COPs – this being similar to the results reported by Alemany et al. (2013).

The total POP and COP contents in the BF – expressed as micro-molarity considering the mean molecular weights for the β-sitosterol and cholesterol oxides – ranged between 1.97–2.17 µM and 1.48–1.96 µM, respectively. Compared to the cytotoxic effects indicated in Caco-2 cells (a validated model of intestinal epithelial cells), for POPs (from 60 µM) and COPs (from 30 µM) (Ryan et al., 2009; Alemany-Costa et al., 2012; Laparra et al., 2015), the values found in the present study were far lower than those concentrations that may be deleterious for health, though it cannot be ruled out that continued ingestion of these oxides may lead to degenerative diseases (Kulig et al., 2016).

4. Conclusions

The analyzed beverage, where only cholesterol and β-sitosterol oxidation products were detected, offers sterols stability at room temperature throughout the studied storage period, since no modification of total SOP contents and their BA were observed – only minor changes in some individual SOPs being recorded. The presence of MFGM and the higher fat milk contents in the beverage of this study did not imply greater SOPs formation compared with similar milk-based fruit beverages containing no MFGM. Possibly the presence of saturated fat profiles added to the incorporation of free PS in microencapsulated form would not favor sterol oxidation. In addition, the low estimated intake of POPs (0.5 mg/day) and COPs (0.25 mg/day) from the

ingestion of 250 g of beverage (daily portion), and the low presence of SOPs in BF (1.5–2.2 µM) – far from cytotoxic effects reported in cell cultures – demonstrated that milk-based fruit beverages with milk fat and whey protein concentrate enriched with MFGM would be a suitable matrix for the formulation of PS-enriched beverages, with a view to minimizing possible oxysterol formation during the shelf-life of the product. Therefore, the intake of this functional beverage with the purpose of achieving the recommended PS-intakes for obtaining a hypocholesterolemic effect may be considered safe.

Contributions

Andrea Alvarez-Sala and Virginia Blanco-Morales contributed with the analytical assays and to data acquisition and interpretation. Antonio Cilla, Guadalupe Garcia-Llatas, Reyes Barberá and María Jesús Lagarda contributed with the conception and design of the study, and inter-pretation of data. Luis Manuel Sánchez-Siles collaborated in the formulation, elaboration and provision of the beverage of this study. All authors have collaborated in the drafting and revision of the article, and have approved the final version of the manuscript for submission.

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Effects of plant sterols and/or β-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death

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Effects of Plant Sterols or β-Cryptoxanthin at Physiological Serum Concentrations on Suicidal Erythrocyte Death

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ABSTRACT: The eryptotic and hemolytic effects of a phytosterol (PS) mixture (β-sitosterol, campesterol, stigmasterol) or β-cryptoxanthin (β-Cx) at physiological serum concentration and their effect against oxidative stress induced by *tert*-butylhydroperoxide (tBOOH) (75 and 300 μM) were evaluated. β-Cryptoxanthin produced an increase in eryptotic cells, cell volume, hemolysis, and glutathione depletion (GSH) without ROS overproduction and intracellular Ca^{2+} influx. Co-incubation of both bioactive compounds protected against β-Cx-induced eryptosis. Under tBOOH stress, PS prevented eryptosis, reducing Ca^{2+} influx, ROS overproduction and GSH depletion at 75 μM, and hemolysis at both tBOOH concentrations. β-Cryptoxanthin showed no cytoprotective effect. Co-incubation with both bioactive compounds completely prevented hemolysis and partially prevented eryptosis as well as GSH depletion induced by β-Cx plus tBOOH. Phytosterols at physiological serum concentrations help to prevent pro-eryptotic and hemolytic effects and are promising candidate compounds for ameliorating eryptosis-associated diseases.

KEYWORDS: *phytosterol, β-cryptoxanthin, eryptosis, hemolysis, oxidative stress*

■ INTRODUCTION

Eryptosis is defined as programmed erythrocyte death (similar to apoptosis in nucleated cells) and is characterized by cell shrinkage and membrane blebbing with phosphatidylserine exposure at the erythrocyte surface. Eryptosis is the principal form of erythrocyte death, though there are also other death processes such as hemolysis, with the subsequent release of hemoglobin into the bloodstream.[1] Excessive eryptosis is observed in several clinical conditions including anemia, atherothrombosis, atherosclerosis, thalassemia, hemolytic uremic syndrome, cardiac and renal failure, Wilson's disease, obesity, diabetes, chronic inflammatory diseases, and malignancies as well as in the elderly. However, eryptosis can also contribute to protect against malaria disease, attenuating parasitemia.[2−9]

Eryptosis is triggered by several stressors such as osmotic shock, energy depletion, hyperthermia, and oxidative stress. The latter is a powerful trigger of eryptosis,[10,11] and it is assumed that the use of antioxidant compounds may prevent this phenomenon. *In vitro* studies have demonstrated the antiapoptotic and antioxidant effects of phytosterols (PS)[12,13] and β-cryptoxanthin (β-Cx)[14,15] on human colon adenocarcinoma cells. The PS, naturally occurring mainly in vegetable oils, margarines, and nuts, are steroids associated with health benefits such as cholesterol-lowering, antiproliferative, and anti-inflammatory effects.[16] While β-Cx is a xanthophyll, contained primarily in citrus fruits, related with protective effects against osteoporosis, inflammatory diseases, and oxidative stress.[17,18] However, their activity in relation to erythrocyte death has not been studied to date. Compounds structurally similar to PS such as whitaferin A at concentrations

effective against breast cancer cells (1−10 μM)[19] or oxysterols at concentrations occurring in hyper-cholesterolemic subjects (20 μM)[20] have shown a pro-eryptotic effect associated with oxidative stress, which could contribute to the development of atherosclerosis and thrombosis. Likewise, dietary compounds structurally similar to β-Cx, such as retinoic acid (1−10 μM)[21] and fucoxanthin (25−75 μM),[22] produced pro-eryptotic effects through intracellular Ca^{2+} influx and phosphatidylserine translocation without disturbing the redox balance. Although these compounds led to eryptosis, it is important to underscore that the existing chemical structural differences (keto-, epoxy-, hydroxy-, and ester groups, among others) with respect to PS and β-Cx could imply different biological effects.

A previous study carried out by our group showed that four months of consumption of PS (1.5 g/day)-enriched milk-based fruit beverage added with β-Cx (0.75 mg/day) resulted in an increase in the serum concentration of PS (β-sitosterol, campesterol, and stigmasterol) (18 to 22 μM) and β-Cx (0.3 to 1 μM).[23,24] The increase in serum concentration was associated with a decrease in osteoporosis and cardiovascular risk (decrease in cholesterolemia), suggesting a synergistic effect of both bioactive compounds.[17] With regard to their positive effect on cardiovascular risk, it is possible that these bioactive compounds can to help prevent atherosclerosis induced by eryptosis since it has been reported that phosphatidylserine exposure in eryptotic erythrocytes promotes
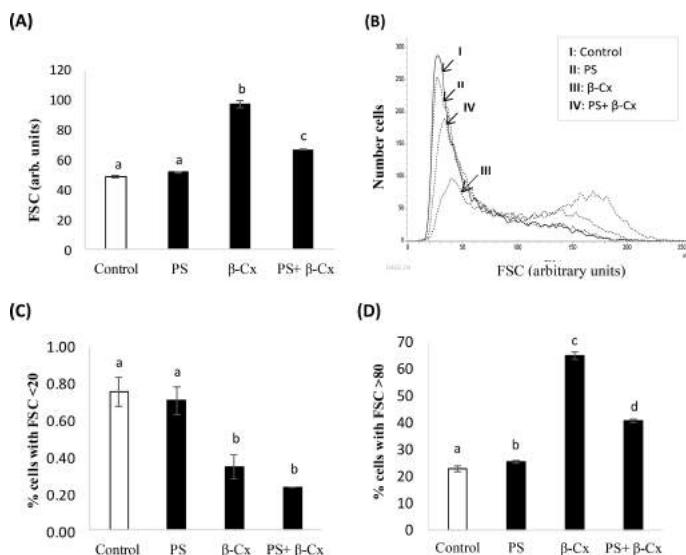
Figure 1. Effect of phytosterols (PS) or β-cryptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on the forward scatter (FSC) after 48 h of incubation with human erythrocytes. (A) Geometric mean $\pm$ SD ($n = 3$) of FSC. (B) Histogram of erythrocyte FSC in a representative treatment. (C) Arithmetic mean $\pm$ SD ($n = 3$) of the percentage of erythrocytes with FSC < 20 (arbitrary units). (D) Arithmetic mean $\pm$ SD ($n = 3$) of the percentage of erythrocytes with FSC > 80 (arbitrary units). Different lowercase letters (a−d) indicate statistical significant differences (p < 0.05) among treatments.

their adhesion to the endothelium, thereby leading to vascular complications.[10,25] In this regard, the present study for the first time has evaluated the pro- or antieryptotic and hemolytic effects of a mixture of PS (β-sitosterol, campesterol, and stigmasterol) or β-Cx at concentrations obtained after the intake of PS-enriched milk-based fruit beverages containing β-Cx, and their cytoprotective effect against oxidative stress induced by *tert*-butyl hydroperoxide (tBOOH), as a novel and hitherto nondescribed mechanism of action complementary to their known hypocholesterolemic and anticancer effects.

■ MATERIALS AND METHODS

Reagents. (24S)-Ethylcholest-5,22-dien-3β-ol (stigmasterol) (purity 97%), (24R)-ethylcholest-5-en-3β-ol (β-sitosterol) (purity 97.3%), and Luperox tBOOH solution were obtained from Sigma Chemical Co. (St. Louis. MO, USA). (24R)-Methylcholest-5-en-3β-ol (campesterol) (purity 96.19%) was purchased from Chengdu Biopurify Phytochemicals, Ltd. (Sichuan, China). β-Cryptoxanthin (purity 97%) was obtained from Cymit Química (Barcelona, Spain).

Preparation of Erythrocyte Cultures and Treatment Conditions. Blood was drawn in K$_2$EDTA tubes from healthy human volunteers ($n = 8$; aged 24−59 years; normal body mass index) at health service of University of Valencia, with informed consent. Erythrocytes were isolated by centrifugation (1000 rpm, 4 °C, 25 min) and washed with PBS according to Tesoriere et al.[20] The cells (0.4% v/v hematocrit) were incubated for 48 h (37 °C, 5% CO$_2$, 90% humidity) in the absence or presence of PS or β-Cx (alone or in combination) prepared at 0.2% (v/v) ethanol final concentration in Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 32 mM HEPES, 5 mM glucose, and 1 mM CaCl$_2$.[26] The PS mixture [22 μM PS mix: β-sitosterol (13 μM), campesterol (8 μM), and stigmasterol (1 μM)] or β-Cx (1 μM) used were compatible with physiological serum concentrations obtained after the consumption of PS-enriched milk-based fruit beverages containing β-Cx, as reported in previous studies carried out by our group.[23,24] Control cells were

incubated with ethanol 0.2% (v/v). Oxidative stress was induced with tBOOH at 75 and 300 μM, this range of concentrations being similar to those of previous reports,[27,28] during the last 30 min of the 48 h incubation period in the presence or absence of bioactive compounds (37 °C, 5% CO$_2$, 90% humidity).

Measurement of Annexin V and Forward Scatter (FSC). The measurement of eryptosis was performed through the externalization of phosphatidylserine to the cell surface, according the protocol of the manufacturer (annexin V apoptosis detection kit I, BD Biosciences, San Diego, CA, USA). Briefly, cell suspension (30 μL) was added to a new tube and washed with PBS. Then cells were centrifuged (800 rpm/25 °C/5 min) and suspended in binding buffer (100 μL). To the cell suspension 5 μL of annexin V-FITC was added and incubated at room temperature for 15 min in the dark. Then 400 μL of PBS was quickly added, and the cells were analyzed by FSC and annexin V fluorescence intensity by flow cytometry (FACS Canto, BD Biosciences), evaluating 1 × 10^4 events for each sample (λ_{exc} = 488 nm and λ_{em} = 525 nm). The analysis of FSC was displayed as percentage of erythrocytes undergoing a threshold for shrunken (<20) and swollen (>80) erythrocytes. This correlation has been described previously by Jemaà et al.[26]

Determination of Hemolysis. Erythrocytes were centrifuged (1800 rpm/24 °C/5 min) and the concentration of hemoglobin in the supernatant was determined at 405 nm by spectrophotometry (PerkinElmer, Lambda 2) according to Tesoriere et al.[20] The absorbance of the supernatant from analogous erythrocytes lysed in distilled water was defined as 100% hemolysis.

Measurement of Cytosolic Ca^{2+}. Intracellular Ca^{2+} concentration was measured according to Tesoriere et al.[29] using the cell permeable probe Fluo-3 AM (Santa Cruz Biotechnology, Santa Cruz, CA, USA), whose fluorescence is directly representative of the ion concentration. Briefly, cell suspension (55 μL), PBS (445 μL), and Fluo-3/AM at 2 μM final concentration were added to a tube, followed by incubation during 40 min (37 °C, 5% CO$_2$, 90% humidity). After centrifugation (800 rpm, 24 °C, 5 min), erythrocytes were suspended with 500 μL of PBS and analyzed by flow cytometry (FACS Canto,
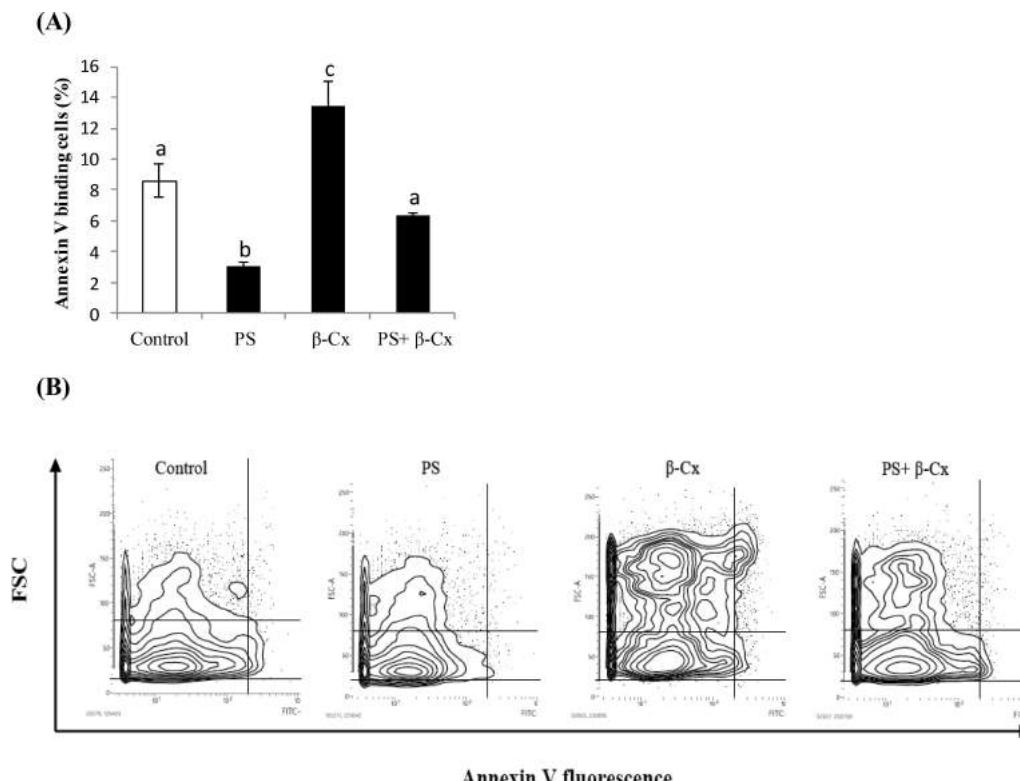
Figure 2. Evaluation of the (A) phosphatidylserine exposure and (B) dot plots of forward scatter (FSC) versus annexin V fluorescence exposure, after 48 h incubation with phytosterols (PS) or β-cryptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on human erythrocytes. Data are expressed as mean $\pm$ SD (n = 3). Different lowercase letters (a−c) indicate statistical significant differences ($p < 0.05$) among treatments.

BD Biosciences), evaluating at least 1×10^4 events for each sample (λ_{exc} = 506 nm and λ_{em} = 526 nm).

Measurement of Intracellular Reactive Oxygen Species (ROS). Intracellular ROS accumulation was measured by changes in fluorescence resulting from the oxidation of 2′,7′-dicholofluorescein diacetate (DCFDA) (Sigma Chemical Co., St. Louis, MO, USA), as previously reported.[20] Cell suspension (55 μL), PBS (445 μL), and DCFDA at 10 μM final concentration were added to a tube and incubated during 40 min (37 °C, 5% CO_2, 90% humidity). Erythrocytes were collected by centrifugation (800 rpm, 24 °C, 5 min), suspended in 500 μL of PBS and subjected to flow cytometric analysis (FACS Canto, BD Biosciences). At least 1×10^4 cells were analyzed for each sample (λ_{exc} = 495 nm and λ_{em} = 529 nm).

Measurement of Intracellular Glutathione (GSH). The evaluation of cellular GSH content was made with 1 μM (final concentration) of 5-chloromethylfluorescein diacetate (Green CMFDA) (Abcam, Cambridge, UK), according to Officioso et al.[28] The CMFDA was added to a tube with 55 μL of cell suspension and PBS (445 μL). Then erythrocytes were incubated during 40 min (37 °C, 5% CO_2, 90% humidity) and collected by centrifugation (800 rpm, 24 °C, 5 min). Cells were suspended in 500 μL of PBS and analyzed by flow cytometry (FACS Canto, BD Biosciences), evaluating at least 1×10^4 events for each sample (λ_{exc} = 492 nm and λ_{em} = 516 nm).

Statistical Analysis. One-way analysis of variance (ANOVA) followed by the Tukey posthoc test was applied to determine differences between the different samples. A significance level of $p <$ 0.05 was adopted for all comparisons, and the Statgraphics Centurion

XVI.I statistical package (Statpoint Technologies Inc., VA, USA) was used throughout.

RESULTS AND DISCUSSION

Effect of PS or β-Cx on Eryptosis. *Forward Scatter, Annexin V Binding, and Cytosolic Ca^{2+}.* Eryptosis is characterized by cell shrinkage. The effect of the bioactive compounds (PS or β-Cx) upon cell volume was therefore evaluated through forward scatter (FSC) detection (see Figure 1). Incubation for 48 h with PS did not produce significant changes ($p < 0.05$) in cell volume, while treatment with β-Cx increased the geometric mean two-fold versus the control. Co-incubation with PS and β-Cx blunted the effect of β-Cx alone (31% decrease in cell volume), suggesting that PS partially prevent the increase in cell volume (Figure 1A,B). However, two differentiated erythrocyte populations were observed under our experimental conditions: one with low FSC (<20) (Figure 1C) and another with high FSC (>80) (Figure 1D). In the case of PS, the erythrocyte population was very similar to the control. β-Cryptoxanthin in turn had a marked effect upon erythrocyte population distribution, decreasing the percentage of cells with low FSC (53%), with a concomitant increase in high FSC cells (143%) versus the control. In agreement with the results referred to β-Cx, erythrocyte exposure during 48 h to fucoxanthin (25−75 μM)[22] produced a subpopulation of
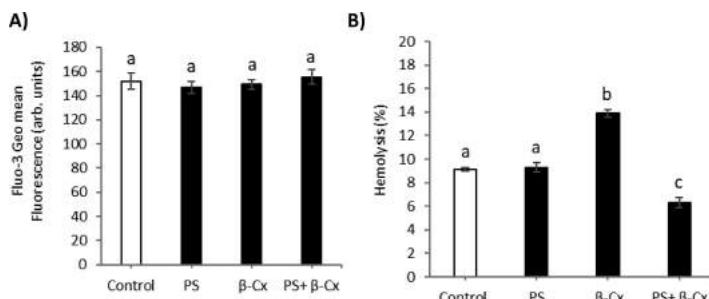
Figure 3. Evaluation of the (A) Ca^{2+} influx and (B) hemolysis after 48 h incubation with phytosterols (PS) or β-cryptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on human erythrocytes. Data are expressed as mean $\pm$ SD ($n = 3$). Different lowercase letters (a–c) indicate statistical significant differences ($p < 0.05$) among treatments.

erythrocytes exhibiting cell shrinkage (FSC < 200), and another with signs of swelling (FSC > 800). However, the proportions of both erythrocyte subpopulations (12−20% and 1.5−3%, respectively) reported by the authors differed from those observed with β-Cx, where high FSC cells predominated. This behavior may be related to two possible death pathways of the erythrocytes. It has been reported that a reduction in cell volume is associated with eryptosis, while a swelling cell is associated with hemolysis.[1] Therefore, β-Cx may partially act through hemolytic effects in its erythrocyte death-inducing action. The simultaneous presence of both bioactive compounds (PS and β-Cx) significantly reduced the percentage of cells with low FSC (69% versus control); it is therefore suggested that PS blunted (37%) the increase in erythrocytes with high FSC induced by β-Cx alone.

One of the main characteristics of eryptosis is cell membrane scrambling, with the exposure of phosphatidylserine at the external cell surface.[30] As illustrated in Figure 2A, β-Cx unlike PS induced a pro-eryptotic effect through an increase in the percentage of annexin V-binding erythrocytes (56% with respect to control cells), this implying the translocation of phosphatidylserine to the external cell membrane surface. The dot plot analysis (FSC versus annexin V) indicates that FSC > 80 cells show translocation of phosphatidylserine after β-Cx treatment (see Figure 2B), suggesting an eryptotic-hemolytic effect. Nevertheless, coincubation with both bioactive compounds prevented the eryptosis induced by β-Cx, indicating a cytoprotective effect of PS. On the other hand, an increase in intracellular Ca^{2+} is considered a key event in the eryptotic process,[28] though it does not always account for the triggering of cell membrane scrambling.[19] Figure 3A shows that none of the treatments with bioactive compounds produced an increase in intracellular Ca^{2+}, despite activation of the translocation of phosphatidylserine by β-Cx. This fact suggests that eryptosis induction by β-Cx is triggered through Ca^{2+}-independent mechanisms. In agreement with these results, it has been reported that some carotenoids such as fucoxanthin (50−75 μM)[22] and retinoic acid (1−10 μM)[21] trigger phosphatidylserine translocation to the cell surface, though unlike in our study, an increase in intracellular Ca^{2+} was observed. It has been described that the activation of protein kinase C[31] or caspase-3[32] triggers membrane phospholipid scrambling in the absence of Ca^{2+} in healthy human erythrocytes. The authors found no clear correlation between elevated calcium levels and phosphatidylserine exposure, suggesting that more molecular pathways are involved.

In contrast to our results with PS, the incubation of human erythrocytes with steroidal compounds such as whitaferin A (5 and 10 μM)[19] or oxysterols (20 μM)[20] during 48 h showed pro-eryptotic effects through an increase in phosphatidylserine translocation ($\approx$ 2.5−5- and 50-fold, respectively) and intracellular Ca^{2+} influx ($\approx$ 1.3−1.6- and 20-fold, respectively). The different behavior observed could be due to the absence in PS of reactive functional groups based on oxygen, such as keto-, epoxy- and ester groups, which have been reported to promote lipid membrane peroxidation and apoptosis in endothelial cells.[33] Moreover, *in vivo* studies have demonstrated that PS incorporation into the erythrocyte membrane does not alter membrane properties such as rigidity,[34] osmotic fragility, and deformability,[35] which may justify the observed neutral effects of PS on the eryptotic process.

Evaluation of Hemolysis. The hemolytic effects derived from our bioactive compounds are shown in Figure 3B. No significant changes in the percentage of hemolytic cells were observed after incubation with PS, though as expected and correlated with the increase in cell volume (Figure 1A), β-Cx showed a marked hemolytic effect, increasing the percentage of hemolytic cells 52% versus the control. In the same way as previously seen in relation to eryptosis, PS prevented hemolysis induced by β-Cx (55%). In line with our results referred to PS, no statistically significant increase in hemoglobin concentration was recorded after treatment during 48 h with the antioxidant apigenin (1−15 μM)[36] or whitaferin A at low concentration (1−2.5 μM).[19] In addition, the absence of hemolytic effects was also revealed from the individual sterol oxidation products ($7\alpha/\beta$-hydroxy-, α/β-epoxy-, or 7-ketocholesterol) at physiological concentrations (1−7 μM, respectively); however, cholestane-triol (2 μM) or a mixture of oxysterols at a concentration similar to that of our own study (20 μM) resulted in an 8% of lysed cells, though the different hemolytic effects of the compounds assessed was not explained by the authors.[20]

To our knowledge, only Briglia et al.[22] have observed a significant increase in the percentage of hemolytic erythrocytes ($\sim$3−8%) after 48 h of treatment with fucoxanthin (25−75 μM) (a compound structurally similar to β-Cx). According to the authors, β-Cx may have a positive effect since it could allow the clearance of erythrocytes when these are infected with the malaria pathogen *Plasmodium*.

Effect on Cellular Redox State: ROS and GSH. Eryptosis is also stimulated by oxidative stress. Accordingly, to evaluate the impact of PS or β-Cx upon the cellular redox state, intracellular ROS and GSH contents were evaluated. As seen in Figure 4A,
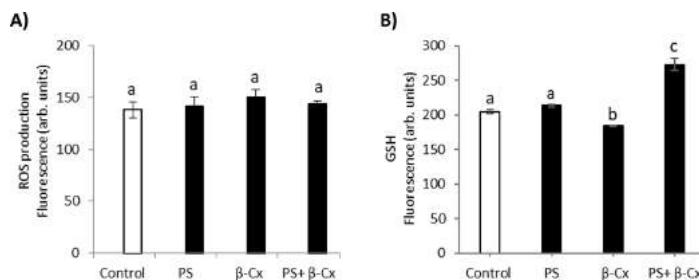
Figure 4. Effect of phytosterol (PS) or β-cryptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on (A) reactive oxygen species (ROS) production and (B) intracellular reduced glutathione (GSH) content after 48 h of incubation on human erythrocytes. Data are expressed as geometric mean $\pm$ SD ($n = 3$). Different lowercase letters (a–c) indicate statistical significant differences ($p < 0.05$) among treatments.

I: Control II: tBOOH III: tBOOH+PS IV: tBOOH+β-Cx V: tBOOH+PS+β-Cx

Without stress ☐   tBOOH 75 μM ■   tBOOH 300 μM ▨

Figure 5. Changes on forward scatter (FSC) under oxidative stress induced by tBOOH and potential preventive effect of preincubation for 48 h with or without phytosterols (PS) or β-cryptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on human erythrocytes. (A, B) Histograms of erythrocyte FSC in a representative treatment with tBOOH at 75 and 300 μM, respectively; (C, D) arithmetic mean $\pm$ SD ($n = 3$) of the percentage of erythrocytes with FSC < 20 or with FSC > 80 (arbitrary units), respectively. Different capital letters (A–C) indicate statistical significant differences ($p < 0.05$) among the different samples exposed to tBOOH at 75 μM and the lowercase letters (a–d) with respect to tBOOH at 300 μM.

Figure 6. Analysis of (A) phosphatidylserine exposure and (B) dot plots of forward scatter (FSC) versus annexin V fluorescence exposure, after 30 min of incubation with tBOOH (75 or 300 μM) with or without preincubation for 48 h with phytosterols (PS) or β-cryptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on human erythrocytes. Data are expressed as mean $\pm$ SD (n = 3). Different capital letters (A, B) indicat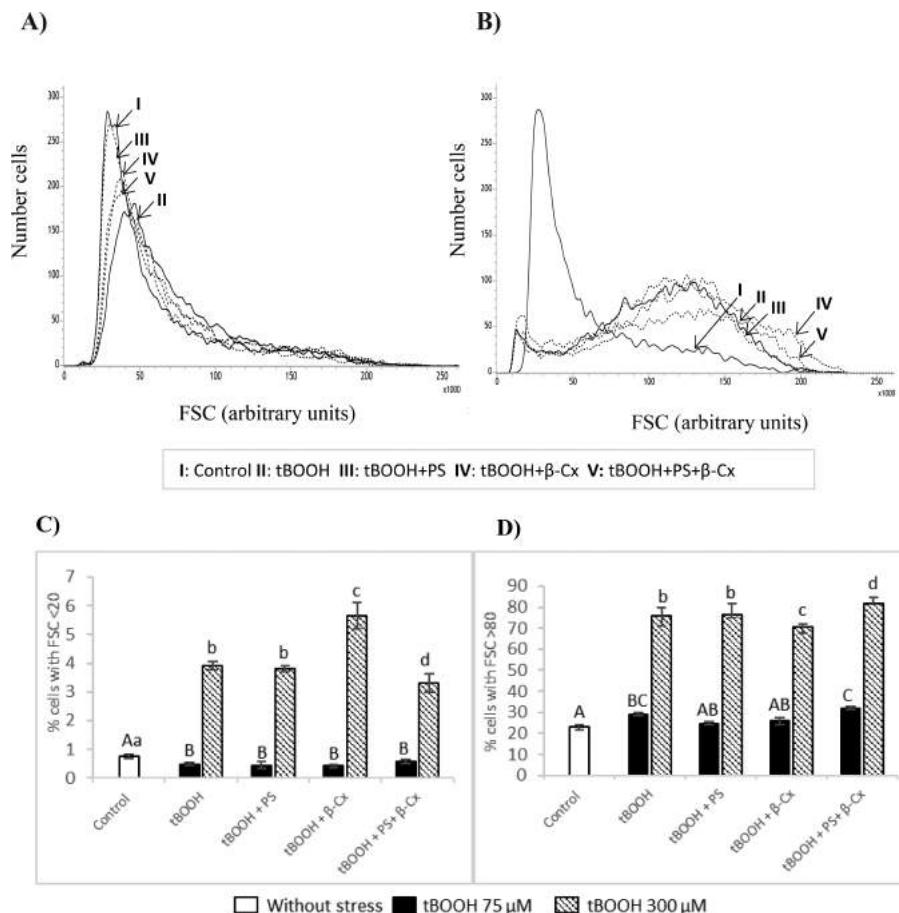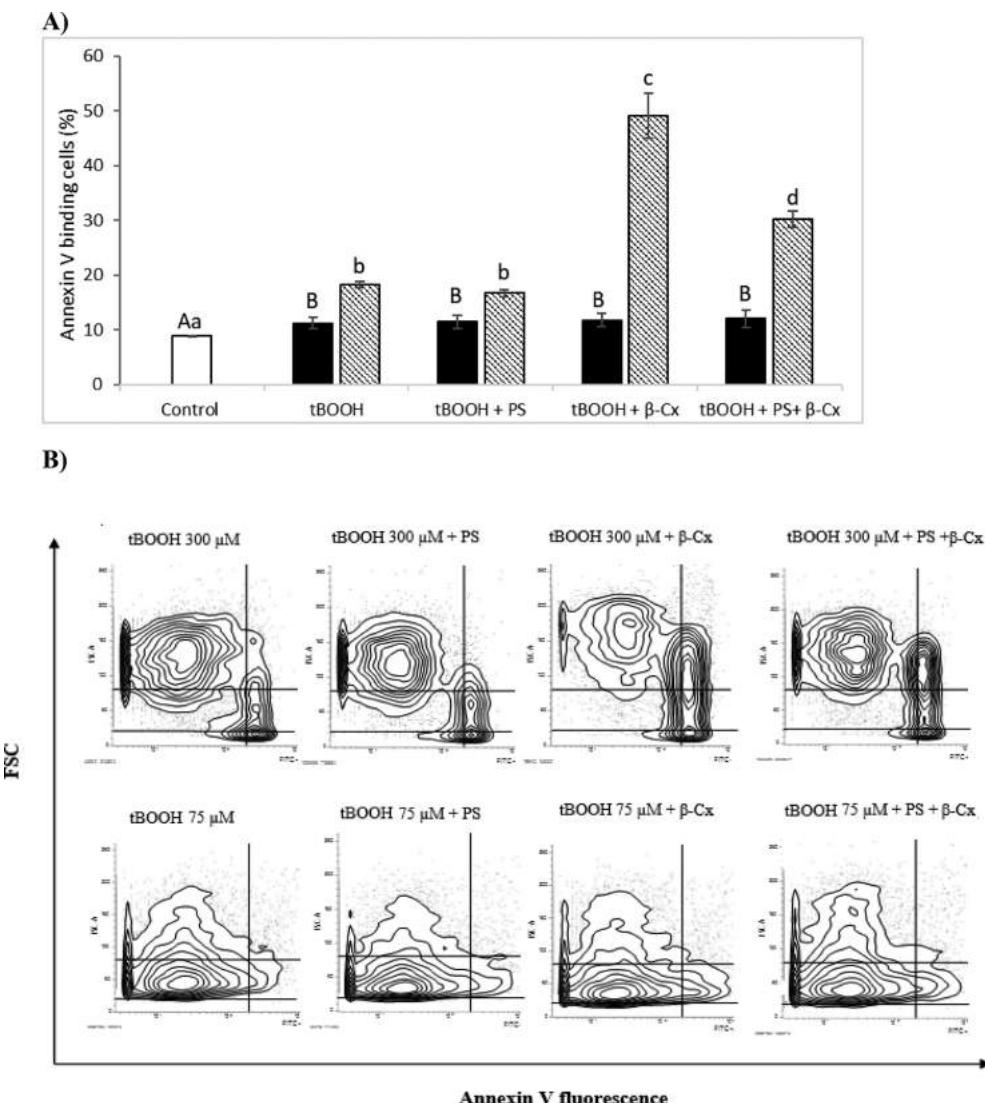e statistical significant differences ($p < 0.05$) among the different samples exposed to tBOOH at 75 μM and the lowercase letters (a–d) for tBOOH at 300 μM.

none of the treatments with the bioactive compounds promoted significant ROS overproduction, possibly due to their potential antioxidant character.[12] In the same way, PS did not produce significant changes in GSH content (see Figure 4B), which is consistent with the other previously evaluated parameters (annexin V, Ca^{2+}, and hemolysis) or its neutral effects upon the healthy erythrocyte membrane.[34,35] β-Cryptoxanthin showed a significant decrease in intracellular GSH content (10% versus the control), indicative of detrimental effects upon redox state. This GSH depletion was

restored when both bioactive compounds were coincubated, showing a cellular response against oxidative stress. In agreement with the behavior referred to the cellular redox state observed in our study, different authors[28,37] have reported the absence of increased ROS production with concomitant GSH depletion and suggest that erythrocyte ROS generation is a late event occurring after severe GSH depletion.

Effect of PS or β-Cx on Oxidative Stress-Induced Eryptosis. *Forward Scatter, Annexin V Binding, and Cytosolic Ca^{2+}.* It has been reported that oxidant agents such

as tBOOH are effective in inducing eryptosis.[27] We therefore evaluated the action of tBOOH at two concentrations (75 and 300 μM). As illustrated in Figure 5A and B, treatment with both tBOOH concentrations produced an alteration in cell volume, particularly at 300 μM. The analysis of the percentage of cells (Figure 5C,D) revealed a limited effect at lower induced stress, while an increase in the number of cells with FSC > 80 (associated with a preliminary step of hemolysis) and FSC < 20 (cell size associated with eryptosis) at the higher concentration (300 μM) was observed. The effect of this stressor upon phosphatidylserine translocation showed that tBOOH at 75 and 300 μM led to a significant increase in the percentage of annexin V-binding erythrocytes (1.3- and 2-fold versus control, respectively) (Figura 6). Interestingly, dot plot graphics (FSC versus annexin V) revealed that both erythrocyte populations (FSC < 20 or >80) presented phosphatidylserine translocation, being stronger under high stress levels (see Figure 6B). Regarding intracellular Ca^{2+} analysis, an activation of Ca^{2+} influx in a dose-dependent manner (26% and 78% versus control, respectively) was observed (see Figure 7A). In
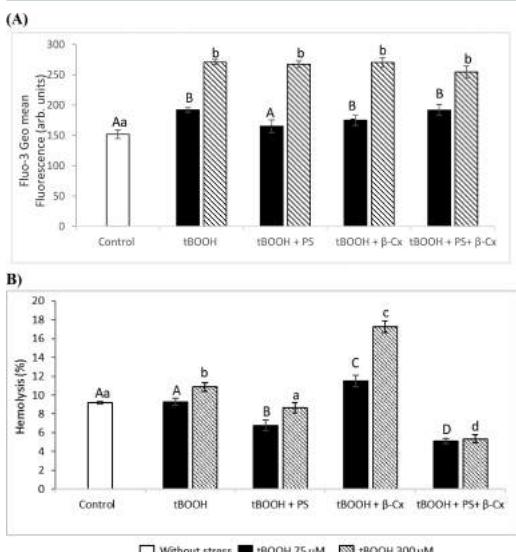

activity.[40] Moreover, other molecular pathways such as the heterotrimeric G protein subunit Gαi2,[41] mitogen- and stress-activated kinases MSK1 and MSK2,[42] p38 mitogen-activated protein kinase (MAPK),[43] which promote the eryptosis induced by hyperosmotic shock, cannot be ruled out.

Because of the oxidative stress induced by tBOOH, we evaluated the effect of our bioactive compounds (PS or β-Cx). Preincubation during 48 h of erythrocytes with PS did not modify eryptotic tBOOH action, though a nonsignificant protective tendency at high-stress concentration was observed (see Figure 6A), in addition to a preventive effect upon Ca^{2+} entry only under low-stress conditions. Regarding β-Cx, an increase in cells with FSC < 20 (45% versus tBOOH 300 μM), with a concomitant decrease in cells with FSC > 80 (8% versus tBOOH 300 μM), was observed (Figure 5C,D). At high-stress concentration, β-Cx produced a marked pro-eryptotic effect (annexin V assay) (3-fold versus tBOOH 300 μM), without altering the intracellular Ca^{2+} levels. Moreover, Figure 6B shows that the presence of β-Cx increases erythrocytes population FSC > 80 (versus control) positive to annexin V, suggesting its role as a hemolytic and eryptotic compound. Finally, coincubation with both bioactive compounds (PS and β-Cx) partially prevented the action of tBOOH and tBOOH plus β-Cx upon low-FSC cells (Figure 5C). Similarly to the situation observed without oxidative stress, coincubation with both bioactive compounds significantly ($p < 0.05$) blunted the increase in annexin V-binding erythrocytes (38% versus tBOOH + β-Cx) (see Figure 6A,B), and tended to reduce Ca^{2+} influx promoted by β-Cx under high-stress conditions (tBOOH 300 μM) (see Figure 7A).

Evaluation of Hemolysis. As is shown in Figure 7B, tBOOH treatment produced a limited hemolytic effect that proved statistically significant ($p < 0.05$) only at 300 μM (18% higher versus control). Therefore, considering the annexin binding results, it is concluded that tBOOH at low concentrations (75 μM) induces erythrocyte death mainly via eryptosis through phosphatidylserine exposure, while at high concentrations (300 μM), eryptosis and hemolysis occurred simultaneously. The induction of hemolysis by tBOOH at 300 μM would explain the lack of effect on FSC reduction (see Figure 5) because hemolysis is associated with an increase in swelling cells.[1] Such swelling is possibly due to a decrease in Na^+/K^+ ATPase activity in the erythrocytes, together with an increase in Na^+, loss of K^+, depolarization and entry of Cl^-, leading to an electrolyte increment that promotes water entry accompanied by cell swelling.[39] Preincubation with PS completely prevented hemolysis produced by tBOOH. However, no preventive effect on hemolysis was observed with β-Cx, producing a significant ($p < 0.05$) increase in hemolytic erythrocytes under oxidative conditions of 24% and 59% versus tBOOH control at 75 and 300 μM, respectively. Nevertheless, coincubation with both bioactive compounds yielded interesting results since the presence of PS completely avoided erythrocyte damage induced by tBOOH and tBOOH plus β-Cx, and even improved the erythrocyte state, with hemolysis values lower than in the control (45% and 51%, respectively).

Effect on Cellular Redox State: ROS and GSH. As illustrated in Figure 8A, treatment with tBOOH 75 and 300 μM triggered significant ($p < 0.05$) ROS overproduction, particularly at 300 μM (1.6- and 12-fold compared to control, respectively), and only at lower induced stress (75 μM) was the addition of PS or β-Cx to erythrocytes able to blunt ROS overproduction. Intracellular GSH depletion (see Figure 8B) was also lower at

Figure 7. Evaluation of (A) Ca^{2+} influx and (C) hemolysis after 30 min of incubation with tBOOH (75 or 300 μM) with or without preincubation for 48 h with phytosterols (PS) or β-cryptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on human erythrocytes. Data are expressed as mean ± SD (n = 3). Different capital letters (A−D) indicate statistical significant differences ($p < 0.05$) among the different samples exposed to tBOOH at 75 μM and the lowercase letters (a−d) for tBOOH at 300 μM.

agreement with our results, other authors have reported that tBOOH at 300 μM is an excellent oxidative agent and inducer of eryptosis, promoting phosphatidylserine translocation (15- to 55-fold, compared with untreated cells)[27,38,39] and increasing the intracellular Ca^{2+} levels (36% compared with untreated cells).[27] However, the exact mechanism is not well-known yet. It has been reported that protein kinase CK1 (casein kinase I) isoform α has a role in the regulation of erythrocyte suicide-induced by tBOOH through modulation of cytosolic Ca^{2+}
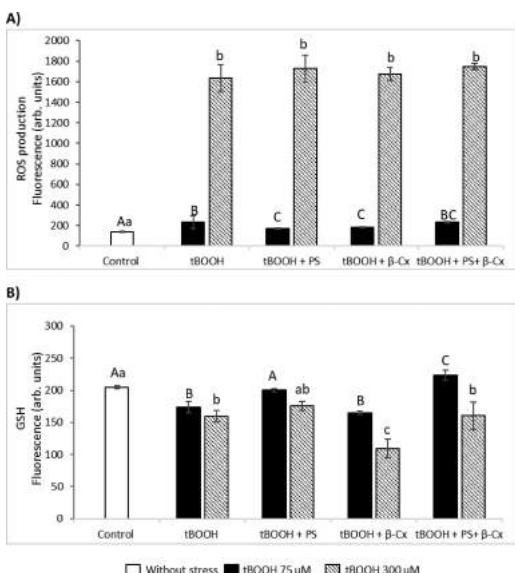
Figure 8. Changes on (A) reactive oxygen species (ROS) production or (B) intracellular reduced glutathione (GSH) content after treatment with 30 min tBOOH at 75 or 300 μM with or without previous preincubation (48 h) with plant sterols (PS) or β-criptoxanthin (β-Cx) at serum concentrations (22 and 1 μM, respectively) on human erythrocytes. Data are expressed as geometric mean $\pm$ SD ($n = 3$). Different capital letters (A–C) indicate statistical significant differences ($p < 0.05$) among the different samples exposed to tBOOH at 75 μM and the lowercase letters (a–c) for tBOOH at 300 μM.

tBOOH 75 μM than at 300 μM, but a dose-dependent relationship was not observed (15% and 22% decrease versus control at 75 and 300 μM, respectively). Preincubation with PS blunted GSH depletion produced by tBOOH at 75 μM, but only a trend to recover GSH depletion was observed at 300 μM. It is thus suggested that when erythrocytes are damaged by mild oxidative stress, PS are able to protect the cells against it. The fact that PS completely prevented GSH depletion at lower stress concentrations, and partially prevented depletion at higher stress concentrations, could suggest that the cell redox balance is preserved as a primary event since it has been observed that ROS generation is a late event in stressed erythrocytes and that eryptosis can occur via primary GSH depletion independently of ROS generation.[28]

On the other hand, the addition of β-Cx with tBOOH to the erythrocytes was not effective in preventing GSH depletion with the lowest stress levels, and even marked GSH depletion was observed at higher induced stress (300 μM). Therefore, the GSH depletion induced by β-Cx could represent a key mechanism of its eryptotic effect, mainly when stress was higher (Figure 8B). Co-incubation with both bioactive compounds (PS and β-Cx) showed no effect on GSH versus their respective tBOOH control at 300 μM, except when coincubated with tBOOH 75 μM, where the GSH levels were completely restored, avoiding erythrocyte oxidative damage.

In conclusion, physiological serum PS concentration (22 μM) exerted no eryptotic or hemolytic effect on human erythrocytes. However, under conditions of oxidative stress

(tBOOH), a cytoprotective effect upon the hemolytic process was observed, with partial prevention of eryptotic cell death at low stress levels. In contrast, β-Cx (1 μM) produced an increase in hemolytic and eryptotic cells, these effects being exacerbated in the presence of induced stress. Co-incubation with both bioactive compounds showed the presence of PS to mitigate eryptosis and hemolysis induced by β-Cx, with or without induced oxidative stress. Considering the results globally, PS would help prevent the pro-eryptotic and hemolytic effect induced by β-Cx and hydroperoxide–induced oxidation, thereby possibly affording protection against diseases associated with eryptosis, such as anemia, atherosclerosis, malignancy, obesity, diabetes, and chronic inflammatory diseases, among disorders. It is important to highlight that this novel and not previously described antieryptotic effect of PS (complementary to their well-known cholesterol-lowering and anticancer effects) can be achieved in the range of concentrations effective against tumor cells.[14] This is of paramount relevance since a recent review[44] has searched for substances that stimulate tumor cell apoptosis and by the same token inhibit eryptosis, which may avoid side effects such as anemia found in cancer patients subjected to chemotherapy. On the other hand, β-Cx exhibited pro-eryptotic and hemolytic effects, a fact that could accelerate the death of erythrocytes infected by plasmodium and help fight malaria. This study therefore opens a window for new potential beneficial applications of two antioxidant phytochemicals (PS and β-Cx), which are commonly present in well-balanced diets or in functional foods.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ANOVA, analysis of variance; CMFDA, 5-chloromethylfluorescein diacetate; β-Cx, β-cryptoxanthin; DCFDA, 2′,7′-dichlorofluorescein diacetate;; FSC, forward scatter; GSH, glutathione; PS, phytosterols; ROS, reactive oxygen species; tBOOH, *tert*-butylhydroperoxide

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Anexo 2. Artículos en revisión
Annex 2. Articles under review

Positive impact on serum lipid profile and cytokines after consumption of a plant sterol-enriched beverage with milk fat globule membrane: a clinical study

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Abstract

The hypocholesterolemic effect and the modification of serum biomarkers of dietary plant sterols (PS) intake, cholesterol precursors and cytokines after the consumption of milk-based fruit beverages with milk fat globule membrane were evaluated with a randomized, double-blind, crossover, multiple-dose bioavailability study. Postmenopausal women (n=38) consumed daily 250 mL of a beverage with or without 2 g of PS added during 6 weeks in each of the study periods. With the intake of the PS-added beverage, significant decreases (mg/dL) in serum total cholesterol (pre-treatment: 220.2±27.8 vs. post-treatment: 212.9±25.8; $p<0.05$) and LDL-cholesterol (129.4±28.5 vs. 121.7±24.4; $p<0.05$) were detected. Cholesterol precursor lathosterol (11.2%), markers of dietary PS intake (campesterol 43.1% and β-sitosterol 32.5%), and antiinflammatory IL-10 cytokine (22.5%) increased significantly, with a concomitant significant reduction in proinflammatory IL-1β (6.7%). No variations in HDL-cholesterol, other sterols (desmosterol and stigmasterol) or cytokines (IL-6, IL-8, IL-12p70 and TNF-α) were detected. Results indicated that this kind of PS-enriched milk-based fruit beverage is suitable during the period of clinical intervention, and its consumption may be an adequate way to improve PS functionality since a significant reduction in cholesterol levels has been observed. Therefore, the intake of this beverage could contribute to reduce the risk of cardiovascular diseases obtaining also a beneficial effect on serum inflammatory status in postmenopausal women.

1. Introduction

An average plant sterols (PS), comprising phytosterols and phytostanols, intake of 2.1 to 3 g/day can gradually reduce low density lipoprotein (LDL)-cholesterol levels by 6-12%,[1] and the European Atherosclerosis Society has reported an absence of adverse effects associated with a PS intake of 2 g/day.[2] Thus, PS-enriched foods can be a good vehicle for reaching an effective PS dose (1.5-3 g/day) considering that the typical Western diet alone provides a maximum of 440 mg PS/day from natural sources.[3] In this sense, PS-enriched milk-based fruit beverages (where the addition of PS is allowed in the European Union[4]) are a good option for obtaining the daily recommended amount of PS in subjects with moderate hypercholesterolemia, and are also good sources of other bioactive compounds.

LDL-cholesterol has a causal relation to atherosclerosis, and reductions in its levels have been demonstrated to decrease the risk of cardiovascular events. However, a direct relation between plasma PS and decreasing of cardiovascular risk is not clearly stablished, despite it is well known that PS can decrease LDL-cholesterol levels.[5] Different clinical studies have observed hypocholesterolemic effect (reduction of total cholesterol and LDL-cholesterol) and reported sterol (cholesterol precursors such as desmosterol and/or lathosterol and PS such as β-sitosterol, campesterol and stigmasterol) levels in plasma or serum after the consumption of PS-enriched foods, including dairy products and beverages, which are contemplated in the metaanalysis by Ras et al.[6] and in further studies (milk,[7] yoghurt,[8,9] soy milk powder,[10] beverage[11] or soy milk-based minidrink[12]).

Previous studies of our research group[13,14] evaluated serum concentrations of PS (β-sitosterol, campesterol and stigmasterol) and cholesterol precursors (desmosterol and lathosterol) after the consumption of PS-enriched milk-based fruit beverages rich in β-cryptoxanthin – demonstrating improvements in cardiovascular risk biomarkers (reduction of total cholesterol and LDL-cholesterol) and bone turnover in postmenopausal women, suggesting a synergistic effect of both bioactive compounds. Moreover, due to the lower absorption of PS (0.5-2%),[2] these may reach the colon and exert local actions to gastrointestinal level, such as antiproliferative[15,16] and cytoprotective effects.[17]

On the other hand, cytokines are involved in all stages of atherosclerosis (a process associated with inflammation), playing dual roles: proinflammatory cytokines (such as interleukins (ILs) IL-1β, IL-6, IL-8, IL-12, or tumor necrosis factor (TNF-α)) promote the development and progression of atherogenic activities, while antiinflammatory cytokines (IL-10) exert antiatherogenic responses.[18,19] Due to the expected beneficial effect of PS on atherosclerosis as a result of the lowering of LDL-cholesterol, these compounds may attenuate cytokine-induced inflammation. Nevertheless, Othman & Moghadasian[20] reported variability in the effects of PS on the release of cytokines, since several animal and human studies reflected anti-inflammatory effects of PS, but some studies have also shown no effect on inflammatory markers – thereby precluding the drawing of firm conclusions – and noted that the impact of dietary PS upon the antiinflammatory process might not be cholesterol-dependent. Studies reporting a relationship between serum/plasma cytokines and the consumption of PS-enriched dairy products or beverages such as milk,[21] soy milk powder,[10] yoghurt[9,22] and orange juice[23] are scarce. A recent review has found that the impact of PS-enriched food consumption upon inflammatory biomarkers remains unclear, and that more carefully controlled clinical trials are required.[5,24]

The food matrix to which PS are added could play an important role in mediating the cholesterol-lowering effect. A recent review[25] has suggested that milk fat globule membrane (MFGM) compounds from bovine milk, particularly phospholipids and gangliosides, may play a role in promoting metabolic health and preventing chronic metabolic diseases, in addition to the cholesterolemia-lowering effects. Several clinical studies involving the consumption of dairy products[26-28] have reported that the presence of MFGM in these kinds of foods may play a role in the inhibition of the intestinal absorption of cholesterol, contributing to a decrease in cholesterolemia. Moreover, recently, we have observed that the addition of milk fat and whey proteins, enriched with MFGM, to PS-enriched milk-based fruit beverages improves the PS bioaccessibility (BA) after simulated gastrointestinal digestion.[29] With this same sample containing MFGM, a human clinical study (NCT 02065024) has been carried out to assess the beneficial effects upon cardiovascular and bone remodeling

markers. In this context, the aim of the present study was to evaluate the *in vivo* responses, beyond its hypocholesterolemic effect, after the consumption of PS-enriched milk-based fruit beverages (with the addition of milk fat and whey proteins enriched with MFGM) using serum PS as marker of dietary PS intake, cholesterol precursors as biomarkers of cholesterol synthesis, and serum cytokines as biomarkers of inflammation. Additionally, PS stability and BA from these beverages were assessed throughout their shelf-life.

2. Materials and methods

2.1. Beverages

Two milk-based fruit beverages containing skimmed milk with the addition of milk fat and whey protein concentrate enriched with MFGM (Lacprodan® 144 MFGM-10, Arla Foods Ingredients) (50%), mandarin juice as source of β-cryptoxanthin (48%), banana puree (1%), grape juice (1%) with (PS-added beverage) or without (control beverage) free microcrystalline PS-added (Lipohytol® 146 ME Dispersible, Lipofoods) (2 g PS/250 mL beverage) from tall oil in powder form were used in this study. Energy and nutritional information per 250 mL of PS-added and control beverages, respectively, were: energy (kcal) 183 and 151; protein (g) 7.5 and 6.5; carbohydrates (g) 27.5 and 25; fat (g) 4.8 and 2.8. Beverages were prepared by the Hero Global Technology Center (Alcantarilla, Murcia, Spain) specifically for this study (products not commercially available), and their manufacturing process have been described by Alvarez-Sala et al.[29] These beverages were stored at room temperature for 6 months.

2.2. Stability study

Sterols determination in the beverages used for the clinical trial and calculation of their BA were carried out just after manufacture (time 0) and after 3 and 6 months of storage. The methodologies applied for obtaining the bioaccessible fraction (BF) (soluble fraction that is released in the gastrointestinal tract and is available for absorption) and sterols determination (in beverage and BF) have been described elsewhere.[29]

2.3. Human intervention trial

A randomized, double-blind, cross-over, multiple-dose bioavailability study (NCT 02065024) was carried out in 40 healthy postmenopausal women presenting untreated mild hypercholesterolemia[30] (total, LDL-, and HDL-cholesterol baseline values were: 220.6±23.6, 128.7±26.2, 72.2±17.8 mg/dL, respectively), a mean age of 55.7 ± 3.4 years and a mean body mass index (BMI) of 24.6 ± 4.7 kg/m². Two volunteers dropped out the study (one due to loss of contact, and another due to medical counseling during the study) (n= 38). Postmenopausal women are considered to be a higher risk of bone demineralization, and hypercholesterolemia is a risk factor for osteoporosis in this group.[13] A previous study has demonstrated in this population a positive effect of β-cryptoxanthin plus PS on cardiovascular risk and bone turnover markers.[13] Moreover, increments in proinflammatory cytokines in postmenopausal women has been described.[31]

The habitual diet of the volunteers was supplemented with 1 x 250 mL beverage/day (preferably before lunch) for 6 weeks in random order by using a computer-based table of pseudo-random numbers with a washout period of 4 weeks between the interventions. This washout period is considered enough to recover basal levels of the studied parameters. It should be noted that the last day of each intervention period all subjects took the beverage in the morning in order to standardize the time between the last intake and the blood extraction. Overnight fasting blood samples were collected before (pre-treatment) and after (post-treatment) each intervention period for the analysis of serum lipid profile and cytokines in serum (Figure 1).
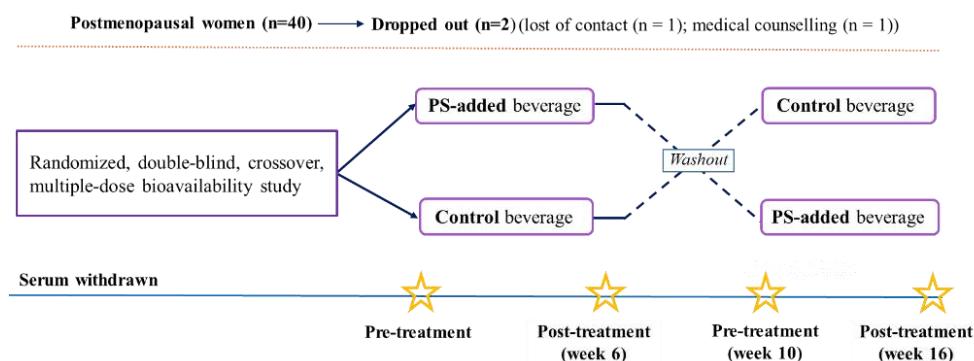

Figure 1. Schematic representation of the clinical trial

Inclusion criteria were: Age (45-65 years), BMI<35 Kg/m^2, amenorrhea over 12 months, non-dieting and non-intake of vitamin D, calcium and ω-3 fatty acids and PS or vitamin-enriched foods or supplements or other dietary bioactive components as phytoestrogens. The use of vitamins, hormone replacement therapy, cholesterol-lowering drugs, and a weight losing diet, as well as acute inflammation, chronic medication and infection or intercurrent illness capable of affecting the bioavailability or status of the compounds of interest, were regarded as exclude criteria. Subjects were asked not to change their usual diet and physical activity. They were also instructed to record any effects during study, and to complete a semiquantitative food frequency questionnaire at the end of each intervention period.

The clinical study took place at the Vitamins Unit of the Clinical Biochemistry Service of the Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain). The study protocol was approved by the Clinical Research Ethics Committee of this hospital, and all subjects gave written consent to participate in the trial. Biological samples were managed and controlled by the BioBank of the Fundación para la Investigación del Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain).

2.4. Serum biomarkers

Serum levels of total cholesterol and high density lipoprotein (HDL)-cholesterol were measured by routine quality controlled method in an Advia 2400 Clinical Chemistry system (Siemens Healthineers). Serum LDL-cholesterol was calculated by Friedewald equation.

Sterols in serum samples were analyzed by gas chromatography - flame ionization detection, according to the method validated by Garcia-Llatas et al.[32] Direct alkaline hydrolysis, without lipid extraction, followed by unsaponifiable extraction and further derivatization were applied prior to determination of the serum levels of PS (campesterol, stigmasterol and β-sitosterol) and cholesterol precursors (desmosterol and lathosterol). The calibration curves employed were: desmosterol (0.031-0.408 µg; $y = 0.4747x - 0.0022$, $r = 0.999$; retention time (RT): 20.23 min), lathosterol (0.017-0.396 µg; $y = 0.8021x + 0.0066$, $r = 0.999$; RT: 20.72 min), campesterol (0.056-0.765 µg; $y = 0.5361x + 0.0065$, $r = 0.999$; RT: 22.07 min);

stigmasterol (0.012-0.116 µg; y = 0.4109x + 0.0017, r = 0.999; RT: 23.33 min); β-sitosterol (0.117-1.508 µg; y = 0.3497x + 0.0139, r = 0.998; RT: 25.33 min), where y = Area$_{sterol}$/Area$_{Internal\ Standard}$ and x = µg of sterol. The RT of the internal standard (epicoprostanol) was 17.68 min. Serum sterol (PS and cholesterol-precursors) concentrations corrected as the ratio to cholesterol (µmol/mmol) were calculated using the cholesterol values obtained with the aforementioned routine method.

Serum proinflammatory (IL-1β, IL-6, IL-8, IL-12p70 and TNF-α) and antiinflammatory cytokines (IL-10) were measured by flow cytometry with the BD Cytometric Bead Array Kit (BD Biosciences, San Jose, CA, USA), following the instructions of the manufacturer.

2.5. Statistical analysis

For the study of sterol stability in beverages, BF or BA, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were applied to assess statistically significant differences as a function of storage time (0, 3 or 6 months).

The sample size of the clinical study was calculated to provide 80% statistical power with an alpha value of 0.05 in the detection of a decrease of 7% in cholesterol levels in mildly hypercholesterolemic subjects. Furthermore, taking into account that about 45% of the Western population may present polymorphisms implicated in the cholesterol absorption process, and assuming a drop-out rate of 10%, the final required sample size was set at 40 subjects. In order to detect statistically significant differences between pre-treatment serum lipid profile (total cholesterol, HDL- and LDL-cholesterol, cholesterol precursors and PS) or cytokines levels and the levels after intervention for each of the beverages, and the changes values (absolute and expressed as percentage) between beverages consumed, we used paired data testing with the t-test for normally-distributed data and Wilcoxon signed-rank test for no normally-distributed data. A significance level of $p < 0.05$ was adopted for all comparisons, and the Statgraphics® Centurion XVI.I statistical package (Statpoint Technologies Inc., VA, USA) was used throughout. The analysis of all samples was performed in triplicate.

3. Results and Discussion

3.1. Stability of sterols in beverages and bioaccessible fractions

Total PS and cholesterol contents in PS-added beverage ranged from 824 to 856 and 7.6 to 8.2 mg/100 g of beverage, respectively (Table 1). The relative percentages of each PS with respect to total content have been previously reported (β-sitosterol ≈ 79%, sitostanol ≈ 12%, campesterol ≈ 7%, campestanol ≈ 1%, stigmasterol ≈ 0.8%).[29] These values comply with the PS profiles specified by the European Commission for milk products and milk-based fruit beverages.[4] At all timepoints (0, 3 and 6 months) there were no significant differences referred to total PS and individual sterols (PS and cholesterol). Thus, no loss in initial sterol content occurred during 6 months of storage, demonstrating PS stability during the intervention period of the *in vivo* study. These results are in agreement with our previous studies in PS-enriched beverages analyzed after 6 months of storage.[14,33] The presence of natural antioxidants from fruit juices, and an increase in total antioxidant capacity probably due to later Maillard reaction products formed during storage, and the antioxidant activity of casein and whey proteins[33] could contribute to the PS stability observed in the PS-added beverage. Besides, we have previously reported a scarce production of sterol oxidation products during 6 months of storage at room temperature in these kinds of beverages.[34] Cholesterol content in control beverage (without PS-enrichment) proved stable during the storage period (t = 0 months 7.42 ± 0.13 mg/100 g beverage, t = 6 months 7.42 ± 0.26 mg/100 g beverage).

The stability of sterol contents (PS and cholesterol) in the BF of PS-added beverage, and their corresponding BA, are shown in Table 1. Total and individual PS contents in BF and their BA were stable at time 0 and after 3 and 6 months of storage. The total PS content in the BF ranged from 243-267 mg/100 g beverage. In relation to the individual PS, the β-sitosterol content was the highest, followed by sitostanol and campesterol, with lesser amounts of campestanol and stigmasterol, as occurred in the beverage. The BA of total PS (29-32%) also remained stable during the storage period, with generally similar BA values of individual PS within the same month. Cholesterol content in BF ranged from 4.9-6 mg/100 g beverage, while BA ranged from 64-75%. The higher BA of cholesterol vs. PS could be ascribed to

greater micellar solubility of cholesterol,[35] as it has been previously discussed for these kinds of beverages.[29]

In control beverage, cholesterol content in BF (6.79 ± 0.24 mg/100g beverage) and the corresponding BA (91.50 ± 3.20%) at 6 months were analyzed. Therefore, the mean BA of cholesterol in PS-added beverage (Table 1) was 15-25% lower than in control beverage, showing that the presence of PS reduces the incorporation of cholesterol to the micellar phase, probably by a competitive behavior in *in vitro* digestion. This dynamic competition during the formation of dietary mixed micelles has been reported previously in model bile salt systems[36-38] and proposed as one of the mechanisms of action of PS in the reduction of cholesterol absorption in the gut.[39] Recently, Gleize et al.,[40] using an *in vitro* digestion model, observed that a mixture of PS as esters or in free form incorporated into a low-fat yoghurt reduced cholesterol micellarization by 15-40% – being at the same order of our results.

Table 1. Sterol contents in PS-added beverage, and in bioaccessible fraction (mg/100 g beverage), and their bioaccessibility during storage.

Sterols	0 months[a]			3 months			6 months		
	Beverage	BF	BA	Beverage	BF	BA	Beverage	BF	BA
Cholesterol	8.15 ± 0.41^a	5.32 ± 0.33^a	$65.2 \pm 4.0^{a,x}$	7.62 ± 0.31^a	4.87 ± 0.07^b	$63.9 \pm 0.9^{a,x}$	8.00 ± 0.82^a	5.99 ± 0.12^a	$74.9 \pm 1.5^{b,x}$
Campesterol	34.70 ± 4.11^a	11.63 ± 0.18^a	$33.5 \pm 0.5^{a,y}$	33.37 ± 2.72^a	10.72 ± 0.45^a	$32.1 \pm 1.4^{a,y,z}$	34.30 ± 3.14^a	11.63 ± 1.03^a	$33.9 \pm 3.0^{a,y}$
Campestanol	9.46 ± 0.82^a	3.55 ± 0.41^a	$37.6 \pm 4.3^{a,z}$	10.06 ± 0.66^a	3.54 ± 0.11^a	$35.2 \pm 1.1^{a,z}$	10.21 ± 0.82^a	3.77 ± 0.26^a	$36.9 \pm 2.5^{a,y}$
Stigmasterol	5.19 ± 0.38^a	1.69 ± 0.05^a	$32.5 \pm 1.0^{a,y}$	5.39 ± 0.39^a	1.65 ± 0.13^a	$30.6 \pm 2.3^{a,y}$	5.54 ± 0.49^a	1.74 ± 0.16^a	$31.5 \pm 2.9^{a,y}$
β-Sitosterol	704.72 ± 70.60^a	215.12 ± 3.75^a	$30.5 \pm 0.5^{a,y}$	675.30 ± 31.05^a	194.72 ± 8.39^a	$28.8 \pm 1.2^{a,y}$	675.23 ± 49.42^a	212.72 ± 19.28^a	$31.5 \pm 2.9^{a,y}$
Sitostanol	102.77 ± 9.10^a	35.13 ± 0.46^a	$34.2 \pm 0.5^{a,b,y}$	105.72 ± 12.86^a	32.26 ± 1.42^a	$30.5 \pm 1.4^{a,y}$	98.66 ± 7.08^a	35.27 ± 2.80^a	$35.8 \pm 2.8^{b,y}$
Total PS	856.29 ± 84.41^a	267.11 ± 3.85^a	31.2 ± 0.5^a	831.09 ± 32.93^a	242.89 ± 10.47^a	29.2 ± 1.3^a	823.94 ± 57.86^a	265.14 ± 23.53^a	32.2 ± 2.9^a

Values are expressed as mean ± standard deviation of three replicates. BF: Bioaccessible fraction content. BA: Bioaccessibility, calculated as [BF content (mg sterol/100 g beverage)/total content in undigested beverage (mg sterol/100 g beverage)] × 100. PS: plant sterols. Different superscript letters denote significant differences ($\rho < 0.05$) in the same compound (individual sterol or total PS content) and in the same kind of sample (beverage or BF or BA, within lines) (a,b) or in the BA of the same month of storage (x-z). [a]Data corresponding to 0 months were previously reported in Alvarez-Sala et al.[29]

3.2. Intervention trial

3.2.1. Serum lipid profile

The intake of PS-added beverage induced significant reductions (post-treatment vs. pre-treatments values) in serum total cholesterol (212.9±25.8 vs. 220±27.8 mg/dL; $p<0.05$) and LDL-cholesterol (121.7±24.4 vs. 129.4±28.5 mg/dL; $p<0.05$), which meant changes of -2.9% and -5.1%, respectively. HDL-cholesterol levels after consumption of this beverage were comparable to levels prior the intervention (Table 2). Intake of control beverage did not significantly modify the parameters aforementioned.

Table 2. Serum lipid profile response upon regular consumption of the beverages (n=38). Results are expressed as Mean±SD.

(mg/dL)	PS-added beverage		Control beverage	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Total cholesterol	220.0±27.8[a]	212.9±25.8[b]	219.4±24.0[a]	220.0±25.4[a]
LDL-cholesterol	129.4±28.5[a]	121.7±24.4[b]	128.8±27.1[a]	129.3±24.9[a]
HDL-cholesterol	71.7±16.9[a]	71.4±20[a]	72.0±18.4[a]	69.9±17.1[a]

Different superscript letters denote significant differences ($p < 0.05$) in the same kind of beverage (with PS-added or control) among pre-treatment and post-treatment values (within lines) (a,b). Reference range (mg/dL): total cholesterol (150-200); LDL-cholesterol (70-160); HDL-cholesterol (35-75).

Serum cholesterol precursors (desmosterol and lathosterol) and PS (β-sitosterol, campesterol and stigmasterol) concentrations (in µg/mL and corrected as the ratio to cholesterol) at pre-treatment and after 6 weeks of consumption of each type of beverage (with or without added-PS), together with the changes values, are shown in Table 3. As a reflection of the consumption of the PS-added beverage, there were statistically significant increases in serum campesterol (43.1%), β-sitosterol (32.5%) and lathosterol (11.2%) concentrations after the intervention, while the stigmasterol (minor PS in the beverage) and desmosterol concentrations showed no variations. The well-known greater degree of absorption of campesterol among dietary PS was also observed in PS-added beverage, as it is indicated in the percentages of change (Table 3).

Table 3. Sterols response in serum upon regular consumption of the beverages (n=38) (Mean, Confidence Intervals 95%).

Sterols	Pre-treatment		Post-treatment (6 weeks)		Change	
	µg/mL	µmol/mmol cholesterol	µg/mL	µmol/mmol cholesterol	Absolute (µg/mL)	(%)
PS-added beverage						
Desmosterol	2.00[a] (1.82,2.17)	0.91[a] (0.84,0.98)	2.00[a] (1.83,2.17)	0.94[b] (0.86,1.02)	0.00[y] (-0.14,0.13)	1.7[y] (-4.93,8.27)
Lathosterol	1.41[a] (1.21,1.61)	0.63[a] (0.54,0.72)	1.48[b] (1.26,1.70)	0.69[b] (0.59,0.79)	0.07[y] (-0.02,0.13)	11.2[y] (-2.13,24.59)
Campesterol	1.86[a] (1.62,2.11)	0.83[a] (0.71,0.94)	2.60[b] (2.21,3.00)	1.18[b] (1.00,1.36)	0.736[y] (0.46,1.01)	43.1[y] (26.19,59.94)
Stigmasterol	0.37[a] (0.31,0.43)	0.16[a] (0.13,0.19)	0.37[a] (0.31,0.43)	0.16[a] (0.14,0.19)	0.00[y] (-0.03,0.04)	3.1[y] (-6.10,12.31)
β-Sitosterol	3.97[a] (3.08,4.87)	1.69[a] (1.39,1.98)	5.08[b] (4.19,5.98)	2.23[b] (1.83,2.63)	1.11[y] (0.50,1.72)	32.5[y] (19.03,45.92)
Control beverage						
Desmosterol	1.95[a] (1.79,2.12)	0.89[a] (0.82,0.95)	1.90[a] (1.72,2.09)	0.87[a] (0.79,0.95)	-0.05[y] (-0.19,0.09)	-1.2[y] (-8.13,5.73)
Lathosterol	1.40[a] (1.20,1.60)	0.67[a] (0.53,0.81)	1.39[a] (1.19,1.58)	0.63[a] (0.54,0.71)	-0.01[y] (-0.12,0.10)	3.3[y] (-5.07,11.66)
Campesterol	1.79[a] (1.43,2.14)	0.77[a] (0.63,0.91)	2.21[b] (1.85,2.57)	0.96[b] (0.81,1.11)	0.42[y] (0.20,0.64)	36.8[y] (20.94,52.63)
Stigmasterol	0.34[a] (0.30,0.38)	0.15[a] (0.13,0.17)	0.35[a] (0.31,0.40)	0.15[a] (0.13,0.17)	0.01[y] (-0.02,0.04)	5.4[y] (-4.56,15.39)
β-Sitosterol	3.50[a] (2.92,4.08)	1.49[a] (1.24,1.74)	3.57[a] (2.89,4.25)	1.52[a] (1.13,1.91)	0.07[z] (-0.28,0.44)	2.7[z] (-8.81,14.20)

Analysis were made in triplicate. Different superscript letters denote significant differences (p < 0.05) in the same kind of beverage (with PS-added or control) among pre-treatment and post-treatment values (within lines) (a,b), or in different beverages among changes (absolute or expressed as percentage) (within columns) (y,z). Absolute change = post-treatment level minus pre-treatment level. Change (%) = Absolute change × 100/ pre-treatment level.

When serum sterol (PS and cholesterol-precursors) concentrations were corrected as the ratio to cholesterol (Table 3), we observed the same increasing trend of lathosterol, campesterol and β-sitosterol after the intervention vs. pre-treatment levels with PS-added beverage. Regarding desmosterol, a slight, but significant increase after the intervention was detected, whereas its absolute values did not change as observed previously by Hallikainen et al.[12] In addition, we observed statistically significant changes (in absolute and percentage values) for β-sitosterol (major PS in PS-added beverage) among beverages, indicating that regular PS-added beverage intake provides a ~ 15-fold greater serum β-sitosterol content vs. control beverage (1.11 vs. 0.07 µg/mL, respectively) (Table 3). Surprisingly, no statistically significant differences for campesterol in the percentages of change between beverages were observed (Table 3).

As described in the literature, the magnitude of serum total cholesterol and LDL-cholesterol reductions and the increase in sterol (cholesterol-precursors and PS) levels, may vary depending on daily amount of PS consumed, the PS-ingredient composition, food matrix, study design, the number of subjects, the duration of the intervention and individual modifiers (such as non-responder subjects and subject baseline characteristics).[1,6] In this sense, on comparing the results obtained in this study with those previously published in our research group[13,14] involving a clinical trial with similar skimmed-milk-based fruit beverages (but without milk fat and MFGM added in their formulation), it was observed that the presence of these ingredients in the PS-added beverage did not improve the hypocholesterolemic effect, since a slight lower reduction in total and LDL-cholesterol (3-5% vs. 5-7%[13]) was detected. Furthermore, these previous studies reported different sterol level modifications (1.4-fold higher increase in β-sitosterol and 2.2-fold lower in campesterol serum levels[14]) and no modifications of lathosterol[13] concentrations. The discrepancies observed after comparing with present study, could be due to the slight differences in the formulation of the beverages, with lower PS dose consumed (1.5 vs. 2 g/day), lesser percentage of campesterol (5.5% vs. 7%), lower fat content (1.3% vs. 1.9% (with addition of milk fat and MFGM)), as well as shorter duration of the study intervention (4 vs. 6 weeks).

Regarding fat content of PS enriched foods, it is observed that could also influence the serum response of lipid profile. In a study involving beverages with different fat content (nonfat beverage (less than 0.1% fat) and low fat-beverage (1%)) with 1.8 g of PS consumption, failed to decrease total and LDL-cholesterol concentrations in moderately hypercholesterolemic subjects; however, showing greater percentages of change for β-sitosterol (-5.6% vs. 37.7%) and campesterol (4.8% vs. 31.5%) in the presence of fat, respectively.[11] Accordingly, an *in vitro* study on the BA of three PS-enriched milk-based fruit beverages with different fat contents (1.1% and 2.4%),[29] suggested that the higher fat content and the presence of MFGM present in our beverage could promote the formation of mixed micelles in the gastrointestinal digestion, thereby improving the availability of PS for decreasing LDL-cholesterol level.

Several studies[41-44] involving parallel designs with variable number of subjects (25-95) that consumed during the same period time (42 days), low-fat fermented milks enriched with lower PS dose (1.6 g), but similar PS mixture (β-sitosterol: 75-80% and campesterol: 8.4-10%) than our work, showed greater reduction in total cholesterol (4.7-12%) and LDL-cholesterol (8.4-11.3%). Possibly, the larger reductions in these parameters could be due to higher respective baseline concentrations of total cholesterol (230-264 mg/dL) and LDL-cholesterol (147-166 mg/dL) in subjects concerned in these studies vs. our own study (221 and 129 mg/dL, respectively). In this sense, the cholesterol concentration at baseline seems to explain part of the heterogeneity observed between different studies as previously reported.[13,45] Moreover, in agreement with our study, any of these studies observed changes in HDL-cholesterol.

The range of increments in serum β-sitosterol and campesterol after the consumption of PS-added beverage were variable, being 0.50-1.72 and 0.46-1.01 µg/mL, respectively, showing an interindividual variability in the response to its intake (Table 3). Higher increase of β-sitosterol than campesterol could due to the higher proportion of this PS (78.86%) vs. campesterol (7.13%)[46] founded in the tall oil PS-ingredient used for the enrichment of this beverage. Moreover, the metaanalysis published by Ras et al.[6] showed that intake of similar PS-enriched

foods than our study involving dairy products (low-fat fermented milk, low-fat yogurt, low-fat milk, skimmed milk and milk) and orange juice with an average PS dose of 1.7 g/day, increases plasma β-sitosterol and campesterol concentrations by 0.6-12 and 0.8-9 µmol/L, respectively after median duration of 40 days – these increments are within levels recorded in our work for β-sitosterol (1.21-4.15 µmol/L) and campesterol (1.15-2.53 µmol/L), with an intake of 2 g/day during 42 days. In contrast, some authors[41,42,44] have observed no significant variations in the levels of campesterol (and neither β-sitosterol in the last study).

With regard to the stigmasterol concentrations, few studies have addressed this aspect,[13,14,41] likewise without recording significant changes after the intervention period – in line with our own findings.

In our trial, the increase in serum PS employed as biomarkers of dietary PS intake, and in lathosterol (cholesterol precursor) as markers of cholesterol synthesis, can be related to a compensatory homeostatic mechanism since PS inhibit cholesterol absorption.[47] Various authors[11,13,26,41,42,44,48] have reported nonsignificant changes in lathosterol after the intake of PS-enriched dairy products (1.5-2.7 g PS/day). Nevertheless, another authors,[7,8] in agreement with our own study, described an increase in plasma lathosterol concentration (13.5-22%) after the intake of dairy product (yoghurt or skimmed milk) enriched with PS (2-3 g/day). These authors linked this increment to the synthesis of endogenous cholesterol to compensate the observed decrease in intestinal cholesterol absorption, which produce a 5.5-8.7% and 4.1-13.7% of serum total and LDL-cholesterol reduction vs. control, respectively. Moreover, it is suggested that PS in plasma are a marker of a healthy diet and a lower cardiometabolic risk associated with a reduced risk for coronary heart diseases.[7,8]

3.2.2. Cytokines in serum

The serum IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α concentrations at pre-treatment and after the intervention period of consumption of each type of beverage (with or without PS-added), together with the changes (absolute and expressed as percentage), are shown in Table 4. After PS-added beverage intake, a statistically significant decrease in proinflammatory IL-1β and concomitant increase in antiinflammatory IL-10 cytokine levels were recorded. During the intervention with

control beverage, we observed an increase in proinflammatory IL-12p70 and a slight increase in IL-10 (< 40% than in the case of PS-added beverage). No variations in other cytokines were detected during the interventions. On comparing both beverages, only IL-1β and IL-10 showed statistically significant differences in percentages of change after 6 weeks for PS-added beverage (-6.7 and 22.5, respectively) vs. control beverage (-5.0 and 13.4, respectively) (Table 4).

To our knowledge, few studies have evaluated the relationship between serum/plasma cytokine levels and the consumption of PS-enriched dairy products similar to our beverages (Table 5), showing that the inflammatory response to PS is not as consistent as the LDL-cholesterol lowering effect. Among them, only two studies have also reported serum PS/stanol measurements after the intervention with enriched products. Specifically, a recently published clinical study[10] has reported that the consumption of soy milk powder (2 g PS/day) during four weeks increased the serum ratio of β-sitosterol, campesterol, and stigmasterol from 3:3:2 to 4:4:3, with no influence on the TNF-α concentrations – probably because this cytokine is undetectable or present in low concentrations in healthy people, as suggested by the authors. Moreover, the intervention neither altered significantly blood cholesterol profile (total, LDL and HDL). However, Brüll et al.[9] recorded a significant increase in serum sitostanol (9.4%) and campestanol (6.8%) concentrations in asthmatic subjects who received plant stanol-enriched yoghurt, with a concomitant decrease in serum total cholesterol (8%), LDL-cholesterol (11%) and in diverse cytokines (IL-1β (0.2%) and TNF-α (0.9%)), with no changes in IL-8 concentrations after 8 weeks. In these studies, although the effect of the PS-enriched food consumption on cytokine level is not very outstanding, authors conclude that dietary PS can alleviate lipid peroxidation events and improve immune function *in vivo* from the observed beneficial effects on other serum biochemical markers– this being more evident in populations with oxidative stress-related risk factors than in healthy populations.

Table 4. Serum concentrations of cytokines (pg/mL) after consumption of the beverages (n=38) (Mean, Confidence Intervals 95%).

Cytokines	Pre-treatment	Post-treatment	Change Absolute (pg/mL)	(%)
	pg/mL			
PS-added beverage				
IL-1β	20.45[a]	17.67[b]	-2.78[y]	-6.7[y]
	(17.30,23.60)	(15.28,20.06)	(-5,-0.56)	(-18.19,4.81)
IL-6	10.21[a]	10.35[a]	0.14[y]	7.7[y]
	(9.57,10.85)	(9.89,10.81)	(-0.52,0.8)	(-7.11,22.53)
IL-8	8.47[a]	8.83[a]	0.36[y]	20.7[y]
	(7.17,9.77)	(7.35,10.31)	(-0.57,1.29)	(-5.68,47.10)
IL-10	11.00[a]	13.10[b]	2.10[y]	22.5[y]
	(9.77,12.23)	(11.69,14.51)	(1.23,2.97)	(11.73,33.17)
IL-12p70	99.18[a]	92.63[a]	-6.55[y]	68.9[y]
	(66.94,131.42)	(58.74,126.52)	(-54.84,41.74)	(-8.55,146.27)
TNF-α	15.35[a]	17.19[a]	1.84[y]	16.6[y]
	(13.52,17.18)	(14.60,19.78)	(-0.06,3.74)	(-3.22,36.34)
Control beverage				
IL-1β	21.75[a]	19.72[a]	-2.03[y]	-5.0[z]
	(18.34,25.16)	(16.94,22.50)	(-3.94,-0.12)	(-11.80,1.78)
IL-6	10.37[a]	10.66[a]	0.29[y]	4.6[y]
	(9.77,10.97)	(9.97,11.35)	(-0.52,1.10)	(-1.88,11.14)
IL-8	8.69[a]	8.96[a]	0.27[y]	9.2[y]
	(7.33,10.05)	(7.41,10.51)	(-0.75,1.29)	(-4.04,22.48)
IL-10	12.01[a]	12.85[b]	0.84[z]	13.4[z]
	(10.65,13.37)	(11.14,14.56)	(-0.68,2.36)	(-1.41,28.29)
IL-12p70	89.58[a]	129.84[b]	40.26[y]	186.0[y]
	(62.12,117.04)	(82.61,177.07)	(-10.74,91.26)	(-30.78,402.86)
TNF-α	17.10[a]	16.99[a]	-0.11[y]	1.2[y]
	(14.78,19.42)	(14.25,19.75)	(-2.00,1.78)	(7.33,9.73)

Analysis were made in triplicate. Different superscript letters denote significant differences ($\rho < 0.05$) in the same kind of beverage (with PS-added or control) among pre-treatment and post-treatment values (within lines) (a,b), or in different beverages among changes (absolute or expressed as percentage) (within columns) (y,z). Absolute change = post-treatment level minus pre-treatment level. Change (%) = Absolute change × 100/ pre-treatment level.

Additionally, other studies (Table 5) only reported cytokine levels describing variable results. In agreement with our study, Devaraj et al.[23] found the consumption of PS-fortified orange juice to result in a decrease in IL-1β (6.4 times higher than in our PS-added beverage), with no variations in the TNF-α and IL-8 levels. However, they reported reductions in IL-6 (27-46%), without changes in IL-10. These differences with respect to our own observations could be explained by the fact that

this study was carried out with a longer intervention period (8 weeks) in healthy volunteers. On the contrary, the study by Plat et al.[22] on patients with metabolic syndrome, did not observe modifications in IL-6 concentrations after the intake during 8 weeks of a low-fat yoghurt drink containing plant stanols. The authors speculated that, although a total cholesterol reduction was detected, the absence of an effect of the plant sterols upon endothelial function could be ascribed to the duration of the intervention, which could have been too short to induce an effect in the studied population. In line with our observations, another study[21] involving milk enriched with PS detected no variations in TNF-α or IL-6 levels in hypercholesterolemic population similar to our subjects. However, when this enrichment was made with triglyceride-recrystallized PS (TRP) an 11% reduction in IL-8 was reported, whereas no effect was shown with free PS (as in our study). Although the mechanism underlying the decrease in IL-8 was unclear, Kunces et al.[21] suggested an indirect effect of the greater reductions in plasma LDL-cholesterol (-15.4%) exerted by the TRP and, thus, in LDL oxidation. This oxidation is also reduced in presence of PS, which would attenuate immune cell recruitment towards the arterial wall, with consequent reduction of cytokine secretion and production. This mechanism has also been proposed by Nashed et al.[49] in apo E-KO mice fed with 2% soybean-derived PS in order to justify the association between IL-10 elevation and the concomitant reduction of atherosclerotic lesions in aortic tissue.

Moreover, regarding mechanistical pathways, the decrease in IL-1β and increase in IL-10 obtained from our PS-added beverage (Table 4) may suggest that its consumption offers antiatherosclerotic effects, since it has been reported that IL-10 promotes expression of the ATP-binding membrane cassette transporter A1 (ABCA1), which play an important role in cholesterol efflux, whereas IL-1β cytokine downregulates its expression.[50]

Table 5. Serum cytokines levels from human studies consuming PS-enriched dairy products and fruit juices.

Study design	Subjects	Subjects status	Food format (g/d)	PS dose (g/d)	Duration (wk)	Inflammatory markers	Results	References
Randomized Double-blind	M (7) F (2) 56-64 y	Metabolic syndrome Family history of CHD and/or overweight	Low-fat yoghurt drink	2*	8	IL-6	=	22
Randomized Double-blind Parallel	M (13) F (23) 19-74 y	Healthy volunteers	Orange juice (NS fat/d)	2$^{(NS)}$	8	IL-1β IL-6 IL-8 IL-10 TNF-α	↓IL-1β (43%) ↓IL-6 (46%)	23
	M (16) F (20) 19-74 y		Orange juice beverage				↓IL-6 (27%)	
Sequential Crossover	M (13) F (7) 35-70 y	Men/postmenopausal women with hypercholesterolemia	Fat-free milk	2¥	12	IL-6 IL-8 TNF-α	=	21
			Fat-free milk	2 (TRP)			↓IL-8 (11%)	
Randomized Double-blind Parallel	M (7) F (22) 18-70 y	Allergic asthma	Soy-based yoghurt	4*	8	IL-1β IL-8 TNF-α	↓IL-1β (0.2%) ↓TNF-α (0.9%)	9
Randomised, Double-blind Crossover	M (6) F (12) 26-45 y	Men/women without hypercholesterolemia	Soy milk powder	2¥	4	TNF-α	=	10
Randomised, Double-blind Crossover	F (38) 45-65 y	Postmenopausal women with moderate hypercholesterolemia	Milk-based fruit beverage (PS-added beverage)	2¥	6	IL-1β IL-6 IL-8 IL-10 IL-12p-70 TNF-α	↓IL-1β (6.7%) ↑IL-10 (22.5%)	Our study

¥ Free PS form; * stanols; = no changes; CHD: coronary heart disease, d: day; F: female; M: male; NS: not specified; PS: plant sterols; TRP: triglyceride-recrystallized phystosterol, wk: weeks; y: year

4. Conclusion

The intake of PS incorporated into milk-based fruit beverages with the addition of milk fat and whey-protein concentrate enriched with MFGM reduced serum levels of total cholesterol and LDL-cholesterol without significantly altering HDL-cholesterol concentrations. Moreover, it resulted in significant increases in serum PS and lathosterol (associated to cholesterol synthesis), with a concomitant increase in antiinflammatory cytokines (IL-10) and a reduction of proinflammatory cytokines (IL-1β). This fact is of interest in postmenopausal women who may suffer sudden increments in the levels of proinflammatory cytokines associated to menopause. Thus, this milk-based fruit beverage is an adequate matrix for enrichment with PS and can be considered a nutritional strategy able to contribute to decrease the risk of cardiovascular disease, although more studies are warranted. In addition, further clinical trials are needed involving different target populations in order to draw broader and more consistent conclusions on its possible health-beneficial effects.

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Antiproliferative effect of a phytosterol-ingredient and its main phytosterol (β-sitosterol) in human cancer cell lines

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Abstract

Dietary interventions may effectively control cancer development, with phytosterols (PS) being a class of cancer chemopreventive dietary phytochemicals. The present study, for the first time, evaluates the antiproliferative effects of a PS-ingredient used for the enrichment of several foods and its main PS, β-sitosterol, at physiological serum levels, in the most prevalent cancer cells in women (breast (MCF-7), colon (HCT116) and cervical (HeLa)). In all three cell lines, these compounds induced significant cell viability reduction without a clear time- and dose-dependent response. Moreover, all treatments produced apoptotic cell death with the induction of DNA fragmentation through the appearance of a sub-G1 cell population. Thus, the use of PS as functional ingredients in the development of PS-enriched foods could exert a potential preventive effect against human breast, colon and cervical cancer.

Keywords: Antiproliferation; Apoptosis; Breast cancer; Cervical cancer; Colon cancer; Plant sterols.

1. Introduction

The global burden of cancer has gained great attention, as it is among the leading causes of morbidity and mortality worldwide. It has been estimated that there were 14.1 million new cancer cases and 8.2 million deaths in 2012, and the numbers are expected to rise significantly over the coming decades. In women, the most prevalent cancers are breast cancer (25.2%), followed by colorectal cancer (9.2%) and cervical cancer (7.9%) (Ferlay et al. 2015). Bioactive food components such as phytosterols (PS) could constitute a new alternative as cancer chemopreventive and therapeutic agents against these kinds of cancers, intervening in several regulatory pathways. Different reviews on the anticancer effects of PS (mainly β-sitosterol) have been published (Woyengo et al. 2009; Bradford and Awad 2010; Shahzad et al. 2017).

The antiproliferative activity of β-sitosterol standards has been studied in breast cancer (MCF-7) (Awad et al. 2007, 2008; Rubis et al. 2010), colon cancer (HT-29, Caco-2 and/or HCT116) (Awad et al. 1996, 1998; Choi et al. 2003; Daly et al. 2009; Montserrat-de la Paz et al. 2015) and cervical cancer (HeLa) cells (Cheng et al. 2015). Likewise, the anticarcinogenic effects of isolated PS from plant extracts have also been evaluated in MCF-7 (Chai et al. 2008; Malek et al. 2009; Rahman et al. 2013; Yaacob et al. 2015; Tahsin et al. 2017), in HCT116 (Malek et al. 2009; Rahman et al. 2013) and in HeLa cells (Block et al. 2004; Csupor-Loffler et al. 2011; Hamdan et al. 2011; Han et al. 2013).

Since dietary PS alone are unable to offer the recommended daily doses (1.5-3 g/day) for lowering LDL-cholesterol (Gylling et al. 2014), several foods are currently enriched with PS. The main sources of PS used for this purpose are vegetable oil deodorizer distillate and *tall oil* (a byproduct of the kraft pulping of wood) (García-Llatas and Rodríguez-Estrada 2011; González-Larena et al. 2011).

Previous studies conducted by our research group have reported a positive effect on cardiovascular risk and bone turnover markers after the regular consumption of milk-based fruit beverages enriched with PS from *tall oil* (Granado-Lorencio et al. 2014); anticarcinogenic effects of PS standards (β-sitosterol, campesterol and stigmasterol, alone or combined) upon Caco-2 cells, at concentrations compatible with physiological serum levels after the intake of such beverages (Cilla et al. 2015),

and cytoprotective effects of the bioaccessible fractions obtained after simulated gastrointestinal digestion of these beverages in Caco-2 cells (López-García et al. (2017a). Moreover, due to the fact that PS undergo less absorption (0.5-2%) (Gylling et al. 2014), these compounds may reach the colon and exert local actions at gastrointestinal level. In this context, a recent study carried out by our research group (López-García et al. 2017b), considering the estimated colonic concentrations of PS capable of reaching the colon after the intake of the aforementioned kind of beverage, revealed antiproliferative effects upon the Caco-2 cell line. However, the anticarcinogenic activity of the *tall oil* PS-ingredient used for food enrichment has not been evaluated so far. Thus, the present work, for the first time, studies the antiproliferative effect of a PS-ingredient from *tall oil* used for the enrichment of milk-based fruit beverage and its main PS (β-sitosterol) at concentrations compatible with physiological serum levels, following its regular consumption, upon human breast (MCF-7), colon (HCT116) and cervical cancer (HeLa) cell lines. In this regard, the present study could complement the well known hypocholesterolemic effect of PS and moreover contribute to extend their use as functional ingredients in the development of PS-enriched foods to maximize their functionality.

2. Materials and methods

2.1. Reagents

Dimethyl-sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), (24R)-ethylcholest-5-en-3β-ol (β-sitosterol), RPMI-1640 medium, propidium iodide (PI) and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V apoptosis detection kit FITC was from eBioscience (San Diego, CA, USA). Antibiotic solution (penicillin–streptomycin), nonessential amino acids, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), phosphate buffered solution (PBS) and trypsin–EDTA solution (2.5 g/l trypsin and 0.2 g/l EDTA) were obtained from Gibco (Scotland, UK).

Phytosterol powder free microcrystalline ingredient from *tall oil*, previously characterized by our research group (β-sitosterol 78.86%; sitostanol 11.95%;

campesterol 7.13%; campestanol 1.20% and stigmasterol 0.82% (González-Larena et al., 2011)), containing maltodextrin, sucrose ester and inulin was purchased from Lipofoods (Lipohytol® 146 ME Dispersible).

2.2. Cell culture and treatments

The human breast (MCF-7), colon (HCT116) and cervical cancer (HeLa) cells (American Type Culture Collection, LGC Promochem, Italy) were used between passages 33 and 47, and were grown in 75 cm^2 Falcon flasks in RPMI-1640 medium containing 4.5 g/l glucose and supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acids, 1% (v/v) HEPES, and antibiotic solution (penicillin–streptomycin). Cells were incubated in a humidified atmosphere (37 ºC, 5% CO_2).

In all experiments, cells were seeded at a density of 5×10^4 cells/cm^2 after trypsin treatment (2.5 g/l trypsin and 0.2 g/l EDTA). For viability assays, cell lines were seeded during 24 h onto 96-well plates with 0.2 ml of RPMI medium, followed by treatment for 24 h or 48 h with the *tall oil* PS-ingredient or β-sitosterol at different concentrations (13, 26 and 52 µM). These concentrations are included in the range of PS physiological serum levels (5-30 µM), reaching values of 50 µM after the consumption of PS enriched foods (Daly et al. 2009; Gylling et al. 2014). The lowest PS concentration assayed in the present work (13 µM), is similar to the serum β-sitosterol concentration (15 µM) obtained after the consumption of milk-based fruit beverages enriched with the *tall oil* PS-ingredient previously evaluated by our research group (Garcia-Llatas et al. 2015). In the case of the PS-ingredient, the selected concentrations were based on the main PS (β-sitosterol).

For cell cycle and apoptosis assays, cells were seeded onto 24-well plates with 1 ml of RPMI medium. Following 24 h from seeding, cell lines were treated with PS-ingredient (13 µM) or β-sitosterol at different concentrations (13, 26 and 52 µM) for 48 h, based on the cell viability results. Control cultures and PS-ingredient were prepared with RPMI medium and β-sitosterol in tetrahydrofuran (0.1%) for all assays. Under the abovementioned conditions, tetrahydrofuran did not affect cell viability (data not shown).

2.3. Cell viability assay

Cells were seeded onto 96-well plates at a density of 18,000 cells per well, and were maintained under appropriate culture conditions (37 ºC, 5% CO_2) for 24 h. After 24 or 48 h of treatment with the three concentrations of PS-ingredient or β-sitosterol (13, 26 and 52 μM), 4 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) (5 mg/ml) was added, followed by incubation at 37 ºC for 2 h. Then, the MTT reaction agent was removed and 100 μl of DMSO was added to allow the dissolution of purple formazan products, as previously described (Girasolo et al. 2014).

2.4. Cell cycle analysis

For cell cycle assay, each cell line was treated and incubated during 48 h (37 ºC, 5% CO_2) with samples. Aliquots of 1×10^6 cells were harvested by centrifugation (125 x g, 5 min), and the cell pellet was washed with PBS and incubated in the dark (30 min at 4 ºC) in a 5 mM sodium phosphate buffer solution containing 20 μg/ml PI, Triton (0.1%, v/v) and 200 μg/ml RNase. DNA fluorescence was measured by cytofluorometry using an Epics XL™ flow cytometer with Expo32 software (Beckman Coulter, Miami, FL, USA). The relative distribution of 1×10^4 events was analyzed for each sample as described by Cilla et al. (2015).

2.5. Assessment of apoptosis through phosphatidylserine exposure

The apoptosis measurement was carried out according to Cilla et al. (2015) through flow cytometry by double staining with Annexin V/PI to detect externalization of phosphatidylserine to the cell surface. Each cell line was treated and incubated during 48 h (37 ºC, 5% CO_2) with samples, and then adjusted to 1×10^6 cells/ml with binding buffer. Cell suspension (100 μl) was added to a new tube and incubated with 5 μl Annexin V and 10 μl of 20 μg/ml PI solution at room temperature in the dark (15 min). Then, for each sample 1×10^4 events were analyzed by flow cytometry with the appropriate two-dimensional gating method.

2.6. Statistical analysis

The analysis of all samples was performed in triplicate. One-way analysis of variance (ANOVA), followed by LSD *post hoc* testing were applied to determine

differences between treated and control cells on the same day of treatment. A paired t-test was used for the MTT assay to detect statistically significant differences between different time periods for one same treatment. A significance level of $p<0.05$ was adopted for all comparisons, and the Statgraphics® Centurion XVI.I statistical package (Statpoint Technologies Inc., VA, USA) was used throughout.

3. Results

The effects of PS treatment with *tall oil* PS-ingredient or its main PS (β-sitosterol) upon cell viability, cell cycle and apoptosis were evaluated at physiological concentrations in cell lines corresponding to the most prevalent cancers in women: breast (MCF-7), colon (HCT116) and cervical (HeLa).
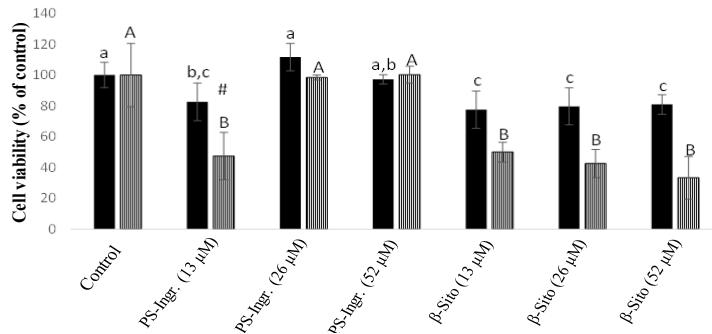
3.1. MCF-7 cells

The effect of PS-ingredient or β-sitosterol (13, 26, 52 μM) after 24 and 48 h of incubation upon MCF-7 cell growth, assessed by MTT assay, is shown in Figure 1A. Only the phytosterol-ingredient at 13 μM and the β-sitosterol standards showed a significant ($p<0.05$) decrease in cell viability, being greater at 48 h. The decrease in cell viability produced by the PS-ingredient (13 μM) was 17% or 53%, which was similar to the results for β-sitosterol (13, 26 and 52 μM), ranging from 19-22% or 50-67% at 24 and 48 h, respectively – with no statistically significant ($p<0.05$) dose-response effect among all the concentrations tested. For this reason, the following assays (cell cycle and apoptosis studies) were carried out only with the physiological PS-ingredient concentration (13 μM) and β-sitosterol (13, 26 and 52 μM) at 48 h.

MCF-7 cell staining with PI was used to evaluate the cell cycle at 48 h by flow cytometry (Figure 1B). The percentage of cells in the sub-G_1 phase (considered as a marker of DNA fragmentation) (Choi et al. 2003) increased with all treatments versus MCF-7 control cells, following the order: β-sitosterol 13 μM (7.1 fold) > β-sitosterol 26 μM (6.3 fold) > PS-ingredient 13 μM (5.4 fold) > β-sitosterol 52 μM (3.5 fold). This was accompanied by a concomitant and statistically significant ($p<0.05$) decrease, in the same order, for the other cell cycle phases (20-58%, 15-51% and 21-55% in phases G_1, S and G_2/M, respectively). These results suggest that

the PS-ingredient and β-sitosterol could exert an antiproliferative effect through apoptosis involving the modulation of cell cycle progression.

The best marker for evaluating cell death through apoptosis is phosphatidylserine externalization, an event occurring in the early phase of apoptotic cell death when the cell membrane is still intact. For this purpose, Annexin V-FITC and PI double labeling was carried out by flow cytometry. As shown in Figure 1C, all treatments induced an increase in early apoptosis versus control MCF-7 cells, with the same behavior of samples (PS-ingredient and β-sitosterol standard) as evaluated in the cell cycle assay. These results again indicated the absence of a dose–response effect upon MCF-7 cells.
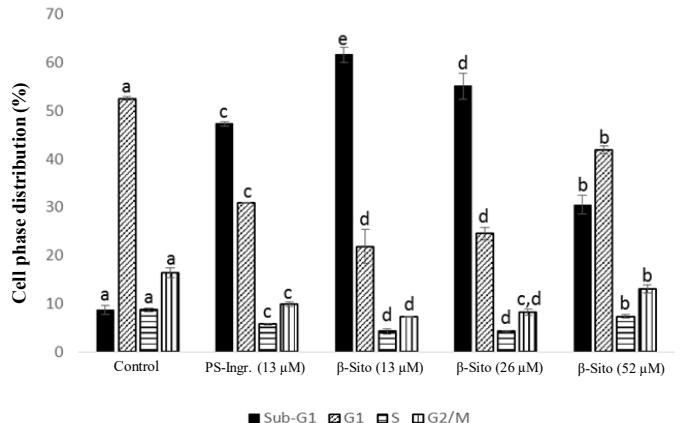
Figure 1. Effect of PS-ingredient (PS-Ingr.) or β-sitosterol (β-sito) on **(A)** viability (MTT assay), **(B)** cell cycle and **(C)** apoptosis of MCF-7 cells. Data are expressed as mean ± SD; MTT assay: different lowercase letters (a-c) indicate statistical significant differences (p<0.05) among the different treatments after 24 h of incubation and the capital letters (A-B) after 48 h. #Denotes statistically significant differences between 24 and 48 h for a same treatment; Cell cycle assay: different lowercase letters (a-e) indicate statistical significant differences (p<0.05) among the different treatments after 48 h of incubation; Apoptosis assay: percentage of Annexin V/propidium iodide (PI) double-stained cells presented are representative images of three experiments in triplicate.

3.2. HCT116 cells

The effect of PS-ingredient or β-sitosterol (13, 26, 52 μM) upon HCT116 cell growth after 24 and 48 h of incubation is shown in Figure 2A. All treatments (except PS-ingredient at 26 μM after 24 h) induced a statistically significant ($p < 0.05$) decrease in cell viability with respect to control cells. The greatest viability reductions were observed with the PS-ingredient at 13 μM (36% or 46% at 24 and 48 h, respectively) and β-sitosterol at 13, 26 and 52 μM (27-42% or 44-49% at 24 and 48 h, respectively), though with no clear time- and dose-response effect.

After 48 h of exposure, an increase in the percentage of cells in sub-G_1 phase was observed with all treatments (Figure 2B), with a significant ($p < 0.05$) higher value for PS-ingredient at 13 μM and β-sitosterol at 13 and 26 μM (6.7-8.8 fold) versus β-sitosterol 52 μM (3.3-fold). At the same time, we observed a significant ($p < 0.05$) decrease only in phase G_1 (19.4-23.9%), with the exception of β-sitosterol at 52 μM. This behavior was also observed after Annexin V/PI assay (Figure 2C), where all treatments significantly ($p < 0.05$) induced an increase in early apoptosis (12.9-20.9%) versus control cells (1%), following the order: PS-ingredient 13 μM ≥ β-sitosterol 13 μM ≥ β-sitosterol 26 μM > β-sitosterol 52 μM. These results again indicated the absence of a dose–response effect upon HCT116 cells, and that the effect of PS-ingredient on this cell line was due to the apparent predominant action of its main PS (β-sitosterol).
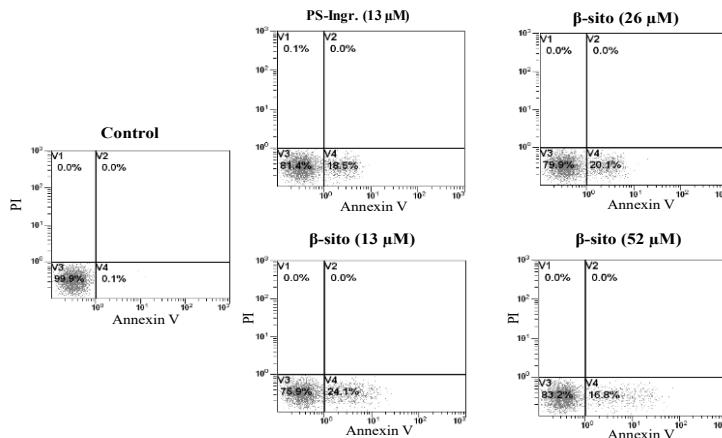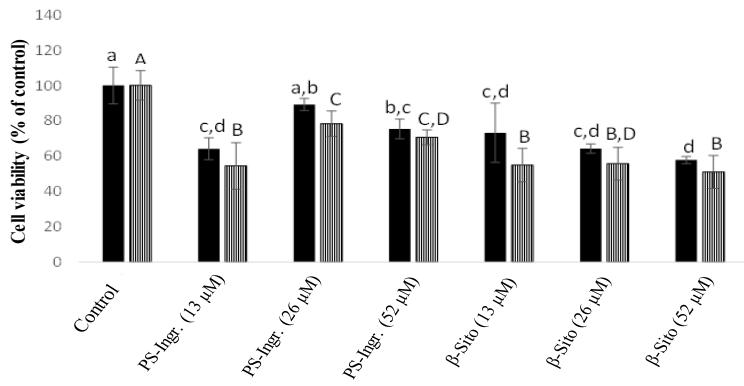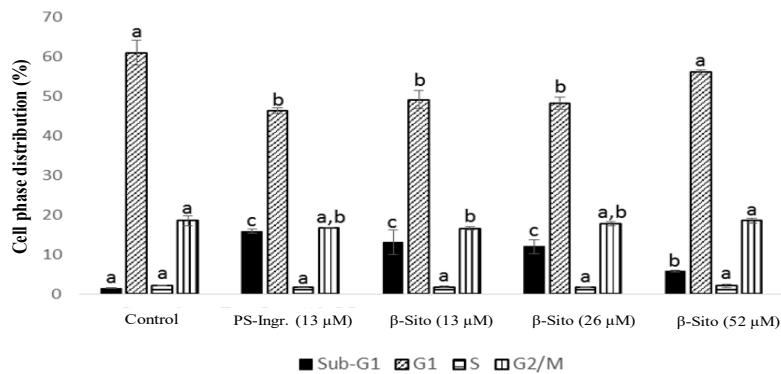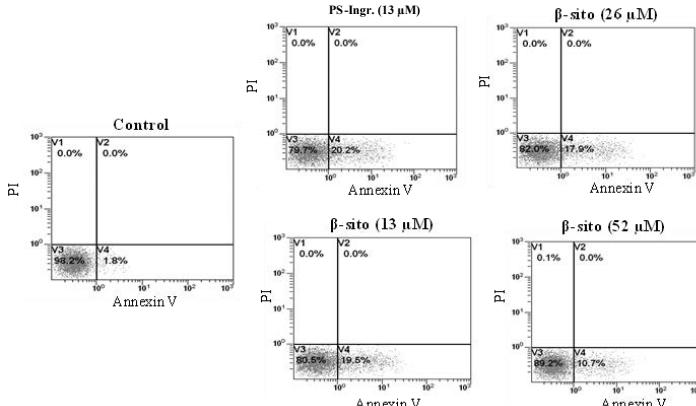
Figure 2. Effect of PS-ingredient (PS-Ingr.) or β-sitosterol (β-sito) on **(A)** viability (MTT assay), **(B)** cell cycle and **(C)** apoptosis of HCT116 cells. Data are expressed as mean ± SD; MTT assay: different lowercase letters (a-d) indicate statistical significant differences (p<0.05) among the different treatments after 24 h of incubation and the capital letters (A-D) after 48 h; Cell cycle assay: different lowercase letters (a-c) indicate statistical significant differences (p<0.05) among the different treatments after 48 h of incubation; Apoptosis assay: percentage of Annexin V/propidium iodide (PI) double-stained cells presented are representative images of three experiments in triplicate.

3.3. HeLa cells

In the HeLa cell line after 24 h of incubation, only PS-ingredient (52 µM) and β-sitosterol (13 µM) showed a significant (p<0.05) decrease of 20.5-21.4% versus control cells (Figure 3A). However, at 48 h, the PS-ingredient at concentrations of 13 and 26 µM (22.6% and 34.1%, respectively) also induced a decrease in HeLa cell viability. Moreover, a greater decrease produced by PS-ingredient at 26 and 52 µM and β-sitosterol (13 µM) at 48 h versus 24 h of exposure was observed (Figure 3A). Regarding cell cycle assay (Figure 3B), and in contrast to the results obtained in the other studied cell lines, the greatest increase in the percentage of cells in the sub-G_1 phase corresponded to PS-ingredient 13 µM (3.4-fold), followed by β-sitosterol 52 µM (1.9-fold) ≥ 26 µM (1.5-fold) ≥ 13 µM (1.3-fold) versus control cells, with a concomitant significant (p<0.05) decrease in phase G_1 (28%, 8.4% and 4.6% for PS-ingredient 13 µM, β-sitosterol 52 and 13 µM, respectively). Moreover, treatment with PS-ingredient (13 µM) also induced a significant (p<0.05) reduction in phases S and G_2/M of 20.6% and 23.7%, respectively. This behavior was also observed after Annexin V/PI assay (Figure 3C), where PS-ingredient 13 µM showed the highest proportion of early HeLa cell apoptosis (24.5%) versus control cells, and a dose–dependent effect of β-sitosterol standards was observed (14.3-20.4%), with a greater apoptotic effect induced by β-sitosterol 52 µM.

(A)

Cell viability (% of control)

120

100

80

60

40

20

0

Control | PS-Ingr. (13 µM) | PS-Ingr. (26 µM) | PS-Ingr. (52 µM) | β-Sito (13 µM) | β-Sito (26 µM) | β-Sito (52 µM)

■ 24 h ▨ 48 h

(B)

Cell phase distribution (%)

60

50

40

30

20

10

0

Control | PS-Ingr. (13 µM) | β-Sito (13 µM) | β-Sito (26 µM) | β-Sito (52 µM)

■ Sub-G1 ▨ G1 ☐ S ▥ G2/M

(C)

Control
AD1 0.0% | AD2 0.0%
AD3 99.8% | AD4 0.2%
PI / Annexin V

PS-Ingr. (13 µM)
AD1 0.0% | AD2 0.0%
AD3 75.6% | AD4 24.4%
PI / Annexin V

β-sito (26 µM)
AD1 0.0% | AD2 0.0%
AD3 82.9% | AD4 17.0%
PI / Annexin V

β-sito (13 µM)
AD1 0.0% | AD2 0.0%
AD3 85.9% | AD4 14.1%
PI / Annexin V

β-sito (52 µM)
AD1 0.0% | AD2 0.0%
AD3 79.7% | AD4 20.3%
PI / Annexin V

Figure 3. Effect of PS-ingredient (PS-Ingr.) or β-sitosterol (β-sito) on **(A)** viability (MTT assay), **(B)** cell cycle and **(C)** apoptosis of HeLa cells. Data are expressed as mean ± SD; MTT assay: different lowercase letters (a-b) indicate statistical significant differences ($p < 0.05$) among the different treatments after 24 h of incubation and the capital letters (A-D) after 48 h; Cell cycle assay: different lowercase letters (a-d) indicate statistical significant differences ($p < 0.05$) among the different treatments after 48 h of incubation; Apoptosis assay: percentage of Annexin V/propidium iodide (PI) double-stained cells presented are representative images of three experiments in triplicate.

4. Discussion

In general, as it will be shown below, the differences observed in comparison with our study may be due to: *(i)* the kind of bioactive compound studied (β-sitosterol standard with or without cyclodextrin complexation, PS mix instead of PS alone, or isolated PS from plant extracts); *(ii)* the use of different incubation times (1-9 days); *(iii)* different concentration of PS (0-400 µM); and *(iv)* the use of different cells lines.

4.1. Breast cancer cells

Regarding the β-sitosterol standards, the growth inhibition percentages observed at 13 µM for 48 h are within levels recorded in previous studies (29-81%) with β-sitosterol at 16 µM tested between 1-5 days of incubation (Awad et al. 2007, 2008; Rubis et al. 2010). On the other hand, it has been observed that β-sitosterol intake is associated to a greater probability of estrogen receptor-positive (related to MCF-7) than estrogen receptor-negative tumors, possibly explaining that MCF-7 cells may be more resistant to this compound (Touillaud et al. 2005; Grattan 2013). These authors suggested biological effects of PS upon estrogen receptor regulation, indirectly influencing cellular processes such as estrogen metabolism, estrogen receptor function and expression, since PS can modify the fluidity of cholesterol-rich cell membranes (without altering their integrity) and inhibit membrane-bound molecules. However, Ju et al. (2004) reported that the reduction of tumor size in ovariectomized athymic mice injected with MCF-7 cells after the addition of β-sitosterol glycoside or β-sitosterol:β-sitosterol glycoside (99:1) to the diet (0.2 and 10 g/kg diet, respectively) was independent of estrogen signaling. These authors therefore suggested that PS may be beneficial for women with breast cancer, though the mechanism involved is unclear. Overall, based on the epidemiological data and studies carried out with cells or animals, it can be concluded that controversy exists regarding the role of PS as a mediator of breast cell growth through estrogen pathways.

Several mechanisms have been proposed for clarifying the action of β-sitosterol (16 µM) in relation to breast cancer cell (MCF-7) proliferation, such as the activation of the extrinsic apoptotic pathway through increased activity of caspase 8 (1.9-fold

increase versus control), and a 30% increase in first apoptosis signal receptor (Fas) (Awad et al. 2007). Moreover, an increase in ceramide (an intracellular modulator of cell growth), which suggests that β-sitosterol activates *de novo* ceramide synthesis by stimulating serine palmitoyl transferase activity (Awad et al. 2008) is also observed. Globally, these results confirm the apoptotic action of β-sitosterol, in agreement with our own results. Regarding cell cycle distribution, and in contrast to our findings, β-sitosterol (1 and 5 µM) did not induce significant changes in the cell cycle of MCF-7 cells at 24 h (Rubis et al. 2010), possibly because of the lower concentrations and shorter incubation times involved.

To the best of our knowledge, no other similar antiproliferative studies have been made with PS-ingredients; however, different studies have evaluated the antiproliferative effect of PS (mainly β-sitosterol or PS mixtures) isolated from plant extracts upon MCF-7 cells, mainly through the determination of IC_{50} (the PS concentration causing 50% inhibition of cell growth), which is directly dependent upon the dose and exposure time involved. There is controversy as to when a plant extract can be considered to have an active cytotoxic effect based on IC_{50}. According to Tahsin et al. (2017), a plant extract is active with $IC_{50} < 500$ µg/ml, while Malek et al. (2009) indicate that a plant extract is regarded as active with $IC_{50} \leq 20$ µg/ml following 48 to 72 h of incubation. Nevertheless, it is recognized that the significant or nonsignificant cytotoxicity reflected by a given IC_{50} is conditioned to the sensitivity of the cell line involved.

Generally, it has been reported that treatment with mixtures of PS (mainly β-sitosterol, campesterol and stigmasterol) from different plant extracts showed no antiproliferative effects upon MCF-7 cells at concentrations of 0.1-100 µg/ml and incubation times of 24, 48 and 72 h (Malek et al. 2009; Rahman et al. 2013; Yaacob et al. 2015). Unlike, the PS-ingredient used in our study, mainly comprising β-sitosterol, showed an antiproliferative effect (17% and 53% at 24 and 48 h, respectively) at the lowest concentration used (5.39 µg/ml, 13 µM). The antiproliferative effect observed with the PS-ingredient could be mainly attributable to the presence of β-sitosterol, since cell viability inhibition values consistent with

our own results, have been reported with similar (10 µM) or higher (174 µM) concentrations of β-sitosterol at 72 h (Chai et al. 2008; Malek et al. 2009).

4.2. Colon cancer cells

As far as we are aware, only one other study (Choi et al. 2003) has been carried out with β-sitosterol standard (2.5-20 µM) in the HCT116 cell line, inducing slightly greater growth inhibition in a dose-dependent manner (50% and 75% with 7.5 and 20 µM, respectively) compared with our results referred to β-sitosterol standard at 48 h (44-49% at 13, 26 and 52 µM, with no clear dose-response). Using different colon cancer cell lines as HT-29, β-sitosterol standard (16 µM) complexed with cyclodextrin, and involving longer treatment periods (3-9 or 5 days), showed similar (~35-67%) (Awad et al. 1996) or lower (19%) (Awad et al. 1998) antiproliferative responses than in our own study (27-49% at 13 µM and at 24 and 48 h). Our results are also within the range of antiproliferative values (~10-50%) reported by Montserrat-de la Paz et al. (2015) with β-sitosterol standard (0-100 µM) at 24 and 48 h upon HT-29 cells. Regarding Caco-2 cells, Daly et al. (2009) found that higher doses of β-sitosterol standard (200 and 400 µM) are necessary in order to obtain similar antiproliferative values (39-47%) at 48 h to our own results. In the same cell line, using serum (6, 12 and 24 µM) (Cilla et al. 2015) or estimated colonic concentrations (115 µM) (López-García et al. 2017b) of β-sitosterol standard or PS mix standards (β-sitosterol, campesterol and stigmasterol) (serum: 6.6, 13.2, 26.5 µM or colonic: 132 µM, respectively), was recorded a decrease in cell viability (21-44% or 57-59%, respectively), similar to that found in our study at 24 h. Moreover, in agreement with our own findings, no clear dose-response relationship was observed between the different concentrations studied, with no evident additive or synergistic effects for the PS mix.

In general, HT-29 and Caco-2 cells seem to be more resistant to antiproliferative PS action than HCT116 cells, since the former needed longer times or higher PS concentrations than in our study to reach the same effects in the latter cells. This may be due to the fact that HT-29 and Caco-2 cells have mutated the tumor suppressor

protein p53, while HCT116 cells have functional p53 (p53 wild type) (Ahmed et al. 2013). Accordingly, tumors with mutated p53 can have a higher proportion of proliferating cells, and may be more metastatic than similar tumors with wild-type p53 (Brown and Wouters 1999).

In agreement with the present work, several studies have demonstrated that the antiproliferative effects of PS may be due to their ability to modulate cell cycle progression and apoptosis; however, variability of response was reported. In coincidence with our findings, Choi et al. (2003) observed in HCT116 cells that β-sitosterol (2.5-20 μM) induces apoptosis by increasing the sub-G_1 cell population (3.8-16.5%) after 48 h, this effect being more accentuated from 7.5 μM. In this regard, Cilla et al. (2015) also observed apoptosis of Caco-2 cells through an increase in the number of cells in sub-G_1 phase (145%) with exposure to standard PS mix at serum concentrations (13.2 μM), while β-sitosterol standard (12 μM) produced no significant change in this phase - though arrest in G_2/M (16%) was recorded at 24 h. In contrast, other authors observed different behavior of the PS upon cell cycle. Montserrat-de la Paz et al. (2015), using only β-sitosterol at 100 μM, have reported statistically significant irreversible arrest in phase G_0/G_1 of the cell cycle in HT-29 cells - this effect not being significant for β-sitosterol at 50 μM. In this same regard, López-García et al. (2017b) showed that standard PS mix (132 μM) and β-sitosterol (115 μM) also increase the number of cells in phase G_0/G_1. This suggests that β-sitosterol or PS mix modulates Caco-2 cell growth by blocking phase G_0/G_1 at high concentrations (100-132 μM).

Other mechanisms involved in the inhibitory effect of PS upon colon cancer cells have been reported, mainly associated with the apoptosis pathway, as in our own study. In the cell line used in the present work (HCT116), Choi et al. (2003) previously showed that the apoptosis mechanism of β-sitosterol (2.5-20 μM) could be mediated by caspase-3 and caspase-9 activation, and can be associated with decreased expression of the anti-apoptotic Bcl-2 protein, with a concomitant increase in pro-apoptotic Bax protein as well as with cytochrome c release from the mitochondria into the cytosol (Choi et al. 2003). In HT-29 cells, β-sitosterol (16 μM) was found to alter membrane lipids - suggesting that the inhibition of cell growth

could be due to activation of the sphingomyelin cycle (involved in physiological parameters of apoptosis and cell growth), increasing ceramide production (50%) (Awad et al. 1996, 1998). Moreover, β-sitosterol at a higher concentration (100 µM), upregulated LXR-α and LXR-β gene expression (nuclear factors crucial for colon cancer progression) (Montserrat-de la Paz et al. 2015). On the other hand, PS anticarcinogenic activity against Caco-2 cells has been reported mediated by the mitochondrial pathway of apoptosis, secondary to an increase in cytosolic Ca^{2+} and oxidative stress (Cilla et al. 2015), or through necrotic cell death (Lopez-García et al. 2017b). In this context, it has been suggested by López-García et al. (2017b) that PS exhibit a biphasic effect in Caco-2 cells, activating different molecular pathways depending on the concentration used, as observed on establishing comparisons with the results obtained in a previous report (Cilla et al. 2015). It seems that apoptosis is the main pathway implicated in cell death at low concentrations, while at high concentrations the apoptotic pathway may be suppressed, and cell death through necrosis prevails (López-García et al. 2017b). In this sense, in our own work, a biphasic effect is also suggested, since we observed greater apoptosis response at lower concentrations (13 and 26 µM) than at higher concentration (52 µM) of β-sitosterol.

The cell line involved in this study (HCT116) has also been used to evaluate the antiproliferative effect of PS isolated from plant extracts. In this regard, it has been reported that β-sitosterol and/or a mixture of PS (campesterol, stigmasterol and β-sitosterol) extracted from *Pereskia bleo* or *Curcuma zedoaria* does not display cytotoxic actions ($IC_{50} > 100$ µg/ml) (Malek et al. 2009; Rahman et al. 2013).

4.3. Cervical cancer cells

To the best of our knowledge, only one study (Cheng et al. 2015) has shown β-sitosterol (20 µM) present in *Pinellas tuber* to exert an antiproliferative effect (40%) upon HeLa cells at 24 h. A comparatively lesser antiproliferative effect was observed in our study (20.5%) for β-sitosterol (13 µM) at 24 h - the effect being similar (44.8%) at 48 h. Cheng et al. (2015) demonstrated that the β-sitosterol antiproliferative effect was due to reduction of the expression of PCNA (proliferating cell nuclear antigen), since β-sitosterol probably inhibits DNA synthesis in cells of

this kind. Alterations in cell morphology were also observed (loss of cell surface microvilli, increased electron density of the cell membrane, and decreased organelle presence), suggesting that these cells gradually lose their malignant tumor characteristics upon treatment with β-sitosterol. The authors concluded that elevated levels of p53 mRNA (2.9-fold) and reduced levels of HPVE6 viral oncogenes (1.6-fold) observed upon treatment with β-sitosterol, could explain the anticancer activity against HeLa cells.

Most of the PS antiproliferative studies have been carried out with PS isolated from plant extracts upon HeLa cells. Han et al. (2013) observed that β-sitosterol isolated from *Benicasa hispida* at different concentrations (2.5-50 µM) did not show cytotoxic activity (inhibitory rate <35%) at 24 h. This behavior is in agreement with our own work, since at 24 h, β-sitosterol at 26 and 52 µM showed no statistically significant (p<0.05) antiproliferative effect, and β-sitosterol at 13 µM only produced 20.5% inhibition of cell growth. Several studies (Block et al. 2004; Csupor-Loffler et al. 2011) have observed that mixtures of β-sitosterol and stigmasterol isolated from *Croton zambesicus* or *Conyza Canadensis*, respectively, exerted no cytotoxic effect (IC$_{50}$ > 30 µg/ml) at 72 h. Moreover, with shorter exposure times (48 h), Hamdan et al. (2011) reported that a mixture of β-sitosterol and stigmasterol isolated from *Citrus jambhiri Lush* exhibited an IC$_{50}$ at 114.2 µM. The authors suggested that the cytotoxic activity could be due to the lipophilic character of these compounds, since they may interact with the lipophilic side chains of phospholipids or cholesterol, and in turn affect membrane fluidity and disrupt cell membrane function by modifying the three-dimensional conformation of membrane proteins. These observations are in agreement with our own results, since PS-ingredient (containing β-sitosterol and stigmasterol) at the concentrations tested (13, 26 and 52 µM) did not reach 50% proliferation inhibition in HeLa cells after 24 or 48 h.

5. Conclusions

The antiproliferative effect of β-sitosterol or PS-ingredient upon MCF-7, HTC116 and HeLa cells was generally evident at the concentration of 13 µM - this being compatible with physiological serum levels after the regular intake of a beverage containing these compounds. A possible biphasic/hormetic effect (with a

greater response at lower concentrations versus higher concentrations) and/or a threshold of optimal action at the lowest assayed concentrations cannot be ruled out. Anticarcinogenic activity was observed through the induction of DNA fragmentation and apoptosis, with no clear time- and dose-dependent effect. Thus, the PS-ingredient used for enrichment of functional beverages and its main PS (β-sitosterol), may be regarded as natural anticarcinogenic compounds against breast, colon and cervical cancer in these preclinical models, though further research is warranted. These observations may be complementary to the decrease in risk of cardiovascular disease and osteoporosis previously reported after the intake of a PS-enriched functional beverage containing the PS-ingredient assayed in this study; moreover, they should be taken into account by the food industry for using this PS-ingredient in the development of PS-enriched food products, with a view to maximizing their functionality.

Disclosure statement

The authors report no conflicts of interest

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Physiological concentrations of phytosterols enhance the apoptotic effects of 5-fluorouracil in colon cancer cells

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Abstract

Combining natural products as co-adjuvants in 5-fluorouracil (5-FU) chemotherapy might enhance the effectiveness of 5-FU by avoiding a high dosage and/or reducing treatment times. We explored the anticancer efficacy of the phytosterols (PS) at concentrations achievable in the human colon, as well as their potential as sensitizing agents of human colon cancer cells (Caco-2 and HT-29) to 5-FU treatment. Cells proliferation, combination index, cell cycle, apoptosis, caspases activation, ROS production, and $\Delta\Psi$m were determined. Co-treatment (PS+5-FU) had an antiproliferative additive effect, and moreover, in general a significantly improved efficacy was observed on cell cycle arrest at S phase, apoptosis induction and increase in caspases activation compared to 5-FU alone, while no significant effects were observed on ROS levels and $\Delta\Psi$m. Our results suggest, for the first time, the ability of PS to sensitize colon cancer cells to 5-FU chemotherapy and warrant further *in vivo* studies to confirm our preclinical findings.

Keywords: Phytosterols; colon cancer cells; 5-fluorouracil; cell cycle; apoptosis; drug sensitivity.

1. Introduction

Colorectal cancer is the third most common cancer worldwide, accounting for almost 10% of all cancers, with nearly 1.4 million new cases diagnosed in 2012 and it is expected that the number of cases annually will rise to 2.4 million by 2035 (Ferlay et al., 2015). A widely used drug against colorectal cancer is 5-fluorouracil (5-FU). This drug exerts anticancer activity via inhibition of the enzyme thymidylate synthase and incorporation of its fluoronucleotide metabolites into RNA and DNA, events that ultimately activate apoptosis (Longley, Harkin & Johnston 2003). However, relative frequent cancer cells resistance and adverse side effects have been associated with 5-FU drug treatment (Walko & Lindley, 2005). The rates of positive response to 5-FU administration as a single agent in advanced colorectal cancer patients are only 10–15%. However, the combination of 5-FU with other anti-tumor drugs has been reported to improve the response rates by 40–50% (Zhang, Yin, Xu, & Chen, 2008). Therefore, further investigations are warranted to explore the possibility of using other compounds as alternatives for the chemotherapy.

In this regard, bioactive food components such as phytosterols (PS) could be a new tool to complement the chemotherapy against cancer. PS can interfere on several cell signaling pathways, including the alteration of phospholipid membrane composition, cell cycle, apoptosis, proliferation, invasion, angiogenesis and immune system, among others, as previously reported (Bin Sayeed & Ameen, 2015; Bradford & Awad, 2007; Shahzad et al., 2017). However, most of the evidence has been found in studies carried out with cells or animal models. In this context, the antiproliferative activity of PS has been reported in different colon cancer cell lines (Caco-2 and HT-29), particularly for β-sitosterol (β-SIT), within the range of physiological plasma concentrations (8-400 µM) at different times of exposure (1-9 days) (Awad, Chen, Fink, & Hennessey, 1996; Awad, von Holtz, Cone, Fink, & Chen, 1998; Choi et al., 2003; Daly, Aherne, O'Connor, & O'Brien, 2009; López-Garcia, Cilla, Barberá, & Alegría, 2017a; Montserrat-de la Paz, Fernández-Arche, Bermúdez, & García-Giménez, 2015). In addition, previous studies conducted by our research group, reported the decrease of cardiovascular risk and bone turnover markers after the regular consumption of PS-enriched milk-based fruit beverages in postmenopausal

women (Granado-Lorencio et al., 2014). In addition, a bioaccessible fraction obtained after simulated gastrointestinal digestion of this beverage showed cytoprotective effects on differentiated Caco-2 cells (López-García, Cilla, Barberá, & Alegría, 2017b) as well as anti-carcinogenic activity on undifferentiated Caco-2 cells upon single or combined PS incubations at physiologically relevant concentrations as those found in circulation (Cilla, Attanzio, Barberá, Tesoriere, & Livrea, 2015). Taking into account that the recommended daily doses of PS to exert systemic effects (2 g/day) are safe and their poor low rates of absorption (0.5-2%) (Gylling et al., 2014), this suggests that PS may reach the colon at higher concentrations than those found in the bloodstream and thus potentially exert colon cancer chemopreventive effects.

To the best of our knowledge, there is only a previous study that described the antiproliferative activity of PS in Caco-2 cells upon incubation with colonic concentrations of β-SIT (115 µM), CAMP (11 µM), STIG (6 µM) and combined PS (132 µM), which were estimated after the intake of a PS-enriched milk-based fruit beverage (López-García et al., 2017a). Moreover, β-SIT (the main dietary PS) has been proposed as a potent co-adjuvant to sensitize cancer cells to various anticancer drugs such as tamoxifen (Awad, Barta, Fink, & Bradford, 2008) or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Park et al., 2008). On the other hand, compounds structurally similar to PS such as vitamin D and analogues (Kotlarz et al., 2016; Liu, Hu, & Chakrabarty, 2010; Milczarek, Filip-Psurska, Świętnicki, Kutner, & Wietrzyk, 2014; Milczarek, Psurski, Kutner, & Wietrzyk, 2013) or protopanaxadiol (Wang et al., 2015) have been reported to enhance the anticancer effect of 5-FU against colon cancer. However, the effect of PS as co-adjuvant of 5-FU in the colon cancer therapy has not yet been evaluated.

Therefore, we aimed at evaluating for the first time whether PS concentrations that can reach the colon after the intake of the main dietary PS (β-SIT, CAMP and/or STIG), either alone or combined as a representative mixture, could enhance the chemopreventive effects of 5-FU in two human colon adenocarcinoma cells (Caco-2 and HT-29), which could be useful to reduce the dose of 5-FU and thus the possible side effects related to this drug.

2. Materials and methods

2.1. Materials and reagents

(24S)-ethylcholest-5,22-dien-3β-ol (stigmasterol; STIG) (purity 97%), (24R)-ethylcholest-5-en-3β-ol (β-sitosterol; β-SIT) (purity 97.3%), 5-fluorouracil (5-FU) (purity 99.8%), 2′,7′-dichlorofluorescein diacetate (DCF-DA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis. MO, USA). (24R)-methylcholest-5-en-3β-ol (campesterol; CAMP) (purity 96.19%) was purchased from Chengdu Biopurify phytochemicals Ltd. (Sichuan, China). Phosphate buffered saline (PBS) was obtained from Fisher Scientific (USA), and DMSO from Panreac (Barcelona, Spain). Ethanol (purity 99.9%) was provided by Merck (Whitehouse Station, NJ, United States). Ultrapure Millipore water was used throughout the study.

2.2. Cell culture conditions and treatments

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, USA) and cultured as recommended by the ATCC. The human colon cancer cell line HT-29 was grown in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L D-glucose) containing 10% v/v fetal bovine serum (FBS), 1% v/v L-glutamine, 1% v/v nonessential amino acids, and 1% v/v antibiotic solution. The human colon cancer cells Caco-2 were grown in minimal essential medium (MEM) supplemented with 10% v/v FBS, 1% v/v L-glutamine, 1% v/v nonessential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen S.A., Barcelona, Spain). Cells were maintained at 37 ºC in a humidified atmosphere (95%) with 5% (v/v) CO_2. Cells were counted using a hemacytometer and were plated at 15,000 cells cm^{-2} for 48 h prior to pure compounds addition. All of the test samples (β-SIT (115 µM), CAMP (11 µM), STIG (6 µM) and a representative mixture of PS (PS Mix: 115 µM β-SIT + 11 µM CAMP + 6 µM STIG; 132 µM) were dissolved in ethanol (EtOH) (<1.15 % in the culture medium) by sonication during 10 min. 5-FU was dissolved in DMSO (<0.5 % in the culture medium) and was assayed at 50 and 25 µM as previously described by González-Sarrías, Tomé-Carneiro, Bellesia, Tomás-Barberán, & Espín (2015). Control cells were also run in parallel and subjected to the same changes in medium with DMSO and/or ethanol and these

concentrations did not significantly affect cells growth or caused cytotoxicity as described elsewhere (López-García et al., 2017a).

2.3. Cell proliferation and viability tests

Cell viability and proliferation were measured in the exponential growth phase by MTT assay according to González-Sarrías et al. (2015). Briefly, cells were seeded in 96-well plates at a density of 3.000 cells/well and were maintained at appropriate culture conditions for 24 h. After incubation of each treatment for 48 and 72 h, a mixture of 50 μL of MTT (5 mg/ml) and 200 μL of serum-free medium was added to the PBS-washed cells, and then maintained at 37 °C for 3 h. After removing MTT reaction agent, 100 μL of DMSO were added to cell culture plates and shaking at room temperature for 5 min to allow the dissolution of purple formazan products. The optical density (OD) of the coloured solution was measured at 570 nm using a scanning spectrophotometer microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The antiproliferative effect was calculated as the percentage of cell proliferation values with respect to the control cells (0.5% DMSO + 1.15% EtOH; 100%). Data are presented as the mean ± SD of at least five separate experiments.

To confirm these data, cell proliferation values were also measured using a TC10™ automated cell counter (Bio-Rad) with the addition of Trypan blue for viability determination.

2.4. Combined effects of phytosterols with 5-FU

The effectiveness of PS either alone or in combination as a representative mixture of PS, and 5-FU to inhibit cell growth of HT-29 and Caco-2 cells was assessed by determining the combination index (CI) adapted from the method described by Chou (2010). CI was calculated as a quantitative measure of combined action using the following equation CI = (%PS+5-FU) / [(%PS) + (%5-FU)], where %PS and %5-FU are the cell growth inhibition percentage of each individual PS and 5-FU, respectively, on cell growth inhibition, and % PS+5-FU is the percentage of the combination on cell growth inhibition. When the ratio (CI) is below, equal to, and above 1 indicate antagonism, addition and synergy, respectively. CI values (mean ± SD) were calculated at each time point and 5-FU doses tested (50 and 25

μM), based on the pooled data from 4 to 6 individual experiments for each PS alone and in combination.

2.5. Analysis of cell cycle assay

Cell cycle distribution was evaluated through DNA content (25.000 cells) using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson, New Jersey, USA) according to González-Sarrias et al. (2015). Briefly, cells (1×10^6) were collected after the corresponding experimental periods, fixed in ice-cold ethanol: PBS (70:30) for 30 min at 4 ºC, further resuspended in PBS with 100 μg/mL RNAse (Sigma) and 40 μg/mL propidium iodide (PI) (Sigma), and incubated at 37 ºC for 30 min. The coefficient of variation, according to the ModFit LT Version 4.1 acquisition software package (Verity Software House, Topsham, ME, USA), was always less than 5%. The analyses of cell cycle distribution were performed at least in triplicate (n = 2 plates per experiment) for each treatment.

2.6. Determination of apoptosis cells assay

Phosphatidylserine, which is normally located on the cytoplasmic surface of cell membranes, is exposed on the cell surface upon induction of apoptosis. Annexin V binds to phosphatidylserine and is used to identify the earliest stage of apoptosis. PI, which does not enter cells with intact membranes, is used to distinguish between early apoptotic cells and late apoptotic cells (Rubino et al., 2016). The annexin V-FITC/PI detection kit (Molecular Probes, Life Technologies Inc.) to identify apoptotic cells was used in accordance to the manufacturer's protocols. At the end of 48 or 72 h of treatment, floating and adherent cells (0.5×10^6) were resuspended in 400 µL of binding buffer and incubated in the dark with both annexin V and PI for 10 min. Afterwards, at least 20,000 cells per sample were analyzed by flow cytometer (as described above) and the percentage of live (negative in both annexin V-FITC and PI), early apoptotic (positive in annexin V-FITC and negative in PI), late apoptotic (positive in both annexin V-FITC and PI), and necrotic cells that show nuclear damage (only positive in PI) were determined. Staurosporine (Sigma, St. Louis, MO, USA) with high apoptotic activity was assayed as a positive control at 5 μM (data not shown). Evaluation of apoptosis was carried out three times for each treatment (n = 2 plates per experiment).

2.7. Evaluation of caspases activation

The activation of caspase-3, -8 and -9 was evaluated by flow cytometry using the carboxyfluorescein (FAM) FLICA apoptosis detection kits, FAM-DEVD-FMK, FAM-LETD-FMK and FAM-LEHD-FMK, respectively (Immunochemistry Technologies LLC, Bloomington, MN, USA) in accordance to the manufacturer's protocols. Briefly, cells (1×10^6 cells/mL) treated were collected after the corresponding experimental periods. Cells were stained with 30× FLICA solution containing fluorescein-labelled inhibitors that bind specifically either to active caspase -3, -8 or -9, and incubated for 1 h (37 °C/5% CO_2) in darkness. Afterwards, cells were washed twice and the apoptotic cells containing active caspase-3, -8 or -9 were detected by flow cytometry (as described above). A minimum of 2×10^4 cells were analyzed for each sample. Experiments were carried out at least twice after 72 h for each treatment (n = 2 plates per experiment).

2.8. Measurement of intracellular reactive oxygen species (ROS)

Determination of intracellular ROS production was performed by measuring the oxidation of the cell permeable non-fluorescent 2′,7′-dichlorofluorescein diacetate (DCF-DA) to fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS according to López-García et al. (2017a). After 48 or 72 h of each treatment, floating and adherent cells (1×10^5) were resuspended in PBS and transferred to a cytometer tube. DCF-DA was added (10 µM, final concentration) and cells were maintained in incubation for 30 min at 37 ºC. Finally, cells were centrifuged and re-suspended in 300 µL PBS and analysed by flow cytometer (as described above). At least 20,000 cells were analyzed for each sample at least in three different experiments for each treatment (n = 2 plates per experiment).

2.9. Mitochondrial membrane potential assessment

The mitochondrial transmembrane potential depolarization, an early process in the apoptotic cell death, was measured using lipophilic, cationic fluorescent redistribution dyes such as ICT's MitoPT® reagents: tetramethylrhodamine ethyl ester (TMRE), tetramethylrhodamine methyl ester (TMRM), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) in accordance to the manufacturer's protocols (Immunochemistry Technologies LLC,

Bloomington, MN, USA). In brief, after 72 h of treatment, cells were harvested with trypsin and cell suspension was transferred to a cytometer tube, where JC-1 solution was added and incubated for 15 min at 37 °C under 5% CO_2 in the dark. After centrifugation, the cells were washed twice with PBS and analyzed by flow cytometry (as described above). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as positive control. At least, 20,000 events were analyzed for each sample. Experiments were carried out, at least twice for each treatment (n = 2 plates per experiment).

2.10. Statistical analysis

Results are presented as mean values ± SD. Two-tailed unpaired Student's t-test was used for statistical analysis of the data using the Microsoft´s Office Excel 2016 software. A significance level of $p < 0.05$ was considered significant. Graphs of the experimental data were performed using Sigma Plot 13.0 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Effect of phytosterols on colon cancer cells proliferation alone or in combination with 5-FU

All PS were stable in the cell media (in presence or absence of cells) without significant degradation, and were identified during the assay times after analysing cell media by gas chromatography−flame ionization detection following the conditions described by Alvarez-Sala et al. (2016) (results not shown).

Inhibition data of proliferation of all treatments on cell cancer lines (Caco-2 and HT-29) tested at 48 and 72 h time of exposure are shown in Table 1. Results showed that Caco-2 cells were more sensitive to PS treatments than HT-29 cells, compared to control cells, showing a significant cell growth inhibition ($p < 0.05$) of 16-22% and 15-30% at 48 and 72 h, respectively. In HT-29 cells, the antiproliferative effect of PS was only significant for STIG and the PS Mix at 48 h and for all treatments after 72 h, except for CAMP.

As expected, 5-FU treatments on both cancer cells showed the highest effect reaching over 50% of cell growth inhibition in Caco-2 cells and over 40% in HT-29

cells in a time-dependent manner, although no clear dose-dependent effect was observed between 25 and 50 µM, showing similar effects.

Regarding the combination of single PS or the PS Mix with 5-FU, these co-treatments were more active against colon cancer cell lines than 5-FU alone (50 µM), reaching significant cell growth inhibition values ($p < 0.05$) around 60-70% and 80-85% against Caco-2 cells at 48 and 72 h, respectively. This sensitization was also evident ($p < 0.05$) when each single PS was combined with 5-FU against HT-29 cells, reaching values of 60-65% and 70-75% at 48 and 72 h, respectively (Table 1). A similar trend was found when comparing co-treatments with 5-FU at 25 µM. On the other hand, no significant differences were observed between PS and the representative PS Mix, on their potential of chemosensitization of both colon cancer cells to 5-FU treatment.

The combination index (CI), which determines whether the interactions between either single PS and 5-FU are synergistic, additive or antagonistic, indicated that the association of 5-FU with each PS had additive effects on both colon cancer cell lines since CI values were approximately equal to 1 (Table 1).

We next evaluated whether these additive effects on the inhibition of cell proliferation upon PS + 5-FU co-treatments were related either to an enhancement of the mechanism of action of 5-FU or to individual and different PS-mediated anticancer mechanisms. To this purpose, we evaluated cell cycle arrest, apoptosis, caspases activation, reactive oxygen species (ROS) production and mitochondrial membrane potential in both cancer cell lines. With the aim to clearly identify the mechanisms of action, we selected, based on the antiproliferative data (Table 1), a single concentration of 5-FU (50 µM) and one-time point (72 h), except for cell cycle and apoptosis analyses that were also checked at 48 h.

Table 1. Antiproliferative effects of PS alone or in combination with 5-FU on human Caco-2 and HT-29 colon cancer cells.

Treatment	Caco-2		HT-29	
	48 h	72 h	48 h	72 h
β-SIT (115 μM)	21.47±2.30[a,b,c]	25.82±8.51[a,b,c]	14.84±16.56 [b,c]	22.49±10.05 [a]
CAMP (11 μM)	16.70±10.67 [a,b,c]	15.41±3.83 [a,b,c]	11.12±11.69 [b,c]	15.70±15.33
STIG (6 μM)	20.24±11.75 [a,b,c]	19.90±7.86 [a,b,c]	24.92±12.46 [a,b]	19.68±12.32 [a,b]
PS Mix (132 μM)	22.19±17.11 [a]	30.85±8.85 [a,b,c]	24.36±14.26 [a]	29.02±15.85 [a]
5-FU (50 μM)	52.20±4.09 [a,c]	58.97±3.66 [a]	44.84±6.45 [a,c]	47.62±17.82 [a,b,c]
5-FU+ β-SIT	65.15±7.17 [a,b,c]	82.15±5.10 [a,b,c]	62.44±6.08 [a,b,c]	70.25±7.59 [a,b,c]
CI	(0.91±0.066)	(0.97±0.13)	(1.06±0.18)	(1.00±0.17)
5-FU+ CAMP	62.98±6.57 [a,b,c]	79.05±5.67 [a,b,c]	60.40±1.99 [a,b,c]	68.15±10.47 [a,b,c]
CI	(0.91±0.14)	(1.06±0.07)	(1.10±0.21)	(1.10±0.19)
5-FU+ STIG	68.23±6.28 [a,b,c]	81.38±5.84 [a,b,c]	59.62±7.68 [a,b,c]	70.68±12.69 [a,b,c]
CI	(0.94±0.12)	(1.03±0.09)	(0.97±0.17)	(1.05±0.31)
5-FU+ PS Mix	71.36±6.33 [a,b,c]	84.51±9.86 [a,b,c]	67.63±2.93 [a,b,c]	73.49±11.80 [a,b,c]
CI	(0.96±0.26)	(0.94±0.15)	(0.98±0.19)	(0.96±0.18)
5-FU (25 μM)	45.69±4.92 [a,b]	53.54±3.65 [a]	35.76±9.61[a,b]	39.27±15.85 [a,b,c]
5-FU+ β-SIT	54.63±6.29 [a]	76.24±3.47 [a,b,c]	54.72±7.05 [a,b,c]	64.81±12.74 [a,b,c]
CI	(0.87±0.09)	(0.96±0.05	(1.09±0.39)	(1.05±0.28)
5-FU+ CAMP	54.52±9.19 [a,c]	69.74±6.90 [a,b,c]	49.17±4.80 [a]	61.45±15.35 [a,b,c]
CI	(0.90±0.13)	(1.01±0.07)	(1.07±0.44)	(1.14±0.44)
5-FU+ STIG	62.06±9.40 [a,c]	72.30±5.02 [a,b,c]	56.95±11.88 [a,c]	68.12±13.53 [a,b,c]
CI	(0.97±0.11)	(0.98±0.04)	(1.09±0.19)	(1.16±0.35)
5-FU+ PS Mix	64.25±10.20 [a,b,c]	76.97±7.78 [a,b,c]	60.95±10.21[a,b,c]	68.75±14.65 [a,b,c]
CI	(0.97±0.35)	(0.91±0.09)	(1.01±0.18)	(1.01±0.13)

Values are shown as cell growth inhibition percentage (mean ± SD) compared to control cells (100%). β-SIT: β-sitosterol; CAMP: campesterol; STIG: stigmasterol; PS Mix: β-SIT, CAMP and STIG. Combination index (CI) was calculated as a quantitative measure of the combined action using the following equation (CI = (%PS+5-FU) / [(%PS) + (%5-FU)]. [a]$p < 0.05$ *versus* the control group; [b]$p < 0.05$ *versus* 5-FU (50 μM); [c]$p < 0.05$ *versus* 5-FU (25 μM).

3.2. Effect of PS alone or in combination with 5-FU on cell cycle distribution

Cell cycle progression was altered by 5-FU (50 μM) in both cancer cell lines showing a significant ($p < 0.05$) arrest at S phase compared to control cells. Similar to antiproliferative data, higher effects were obtained in Caco-2 (Figure 1A-B) than in HT-29 (Figure 1C-D) cells although no clear time-dependent effect was observed.

Regarding PS treatments at colonic concentrations (alone or as mixture), data showed a significant arrest ($p < 0.05$) at S phase after treatment with the PS Mix at 48 h (Figure 1A), as well as for the treatment with β-SIT, STIG and PS Mix at 72 h (Figure 1B) on Caco-2 cells. Meanwhile, the co-treatment of PS plus 5-FU did not

induce a significant higher arrest of cell cycle at S phase when compared to 5-FU alone, although a significant decrease of cells in G_0/G_1 phase was observed at 48 h.

In contrast to Caco-2 cells, the treatments with PS (alone or as mixture) did not exert a significant cell cycle arrest compared to control cells on HT-29 cells (Figure 1C and 1D). However, similar to antiproliferative assay, the combination of PS plus 5-FU showed a higher significant arrest at S phase compared to 5-FU alone ($p < 0.05$), with the exception of the combination 5-FU + CAMP at 48 h. However, this chemosensitization effect in the S phase arrest was not time-dependent ($p > 0.05$).
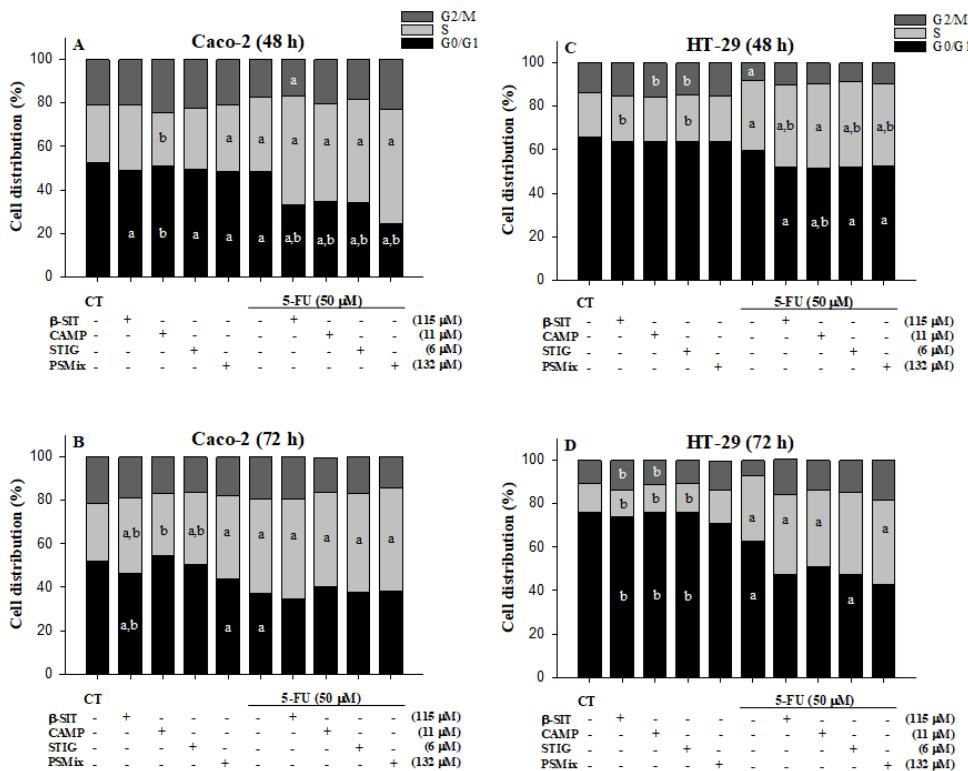

Figure 1. Effect of PS (β-SIT (115 μM), CAMP (11 μM), STIG (6 μM) and PS Mix (132 μM =115 μM β-SIT + 11 μM CAMP + 6 μM STIG; 132 μM) alone or in combination with 5-FU (50 μM) on cell cycle distribution of Caco-2 (**1A-B**) and HT-29 cells (**1C-D**) at 48 and 72 h, respectively. Values (%) are expressed as mean (n = 3). [a]$p<0.05$ *versus* control group; [b]$p<0.05$ *versus* 5-FU.

3.3. Effect of PS and/or 5-FU on apoptosis induction

Figure 2 shows both early and late apoptosis in both cell lines after 48 and 72 h. 5-FU treatments (50 µM) induced a greater increase of the number of early and late apoptotic cells ($p < 0.05$) in both cancer cell lines when compared to control cells (Figure 2A-D) at 48 and 72 h, reaching values similar to those obtained with staurosporine (5 µM) as positive control (data not shown).

Results showed that all treatments with PS at colonic concentrations induced both early and late apoptosis ($p < 0.05$) compared to control cells at both times tested in Caco-2 cells, except CAMP at 48 h for early apoptosis (Figure 2A and 2B). However, PS treatments did not exert a statistically significant increase in the number of apoptotic cells (in early and late phase) in HT-29 cells, except for the PS Mix at 72 h (late apoptosis) (Figure 2C-D). Indeed, viable cells remained over 90% after PS treatments (data not shown).

Regarding the combination of PS + 5-FU in Caco-2 cells, all PS co-treatments enhanced significantly ($p < 0.05$) the number of apoptotic cells (in early and late phases) when compared to 5-FU treatment alone, but only at 72 h, although the combination with PS Mix also was higher in late apoptosis at 48 h ($p < 0.05$) (Figure 2A-B). Surprisingly, the combination of PS plus 5-FU induced a higher increase of the number of mainly early apoptotic cells ($p < 0.05$) in HT-29 cells, compared to 5-FU alone at both times, except for the combination with β-SIT at 48 h (Figure 2C-D). Finally, similar to Caco-2 cells, despite no differences were found between single PS treatments, the combination of 5-FU plus PS Mix showed the highest values in the number of apoptotic cells, reaching significant differences also in the number of late apoptotic cells ($p < 0.05$) in HT-29 cells.
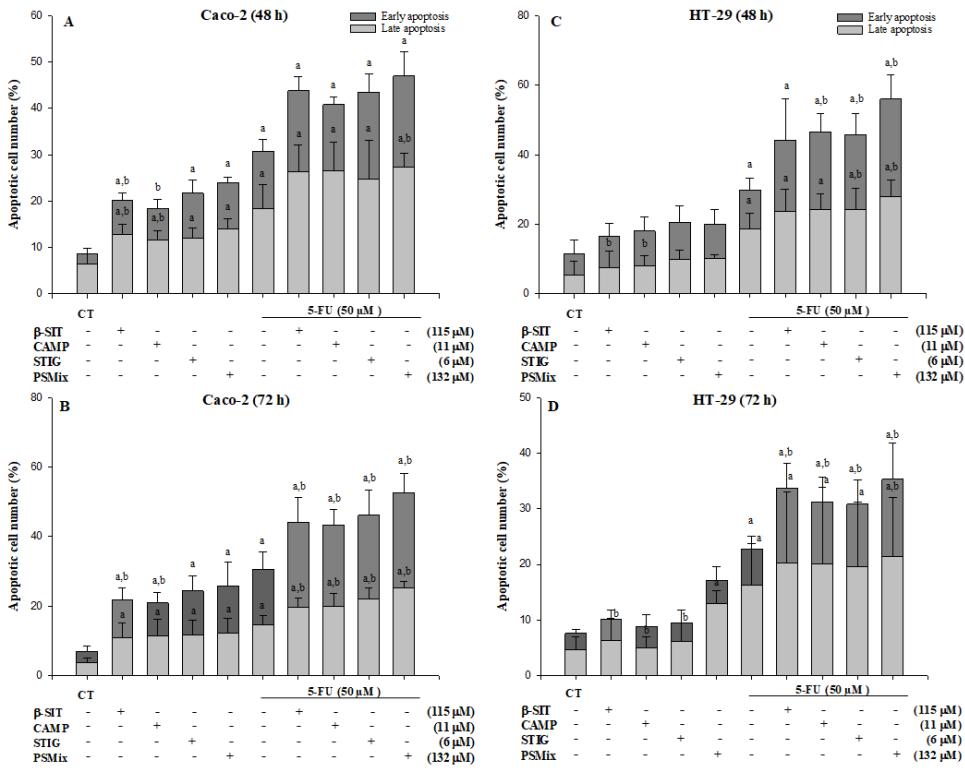
Figure 2. Effect of PS (β-SIT (115 μM), CAMP (11 μM), STIG (6 μM) and PS Mix (132 μM =115 μM β-SIT + 11 μM CAMP + 6 μM STIG) alone or in combination with 5-FU (50 μM) on apoptosis induction (early and late apoptosis) of Caco-2 (**2A-B**) and HT-29 cells (**2C-D**) at 48 and 72 h, respectively. Values (%) are expressed as mean (n = 3). [a]$p<0.05$ *versus* control group; [b]$p<0.05$ *versus* 5-FU.

3.4. Effects of PS on 5-FU-induced caspase activation

Given the apoptosis-inducing actions of the PS treatments at colonic concentrations, mainly in Caco-2 cells, and overall, their potential chemosensitization to both cancer cell lines to 5-FU, we evaluated the activity of caspases-9 and -8, key enzymes in the mitochondrial and cell death receptor apoptotic pathways, respectively. In addition, we wanted to corroborate the apoptosis induction by evaluating the caspase-3 activation.

After 72 h of treatment, 5-FU triggered apoptotic effects via both intrinsic and extrinsic pathways in both colon cancer cell lines ($p < 0.05$) compared to control cells (Figure 3A-B). In agreement with Annexin V-PI data, both caspase-8 and -9 were strongly activated after exposure to all PS treatments ($p < 0.05$) in Caco-2 cells compared to control cells (Figure 3A), while only a slight significant activation (p

< 0.05) of caspase-8 was detected with the PS Mix treatment in HT-29 cells (Figure 3B). The co-treatment of each PS plus 5-FU induced higher activity levels of caspases-8 and -9 when compared to 5-FU alone ($p < 0.05$) in Caco-2 cells, except with the co-treatment with CAMP (in both caspases) and β-SIT (caspase-9) (Figure 3A).

Regarding HT-29 cells, co-treatments with PS plus 5-FU induced a slight increase, although not statistically significant, in both caspase-9 and caspase-8 activation, except in the case of the co-incubation with the PS Mix when compared to 5-FU treatment alone for caspase-8 (Figure 3B). Moreover, with respect to caspase-3 activation, data of PS treatments as well as the 5-FU treatments alone were in agreement with the apoptosis induction patterns shown in Figure 2B and 2D, corroborating a lower effect of PS treatments in HT-29 cells than Caco-2 cells. Notably, co-treatments of PS plus 5-FU were significantly enhanced ($p < 0.05$) compared with 5-FU alone in HT-29 cells, except for CAMP, but not in Caco-2 cells.
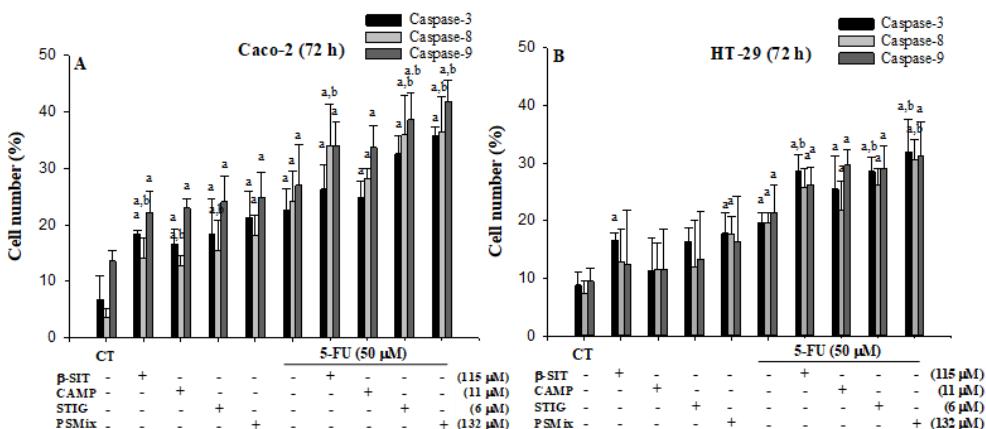

Figure 3. Effect of PS (β-SIT (115 µM), CAMP (11 µM), STIG (6 µM) and PS Mix (132 µM =115 µM β-SIT + 11 µM CAMP + 6 µM STIG) alone or in combination with 5-FU (50 µM) on caspase-3, -8 and -9 activation of Caco-2 (**3A**) and HT-29 cells (**3B**) at 72 h. Values (%) are expressed as mean ± SD (n = 3). [a]$p<0.05$ *versus* control group; [b]$p<0.05$ *versus* 5-FU.

3.5. Effects of PS on 5-FU-induced intracellular ROS levels production and mitochondrial membrane potential (ΔΨm) loss

To elucidate whether oxidative stress was found to be associated with the chemosensitization of both Caco-2 and HT-29 cells to 5-FU by PS (alone or in combination), we checked intracellular ROS production using DCF-DA staining. The effects of the PS and/or 5-FU on intracellular ROS levels are shown in Table 2. Our results show that 5-FU produced a significant increase ($p < 0.05$) of ROS levels in both cancer cells after 48 and 72 h of incubation, being higher in HT-29 compared to Caco-2 cells. However, among PS treatments, only the PS Mix increased ROS production *versus* control cells. Regarding co-treatment with PS plus 5-FU, no significant enhancement of ROS production was observed in comparison to 5-FU alone, except for STIG and PS Mix in Caco-2 cells (Table 2).

Table 2. Intracellular reactive oxygen species (ROS) production from treatments with 5-FU or PS alone or in combination against Caco-2 and HT-29 human colon cancer cells.

Treatment	Caco-2		HT-29	
	48 h	72 h	48 h	72 h
β-SIT (115 µM)	1.25±0.93[b]	1.57±0.26	6.28±1.49[b]	4.09±0.17[b]
CAMP (11 µM)	1.57±0.68[b]	2.71±1.92	6.75±1.05[b]	9.86±2.57[b]
STIG (6 µM)	2.38±1.07	3.24±2.34	9.79±4.01	13.02±2.29[b]
PS Mix (132 µM)	2.71±0.44[a]	1.98±0.12	12.52±2.47[a]	17.07±2.29[a,b]
5-FU (50 µM)	2.97±0.42[a]	2.50±0.94[a]	23.31±6.92[a]	44.04±0.37[a]
5-FU+ β-SIT	2.86±0.34[a]	2.94±0.94[a]	22.21±2.19[a]	37.15±2.57[a]
5-FU+ CAMP	3.46±0.04[a]	2.96±0.25[a]	18.54±0.019[a]	43.81±1.73[a]
5-FU+ STIG	3.92±0.38[a,b]	5.20±2.42[a,b]	26.54±14.13[a]	38.60±3.71[a]
5-FU+ PS Mix	4.27±0.37[a,b]	4.31±1.25[a]	23.67±4.05[a]	41.18±2.08[a]

Values are shown as mean ± SD. β-SIT: β-sitosterol; CAMP: campesterol; STIG: stigmasterol; PS Mix: β-SIT, CAMP and STIG. [a]$p < 0.05$ *versus* the control group; [b]$p < 0.05$ *versus* 5-FU (50 µM).

Similar results were found regarding ΔΨm loss by determining the red/green fluorescence ratio of JC-1 (Figure 4). 5-FU treatment produced a low (~10%) but significant ($p < 0.05$) increase in the ΔΨm loss in both cancer cells after 72 h of incubation, compared to control cells. However, no PS treatment showed any effect on the ΔΨm loss in our experimental conditions. Regarding co-treatment with PS

plus 5-FU, only PS Mix in HT-29 cells showed a significant enhancement of $\Delta\Psi$m loss compared to 5-FU alone (Figure 4B).
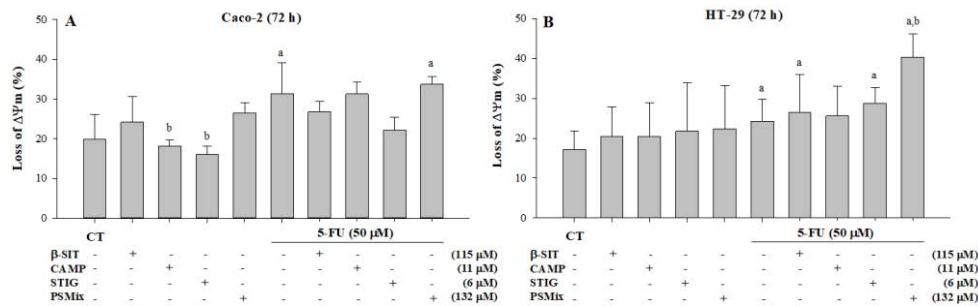

Figure 4. Effect of PS (β-SIT (115 µM), CAMP (11 µM), STIG (6 µM) and PS Mix (132 µM =115 µM β-SIT + 11 µM CAMP + 6 µM STIG) alone or in combination with 5-FU (50 µM) on mitochondrial transmembrane potential depolarization of Caco-2 (**4A**) and HT-29 cells (**4B**) at 72 h. Values (%) are expressed as mean ± SD (n = 3). [a]$p<0.05$ *versus* control group; [b]$p<0.05$ *versus* 5-FU.

4. Discussion

5-FU has been widely used as a chemotherapy drug in the management of colorectal cancer. However, avoiding or reducing the side effects as well as the resistance of malignant cells upon 5-FU treatment is still a major challenge. Therefore, it is desirable to explore novel therapeutic approaches to overcome these issues (Higgins, 2007; Longley et al., 2003; Walko & Lindley, 2005).

Recent studies have reported that dietary PS, mainly β-SIT, exert growth inhibitory effects on colon cancer cells (Cilla et al., 2015; López-García et al., 2017a; Monserrat-de la Paz et al., 2015), without or slightly altering the normal cell growth and viability of non-cancerous cells such as differentiated Caco-2 (a model of normal small intestinal cells) (Awad, Fink, Trautwein, & Ntanios, 2005), COS-1 (fibroblast cells) (Jayaprakasha et al., 2007), VERO (monkey kidney cells) (Baskar, Ignacimuthu, Paulraj, & Al Numair, 2010) or MRC-5 cells (fibroblast derived from lung cells) (Rahman, Wahab, & Malek, 2013) in a range of concentrations (0.6-1000 µM) in which are comprised our own tested concentrations (6-132 µM). However, it is not known whether dietary PS could sensitize cancer cells to improve the effects of conventional anticancer drugs and reduce their side effects. To this purpose, we explored here for the first time whether PS could act as co-adjuvants in 5-FU chemotherapy. Our results suggest an additive effect of PS in the cytotoxic action of

5-FU in colon cancer cells. Interestingly, this effect was exerted at PS concentrations attainable in the colon according to previous studies (López-García et al., 2017a).

Previous studies have reported that 5-FU treatment induced cell cycle arrest at S phase and apoptosis with a concomitant increase in caspases-3, -8 and -9 activation as well as increased ROS levels and loss of mitochondrial membrane potential in Caco-2 and HT-29 colon cancer cell lines (González-Sarrías et al., 2015; Pariente, Bejarano, Rodríguez, Pariente, & Espino, 2018). In the present study, the combination of 5-FU with either individual PS or a representative mixture of PS increased the 5-FU efficacy in the cell cycle arrest at S phase, apoptosis induction and increase in caspases activation in both cancer cell lines compared to 5-FU alone. However, no significant additional effects were found on ROS levels and mitochondrial membrane potential.

It should be noted that HT-29 cells were more resistant to all PS treatments and also to 5-FU treatment than Caco-2 cells. Despite PS Mix treatment showed the greatest additive effect with the combination of 5-FU, no synergistic or additive effect was observed compared to single PS treatments. This was in agreement with previous results, where physiological serum concentrations of a mixture of PS (13.25–26.5 µM in total) and individual PS were compared (Cilla et al., 2015).

To the best of our knowledge, only β-SIT has been investigated as potential co-adjuvant of chemotherapy drugs to sensitize cancer cells. In a previous study, sub-toxic concentrations of β-SIT (8 µM) in combination with TNF-related apoptosis-inducing ligand (TRAIL) sensitized MDA-MB-231 breast cancer cells by a TRAIL-mediated apoptosis (Park et al., 2008). In agreement with our data, β-SIT exerted the activation of caspases-3, -8 and -9. In addition, another *in vitro* study reported that β-SIT (4 and 16 µM) potentiated the inhibition of breast cancer MCF-7 and MDA-MB-231 cells exerted by the antiestrogen drug tamoxifen (Awad et al., 2008).

Although the effect of PS as co-adjuvants of 5-FU in the colon cancer therapy has not been evaluated so far, previous studies have shown the enhanced anticancer effect of 5-FU in combination with compounds structurally similar to PS such as vitamin D and other analogues against different colon cancer cell models (Kotlarz et al., 2016; Liu et al., 2010; Milczarek et al., 2013, 2014; Neska et al., 2016). To the

best of our knowledge, only two *in vivo* studies have shown that synthetic vitamin D analogues enhanced the chemopreventive efficacy of 5-FU on murine models of colorectal cancer (El-Shemi et al., 2016; Milczarek et al., 2013). El-Shemi et al. (2016) observed the reduction of tumors and large aberrant crypts foci in azoxymethane-induced colorectal cancer in rats, whereas Milczarek et al. (2013) reported the decrease of metastasis and, therefore, a prolongation of the survival time of mice, in comparison with the administrations of 5-FU given alone in mice bearing MC38 mouse colon tumors implanted subcutaneously or orthotopically. In agreement with our study, in these works the mechanisms of anticancer action of 5-FU occurred via promotion of cellular apoptosis and inhibition of cell cycle progression. Despite a ROS-mediated antiproliferative mechanism has been suggested for β-SIT (Baskar et al., 2010; Ramprasath & Awad, 2015), we observed that PS anticancer activity was not mediated by enhancement of mitochondrial damage and oxidative stress in both colon cancer cell lines. This is in agreement with previous results (Lopez-García et al. 2017a) that did not find dissipation of the mitochondrial membrane potential or changes in intracellular ROS content in PS-treated Caco-2 cells.

Nevertheless, we cannot discard that other mechanisms of action might be involved in the anticancer and chemosensitization effects of cancer cells by PS such the alteration of the plasma membrane, the activation of sphingolipid metabolism, the stimulation of ceramide formation and immune function, as well as the inhibition of cell invasion, migration and adhesion (Bradford & Awad, 2007; Ramprasath & Awad, 2015; Shahzad et al., 2017). Therefore, further investigation is still required to elucidate the underlying anticancer molecular mechanisms involved in the action of PS.

5. Conclusions

Our findings show for the first time that colonic concentrations of the main dietary PS (β-SIT, CAMP and STIG, either alone or as mixture) efficiently enhanced the effects exerted by 5-FU in colon cancer cells (Caco-2 and HT-29). The additive effects of PS as co-adjuvants to 5-FU treatments suggest that less 5-FU doses would be necessary to achieve a target anticancer action, which could be useful to reduce

potential side effects. Thus, combination therapy of PS and 5-FU could have clinical applications for colon cancer treatment, although further *in vivo* studies must be conducted to confirm our preclinical findings.

Conflict of interest

The authors declare that they have no conflict of interest

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Anexo 3. Comunicaciones a congresos
Annex 3. Congress communications

Autores: **Álvarez-Sala, A.,** Ávila-Gálvez, M. A., Cilla, A., Barberá, R., Garcia-Llatas, G., Espín, J.C., González-Sarrías, A.

Título: Los esteroles vegetales incrementan el efecto anticancerígeno del fármaco 5-fluorouracilo en células de cáncer de colon

Tipo de participación: Póster. Año: 2018

Congreso: VI Jornadas de Formación en Toxicología. Universitat de València (Facultat de Farmàcia)

Publicación: Revista de Toxicología. ISSN: 0212-7113 (página: 55).

Autores: **Álvarez-Sala, A.,** López-García, G., Attanzio, A., Tesoriere, L., Cilla, A., Barberá, R., Alegría, A.

Título: Pro- or anti-eryptotic effect of phytosterols and/or β-cryptoxanthin at serum concentrations with/without induced oxidative stress

Tipo de participación: Póster. Año: 2017

Congreso: 31st EFFoST International Conference. Food Sciences and Technology Challenges for the 21st Century- Research to Progress Society. Sitges, Barcelona.

Autores: **Álvarez-Sala, A.,** Blanco-Morales, V., Cilla, A., Garcia-Llatas, G., Barberá, R.

Título: Influence of the consumption of a plant sterol-enriched beverage on cytokines levels: a clinical trial

Tipo de participación: Póster. Año: 2017

Congreso: 31st EFFoST International Conference. Food Sciences and Technology Challenges for the 21st Century- Research to Progress Society. Sitges, Barcelona.

Autores: **Álvarez-Sala, A.,** Attanzio, A., Tesoriere, L., García-Llatas, G., Barberá, R., Cilla, A.

Título: Antiproliferative effect of microencapsulated plant sterols on human colon cancer cells

Tipo de participación: Comunicación oral. Año: 2017

Congreso: III International & IV National Student Congress of Food Science & Technology. Universitat de València (Facultat de Farmàcia)

Publicación: Book of abstracts "III International & IV National Student Congress of Food Science & Technology". ISSN: 2341-2240 (page: 24).

Autores: **Álvarez-Sala, A.,** García-Llatas, G., Cilla, A., Barberá, R.

Título: Sterol bioaccessibility from plant sterol enriched milk-based fruit beverages added of milk fat globule membrane during its self-life.

Tipo de participación: Póster. Año: 2017

Congreso: III International & IV National Student Congress of Food Science & Technology. Universitat de València (Facultat de Farmàcia)

Publicación: Book of abstracts "III International & IV National Student Congress of Food Science & Technology". ISSN: 2341-2240 (page: 33)

Autores: Blanco, V., **Álvarez-Sala, A.,** Cilla, A., García-Llatas, G., Barberá, R.

Título: Anti-inflammatory effect of plant sterols: clinical studies

Tipo de participación: Póster. Año: 2016

Congreso: III National & II International Student Congress of Food Science & Technology. Universitat de València (Facultat de Farmàcia)

Publicación: Book of abstracts "Bioactive compounds of natural origin in food science and technology". ISSN: 2341-2240 (page: 73)

Autores: **Álvarez-Sala, A.,** Blanco-Morales, V., Cilla, A., García-Llatas, G., Barberá, R., Lagarda, M.J.
Título: Oxysterols in a PS-enriched milk-based fruit beverage: effect of storage and bioaccessibility.
Tipo de participación: Póster. Año: 2016
Congreso: ENOR 6th Symposium. Universidad de Paris. Descartes, Francia

Autores: **Álvarez-Sala, A.,** Cilla, A., Garcia-Llatas, G., Barberá, R., Bermúdez, J.O., Lagarda, M.J.
Título: Effect of phytosterol-enriched milk-based fruit beverages added of milk fat globule membrane on serum levels of phytosterols and cholesterol precursors
Tipo de participación: Póster. Año: 2016
Congreso: The 1st International Conference on Food Bioactives & Health. Norwich, UK
Publicación: Book "1st International Conference on Food Bioactives & Health" (page: 71)

Autores: **Álvarez-Sala, A.,** Barberá, R., García-Llatas, G., Lagarda, M.J.
Título: Stability of sterols in milk-based fruit beverages
Tipo de participación: Póster. Año: 2015
Congreso: 1st International Congress of Students of Food Science and Technology. Universitat de València (Facultat de Farmàcia).
Publicación: Book of abstracts "II National & I International Student Congress of Food Science & Technology". ISSN: 2341-2240 (page: 73)

Autores: **Álvarez-Sala, A.,** Barberá, R., García-Llatas, G., Lagarda, M.J.
Título: Sterol bioaccessibility from milk-based fruit beverages: influence of food matrix
Tipo de participación: Póster. Año: 2015
Congreso: 4th International Conference of Food Digestion. University of Naples, Italia
Publicación: Book of abstracts proceeding of the 4th International Conference of Food Digestion

Autores: **Álvarez-Sala, A.,** Barberá, R. García-Llatas, G. Lagarda M.J.
Título: Determinación de esteroles vegetales en bebidas y derivados lácteos enriquecidos
Tipo de participación: Póster. Año: 2014
Congreso: 1er Congreso Nacional de Estudiantes de Ciencia y Tecnología de los Alimentos. Universitat de València (Facultat de Farmàcia).
Publicación: Libro de abstracts "1er Congreso Nacional de Estudiantes de Ciencia y Tecnología de los Alimentos. Avances en Calidad, Seguridad y Tecnologías Alimentarias". ISSN: 2341-2240 (página: 81).