UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA-FÍSICA APLICADA

TESIS DOCTORAL

CARACTERIZACIÓN Y FUNCIONALIDAD DE LOS FOSFO- Y ESFINGOLÍPIDOS DE LA MEMBRANA DEL GLÓBULO GRASO LÁCTEO PARA SU APLICACIÓN COMO INGREDIENTES BIOACTIVOS EN DERIVADOS LÁCTEOS FUNCIONALES

MARÍA DEL PILAR CASTRO GÓMEZ

MADRID, 2015

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA-FÍSICA APLICADA

CARACTERIZACIÓN Y FUNCIONALIDAD DE LOS FOSFO- Y ESFINGOLÍPIDOS DE LA MEMBRANA DEL GLÓBULO GRASO LÁCTEO PARA SU APLICACIÓN COMO INGREDIENTES BIOACTIVOS EN DERIVADOS LÁCTEOS FUNCIONALES

Memoria presentada por

MARÍA DEL PILAR CASTRO GÓMEZ

Para optar al grado de:

Doctor en Biología y Ciencias de la Alimentación

Trabajo realizado bajo la dirección de:

Dr. Fco. Javier Fontecha Alonso

Dra. María Visitación Calvo Garrido

Y bajo la tutela académica de la Dra. Susana Santoyo Diez

Instituto de Investigación en Ciencias de la Alimentación (CIAL)

FRANCISCO JAVIER FONTECHA ALONSO, DOCTOR EN CIENCIAS QUÍMICAS E INVESTIGADOR CIENTÍFICO Y MARÍA VISITACIÓN CALVO GARRIDO, DOCTORA EN CIENCIAS Y TITULADO SUPERIOR DEL INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

CERTIFICAN:

Que el trabajo titulado: "Caracterización y funcionalidad de los fosfo- y esfingolípidos de la membrana del glóbulo graso lácteo para su aplicación como ingredientes bioactivos en derivados lácteos funcionales", que constituye la memoria que presenta María del Pilar Castro Gómez, ha sido realizada en el Instituto de Investigación en Ciencias de la Alimentación, centro mixto del Consejo Superior de Investigaciones Científicas y la Universidad Autónoma de Madrid, bajo nuestra dirección y cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Autónoma de Madrid y, por tanto, autorizo su presentación.

Y para que conste a los efectos oportunos, firmamos el presente Certificado en Madrid, a 2 de junio de 2015.

Fco. Javier Fontecha Alonso María Visitación Calvo Garrido

Este trabajo ha sido realizada con la financiación aportada por:

- AGL2011-26713. “Fosfolípidos y esfingolípidos de la membrana del glóbulo graso lácteo como ingredientes bioactivos. Estudios de la bioaccesibilidad gastrointestinal en derivados lácteos y fórmulas infantiles enriquecidas”.

- CYTED-110AC0386. “Incorporación de nuevos ingredientes funcionales a alimentos como contribución a la promoción de la salud y/o a la prevención de enfermedades de la población iberoamericana”.

- CENIT-SENIFOOD. “Dietas y alimentos con características específicas para las personas mayores”.

- ALIBIRD-S2013/ABI-2728 “Alimentos funcionales y estrategias nutricionales eficaces para la prevención y tratamientos de enfermedades crónicas”

- FUNCFOOD-CDS-2007-063 CONSOLIDER INGENIO. “Ingredientes Alimentos Funcionales para Mejorar la Salud".

- JAE CSIC– PRE CP. “Mejora de la composición de fosfo- y esfingolípidos de la membrana del glóbulo graso de la leche para su aplicación como ingrediente bioactivo en derivados lácteos funcionales”

Agradecimientos

Mirando atrás, parece mentira que este "viaje" comenzara en el año 2010. De él sólo me puedo llevar buenos recuerdos, buena gente y ¿cómo no?, mucha ciencia.

En primer lugar, me gustaría agradecer a mi director de Tesis, Javier Fontecha, la confianza que ha depositado en mí desde el primer día. Aún recuerdo cómo por puro azar, en unas prácticas de la Universidad, fui a caer en su grupo y hoy me encuentro escribiendo los agradecimientos de mi Tesis Doctoral. Javier, gracias por todo lo que me has enseñado y aconsejado, tu paciencia, tu atención a pesar de tener la agenda siempre llena y por ser un ejemplo a seguir. Sinceramente, espero que haya mucha más gente detrás de mí que pueda disfrutar de un mentor como tú.
A mi co-directora, Mariví Calvo, por sus consejos, su paciencia y su gran esfuerzo en esta última etapa de escritura. Mil gracias por cuidarme, preocuparte por mí y hacerme sentir como en casa desde que entré por esa puerta siendo una "pipiola".

También me gustaría agradecer a Manuela Juárez su presencia y aportaciones a lo largo de mi formación. Siempre recordaré su pasión por el trabajo, su inagotable energía y las buenas palabras que me ha dedicado a pesar de los innumerables compromisos.

Al Departamento de Química-Física Aplicada de la Universidad Autónoma de Madrid, especialmente a mi tutora académica Susana Santoyo, por su disponibilidad y consejos en todo momento que lo he necesitado.

A mis compañeros y amigos que están o han pasado por el laboratorio. A Luis, por la cantidad de horas que ha empleado en mi formación, las neuronas que le he hecho quemar con tantas preguntas y por supuesto por sus buenos consejos. A Paquita, por lo que me enseñó durante nuestra andadura juntas en el laboratorio, pero sobre todo por ser una amiga con mayúsculas y un ejemplo a seguir ante las adversidades ¡no cambies nunca!. A mi otra flor Toñi, porque a pesar de no vernos en exceso sigue contagiándome con esa ilusión por vivir que le caracteriza. A mi amiga y compañera de gremio María Freire, porque esta segunda oportunidad que nos ha dado Madrid me ha servido para confirmar que sigues siendo la misma

buena amiga que encontré en la Universidad. Finalmente, a mis "zanus" Alba y Cristina porque sin ellas la segunda mitad de mi Tesis no hubiera sido igual. A Alba por desprender esa felicidad por los cuatro costados por mínima que sea la razón, por las infinitas risas y nuestras conversaciones de las que arreglan el mundo. A Cristina, porque a pesar de haber sido la última adquisición, desde el primer día supe que era una de esas personas que merece la pena tener cerca. ¡Os quiero mucho chicas!.

A tanta gente que ha pasado por el laboratorio y que han dejado su pequeña huella, su amistad o un lugar que visitar. Es por esta razón que no me puedo dejar en el tintero a Carina por ser la argentina más nórdica que conozco, a Manel por la alegría que irradia más allá de Túnez y a Samira por enseñarme portugués y más cultura brasileña en atascados viajes en coche.

Al doctor João Ernesto Carvalho del Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) de la Universidad de Campinas, Brasil, por darme la posibilidad de introducirme en el mundo de los cultivos celulares en un entorno ambiental y humano espectacular. Me gustaría agradecer también muy especialmente a Karin Maia Monteiro por hacerme sentir en casa desde el primer día de mi estancia en Campinas.

Al doctor Olimpio Montero del Centre for Biotechnology Development (CDB) del CSIC por su paciencia, disponibilidad y buen hacer enseñándome a trabajar con el UPLC-QToF-MS en mis visitas a Valladolid.

A mis "compis" de comida Clara, Elvira, Carlos, Tomás y Alberto por aguantarse el hambre para poder reunirnos los máximos posibles y hablar de la presión a la que trabaja la Nespresso mientras uno se atraganta... Ahora en serio, sólo espero llegar a reírme en cada comida la décima parte de lo que lo hago con vosotros.

A Álvaro, porque si tras más de 20 años de amistad (que se dice pronto) seguimos intentando alargarlo, estoy segura que esto es para toda la vida. Mil gracias por estar siempre, por preocuparte, por cuidar de mí y por escucharme en todo momento. ¡Sólo te mereces cosas buenas!.

A Laura, simplemente por ser especial en mi vida y hacerme mejor persona. Admiro tus valores, tu cariño hacia los tuyos, tu ilusión por el trabajo, tu espíritu inconformista, tu pasión por los animales, etc. Hay tantas cosas, que espero tenerte en mi vida siempre para descubrirlas todas.

A mis hermanas Sonia, Almudena y Macarena porque a pesar de nuestras diferencias siempre están dispuestas a juntarnos el día que haga falta para reírnos las unas con las otras y contarnos nuestras cosas. Por supuesto no olvido a Nacho porque sólo aporta cosas buenas y cariño. A Borja, por aguantar a Sonia ¡es broma!, por ser de esas personas cuya nobleza hace sentir bien al resto. También a mis sobrinos Paula, Lucia, Aitana, Samuel y los que están por venir, por sacarme una sonrisa siempre que nos vemos. A mi abuelo, porque aunque me dé pereza explicarle mis cosas de trabajo, siempre me pregunta por mis "bichos". A mi abuela Pilar, porque a pesar de que nos dejó ya hace un tiempo, no hay un momento especial en mi vida en el que no esté presente en primera fila.

Y muy especialmente quiero agradecerles a mis padres Antonio y Marisa por facilitarme absolutamente todo lo que ha estado en sus manos. Gracias por creer en mí, en mis capacidades y apoyarme incluso en los momentos en los que ni yo me sentía capaz de hacerlo. Gracias por los valores que me habéis trasmitido y por hacerme la persona que hoy se encuentra escribiendo esto. Con perdón al resto de padres y madres que lean esto… ¡sois los mejores!.

"The doctor of the future will no longer treat the human frame with drugs, but rather will cure and prevent disease with nutrition."

Thomas Edison

ACL: cloruro de acetilo

CDP: citidina difosfato

CE: éster de colesterol

CHOL: colesterol

CLA: ácido linoleico conjugado

CM: quilomicrón

CN: número de átomos de carbono

CtM: leche control

CVD: enfermedades cardiovasculares

DAG: diacilglicéridos

DCL: deterioro cognitivo leve

DHA: ácido docosahexanoico

DMA: dimetilacetales

DMF: dimetilformamida

DMSO: dimetilsulfoxido

ELSD: detector evaporativo de dispersión de luz

EPA: ácido eicosapentanoico

ESI: ionización electospray

FA: ácidos grasos

FAME: ésteres metílicos de ácidos grasos

FFA: ácidos grasos libres

FG: grado alimentario

FID: detector de ionización de llama

FM: método Folch *et al. (1957)* modificado

GC: cromatografía de gases

HDL: lipoproteína de alta densidad

HILIC: cromatografía de líquidos de interacción hidrofílica

HPLC: cromatografía de líquidos de alta resolución

IR: infrarrojo

KHS: hidróxido sódico /ácido sulfúrico

KO: aceite de krill

LC: cromatografía de líquidos
LCPUFA: ácidos grasos poliinsaturados de larga cadena
MAG: monoacilglicéridos
MALDI: desorción/ionización láser asistida por matriz
MBF: metilato de sodio / trifluoruro de boro
MetNa: metóxido sódico
MF: microfiltración
MFGM: membrana del globulo graso de la leche
MHS: metóxido sódico / ácido sulfúrico
MS: detector de masas
MUFA: ácidos grasos monoinsaturados
N-3: omega 3
NFG: grado no alimentario
NMR: resonancia magnética nuclear
PA: ácido fosfatídico
PC: fosfatidilcolina
PE: fosfatidiletanolamina
PGE: prostaglandinas
PI: fosfatidilinositol
PL: lípidos polares
PLE: extracción con líquidos presurizados
PS: fosfatidilserina
PUFA: ácidos grasos poliinsaturados
UFA: ácidos grasos insaturados
Q-ToF: cuadrupolo- tiempo de vuelo
RA: ácido ruménico
RF: factor de respuesta
SCFA: ácidos grasos de cadena corta
SFA: ácidos grasos saturados
SFE: extracción con fluidos supercríticos
SM: esfingomielina
SPE: separación en fase sólida
SppM: leche suplementada
TAG: triacilglicéridos

TFA: ácidos grasos *trans*

TLC: cromatografía en capa fina

TNF: factor de necrosis tumoral

UF: ultrafiltración

UPLC: cromatografía de líquidos de ultra resulución

VA: ácido vacénico

VLDL: lipoproteínas de muy baja densidad

ÍNDICE

1. INTRODUCCIÓN

1. Fosfo- y esfingolípidos de la dieta

Los fosfolípidos y esfingolípidos son lípidos polares (PL) que se hallan presentes en todos los tejidos, tanto animales como vegetales, y son constituyentes fundamentales de las membranas celulares. Dentro del grupo de los fosfolípidos se encuentran la fosfatidiletanolamina (PE), el fosfatidilinositol (PI), la fosfatidilserina (PS) y la fosfatidelcolina (PC) y entre los esfingolípidos, destaca la esfingomielina (SM) (Figura 1). Su presencia en las membranas celulares viene justificada por su naturaleza anfifílica, ya que la cabeza hidrofílica y la cola hidrofóbica hacen posible el mantenimiento de la bicapa, tanto en células como en orgánulos. Son bien conocidas las numerosas funciones, además de la estructural, desempeñadas por estas membranas y que se atribuyen principalmente a las numerosas proteínas que se encuentran intercaladas y ancladas entre los PL. Por esta razón los PL pasan a formar parte de manera indirecta de procesos metabólicos, neurológicos y de señalización intracelular esenciales para la vida como son el crecimiento, el reconocimiento y la apoptosis celular, el transporte, la replicación del DNA, la secreción o señalización neuronal, entre otros (Pettus *et al.*, 2004), (Guo *et al.*, 2005) . Sin embargo, aunque los PL y las proteínas asociadas son los componentes mayoritarios de las membranas, otros como los carbohidratos agregados a proteínas (glicoproteínas) o a lípidos (glicolípidos), así como el colesterol (CHOL) resultan también esenciales para el mantenimiento y funcionalidad de la membrana.

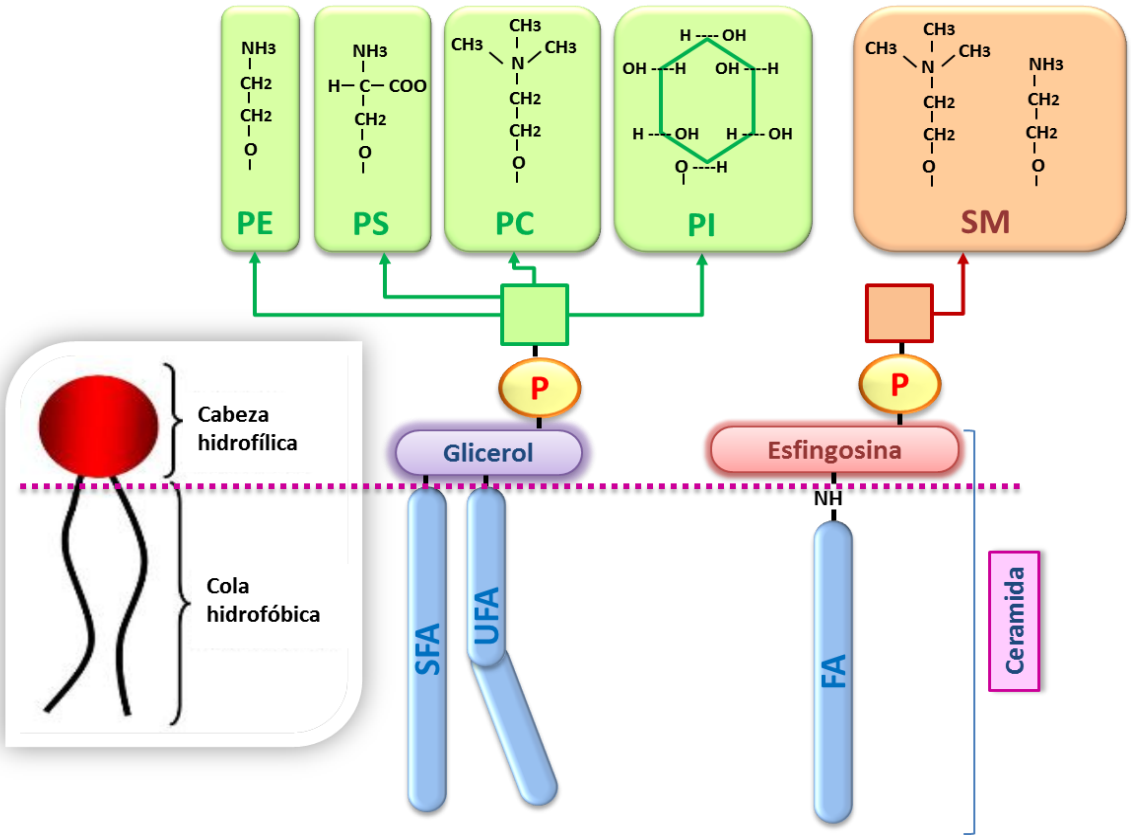
Debido a su presencia en todas las membranas celulares, los PL pueden encontrarse en la gran mayoría de los alimentos que se consumen habitualmente, y son numerosos los potenciales efectos beneficiosos sobre enfermedades y dolencias en humanos que se les atribuyen.

1.1. Estructura química

Los fosfolípidos comparten una estructura común que consiste en dos moléculas de ácidos grasos (FA) esterificados en las posiciones *sn*-1 y *sn*-2 del glicerol atribuyéndose a ellos la hidrofobicidad de la molécula. El componente

que le otorga el carácter hidrofílico (Figura 1), está constituido por el grupo fosfato unido al último –OH de la posición *sn*-3 del glicerol, que mediante un enlace fosfodiéster puede unirse a cuatro posibles grupos como la etanolamina, el inositol, la serina o la colina, y de los que los PL toman su nombre (PE, PI, PC o PC, respectivamente) (Vance y Vance, 2008). Cuando alguno de estos fosfolípidos pierde uno de los FA que forman parte de su estructura, se origina la correspondiente lisoforma del compuesto (liso-PE, liso-PI, liso-PS y liso-PC). Sin embargo, cuando el componente que desaparece es desaparece la cabeza polar, la estructura resultante se denomina ácido fosfatídico (PA).

Figura 1. Representación esquemática de la estructura de los fosfoglicerolípidos y de la esfingomielina



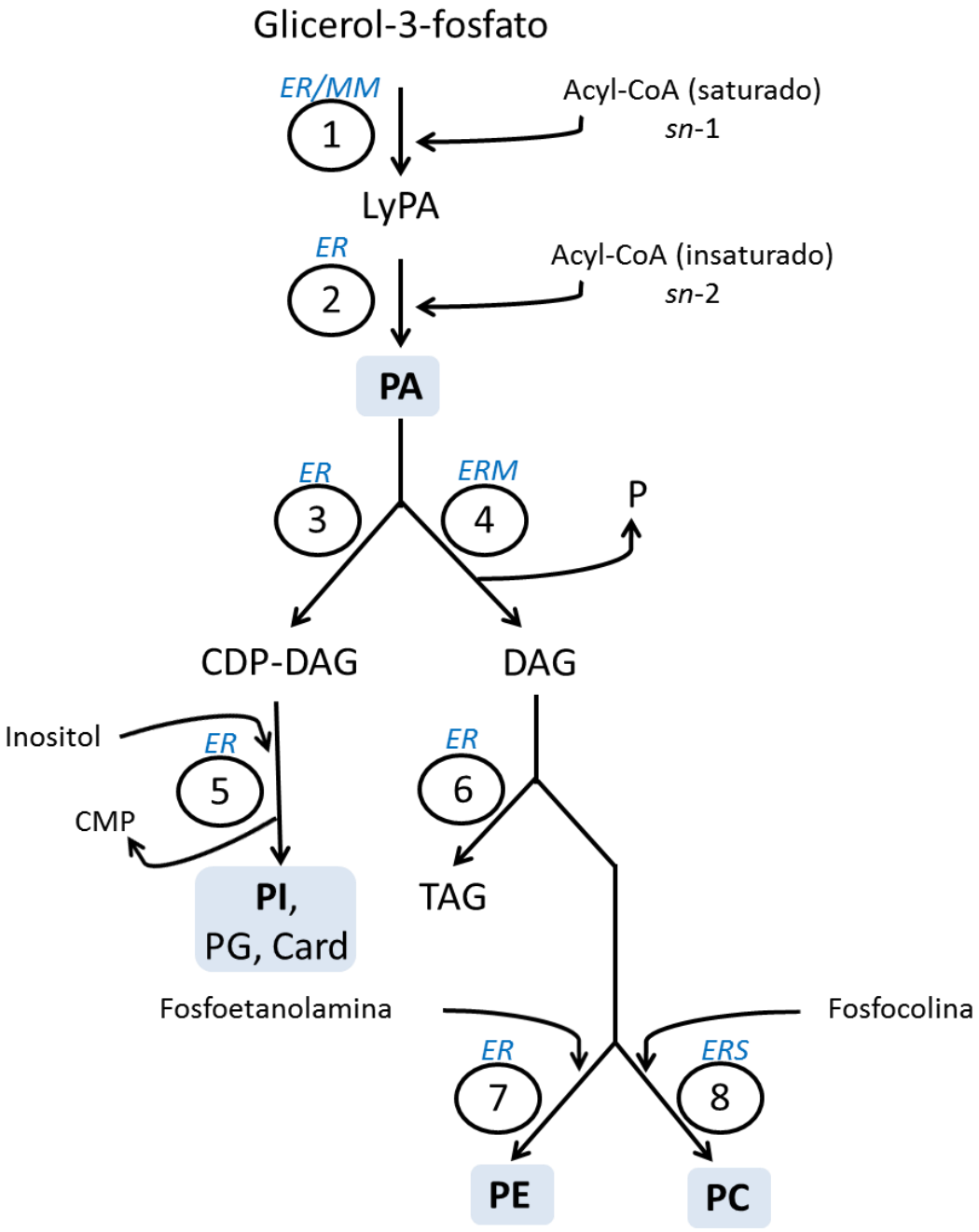
PE: fosfatidiletanolamina, PI: fosfatidilinositol, PS: fosfatidilserina, PC: fosfatidilcolina, SM: esfingomielina

La SM pertenece al grupo de los esfingolípidos, por lo que no comparte una estructura común con los PL. Se encuentra unicamente en las células animales y su análogo en plantas es la ceramida fosforilinositol. La estructura de la SM consiste en una ceramida (FA unido a una esfingosina a través de enlace amida) que lleva incorporada, la mayor parte de las veces, una fosforilcolina, en la posición *sn*-1, aunque también puede llevar unida una etanolamina (Figura 1).

1.2. Biosíntesis y funciones

El ciclo de la biosíntesis de los fosfolípidos comienza con la acción de la enzima glicerol-3-fosfato aciltransferasa (situada en la capa externa de la membrana mitocondrial y en el retículo endoplásmico), que incorpora un acilo graso CoA (normalmente saturado) a la posición *sn*-1 del glicerol-3-fosfato dando lugar al liso-PA. Para la obtención del PA en el retículo endoplásmico, es necesaria la acción de una segunda enzima, la acilglicerol-3-aciltransferasa, que unirá otro acilo graso CoA, generalmente insaturado, en la posición *sn*-2 del glicerol. Posteriormente el PA se convierte en el sustrato de dos vías enzimáticas metabólicamente importantes. Una de ellas, que transcurre en la membrana del retículo endoplásmico, es llevada a cabo por la fosfatasa que actúa sobre el ácido fosfatídico citosólico, separando el grupo fosfato de la posición *sn*-3 del PA y dando lugar a diacilglicéridos (DAG). Mediante la acción de la diacilglicerol aciltransferasa, se puede esterificar otro FA en dicha posición dando lugar a triacilglicéridos (TAG), que constituyen la principal forma de almacenamiento de energía. La otra vía consiste en la síntesis de PC y PE en la superficie y en el interior del retículo endoplásmico, respectivamente (Figura 2). Dicha síntesis se inicia a partir de PA por la unión de una molécula de colina o etanolamina mediante la acción de las enzimas citidina difosfato (CDP)-colina: 1,2-diacilglicerol colina fosfotransferasa y CDP-etanolamina: 1,2-diacilglicerol etanolamina fosfotransferasa, respectivamente (Baenke *et al.*, 2013).

Figura 2. Representación esquemática de las rutas de síntesis de PE, PI y PC.



TAG: triacilglicerido; DAG: diacilglicerido; P: grupo fosfato; CMP: citidina monofosfato; CDP-DAG: citidina difosfato diacilglicerol; LyPA: ácido lisofosfatídico; PA: ácido fosfatídico; PE: fosfatidileanolamina, PI: fosfatidilinositol; PC: fosfatidilcolina, PG: fosfatilglicerol; Card: cardiolipinas. Los números que aparecenrodeados son las enzimas participantes en la reacción: 1: glicerol-3-fosfato aciltransferasa; 2: acilglicerol-3-aciltransferasa; 3: CDP-diacilglicerol sintetasa; 4: fosfatasa del ácido fosfatídico; 5: CDP-diacilglicerol inositol fosfatidiltranaferasa; 6: diacilglicerol aciltransferasa; 7: CDP-etanolamina:1,2-diacilglicerol etanolaminfosfotransferasa; 8: CDP-colina: 1,2-diacilglicerol colinafosfotransferasa. La letra en cursiva corresponde al lugar donde transcurre la reacción: ER: retículo endoplásmico; ERM: membrana del retículo endoplásmico; ERS: superficie del retículo endoplásmico; MM: membrana mitocondrial

Estos PL desempeñan funciones esenciales en la célula. La PC es el más abundante y se encuentra localizada en la parte externa de la membrana celular constituyendo un elemento clave en el mantenimiento de la integridad y función de la misma. Por otro lado, la PC juega un papel esencial en la secreción de lipoproteínas de muy baja densidad (VLDL) en el hígado, participando, por tanto, de manera activa en la distribución de CHOL y grasa hasta los tejidos. Participa también en la formación de micelas en el lumen intestinal y junto con las sales biliares, ayuda a la solubilización de los ácidos biliares en la bilis, contribuyendo de esta manera a la mejor absorción de nutrientes liposolubles de la dieta. El segundo PL más abundante es la PE que puede llegar a representar entre un 20 y 30% del total de PL presentes en la membrana celular. Se localiza principalmente en la capa interna de la membrana (hasta el 80% del total) y destaca su presencia en las mitocondrias celulares donde desempeña una función vital en su crecimiento y estabilidad. Otra de las funciones que se atribuye a la PE, es la de constituir la base para la síntesis del glicolípido glicosilfosfatidilinositol y facilitar el anclaje de proteínas a la membrana, cuya inserción se ve favorecida por el reducido tamaño de este PL, manteniendo de esta manera la estabilidad de la membrana (Vance y Vance, 2008).

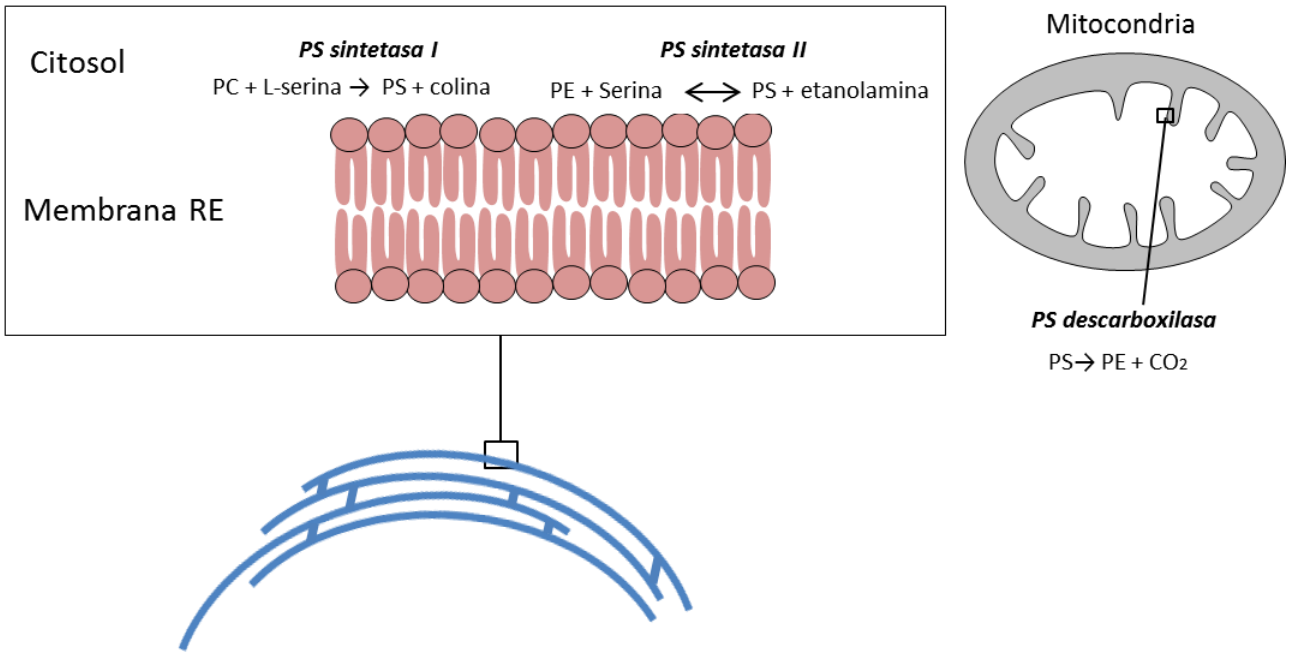
La biosíntesis de la PS se desarrolla por vías diferentes en células procariotas y eucariotas. En el caso de células procariotas, la síntesis de PS, al igual que la de PI, transcurre a través de la ruta CDP-DAG, y se explicará más adelante.
En eucariotas existen dos posibles rutas metabólicas para la síntesis de PS, que emplean diferentes sustratos y enzimas. Dicha proceso de síntesis tiene lugar en el retículo endoplásmico y es dependiente de la presencia de PE y de PC. Al entrar en contacto la PC con una molécula de serina en presencia de la enzima PS sintetasa I, la PC sustituye su grupo colina por el de serina, obteniéndose como productos de la reacción una molécula de PS y una colina. En el caso de la PE el grupo etanolamina es sustituido por una serina, en una reacción catalizada por la PS sintetasa II, originándose una molécula de PS y otra de etanolamina. Esta última reacción tiene la peculiaridad de ser reversible y en presencia de la misma enzima en el retículo endoplásmico, la PS puede perder la serina para sustituirla por etanolamina y así volver a formar PE y

serina como productos de la reacción. Sin embargo, este no es el único modo en el que la PS se transforma en PE ya que en la mitocondria, gracias a la acción de la PS descarboxilasa, la PS pierde un CO_2 para dar lugar a la PE. Para continuar el ciclo, esta PE producida durante la descarboxilación, puede regresar de nuevo al retículo endoplásmico para ser transformado en el PS original. En la Figura 3 se recogen de manera esquemática las reacciones involucradas en la síntesis de PS.

La PS constituye un 5-10% del total de PL presentes en las membranas celulares y se localiza mayoritariamente en la capa interna. No obstante, la PS situada en la capa externa desempeña un papel crucial en los mecanismos de señalización implicados en la coagulación y la apoptosis, siendo capaz de estimular a la proteín-kinasa C, responsable de numerosas respuestas celulares. Por otro lado, la naturaleza aniónica de la cabeza de la PS le permite tener una mayor interacción con proteínas cargadas positivamente promoviendo un mejor contacto entre proteínas y receptores (Mozzi et al., 2003; Leventis y Grinstein, 2010).

Durante la síntesis de PI en el retículo endoplásmico, el PA es inicialmente transformado en CDP-DAG por la acción de CDP-diacilglicerol sintetasa. Posteriormente, en presencia de la CDP-DAG inositol fosfatidiltransferasa, el inositol es capaz de unirse a la molécula de CDP-DAG dando como productos de la reacción el PI y una citidina monofosfato. Esta misma ruta es utilizada en la formación de otras moléculas esenciales para la vida como son las cardiolipinas (Gardocki *et al.*, 2005). El PI representa un 10% del total de PL del interior de la membrana y a pesar de no ser el más abundante, desempeña un papel crítico en la regulación de mecanismos fundamentales para la vida como el dinamismo y mantenimiento de la membrana celular, además de actuar como factor de señalización en varias rutas de transducción. Por otro lado, la hidrólisis de PI por acción de la fosfolipasa A, favorece la disponibilidad de ciertos FA bioactivos como es el caso del araquidónico, con actividad antiinflamatoria (D'Souza y Epand, 2014).

Figura 3. Representación esquemática de las rutas de síntesis de PS



RE: retículo endoplásmico; PE: fosfatidiletanolamina; PS: fosfatidilserina; PC: fosfatidilcolina

De forma general, cuando un fosfolípido pierde uno de los FA se genera una lisoforma capaz de funcionar como mensajero celular. Por su parte, cada una de esas lisoformas, de manera individual, desempeña diferentes funciones. Así, para la liso-PE se han descrito varias propiedades biológicas en tejidos animales *in vitro* (Arouri y Mouritsen, 2013). El liso-PI participa en la remodelación de la composición de los FA y actúa como biomarcador en pacientes de cáncer (Yamashita *et al.*, 2013). La liso-PS interviene como señalizador celular en la activación de neutrófilos, además de ejercer una acción antiinflamatoria (Frasch y Bratton, 2012). Por último, la liso-PC que actúa también como señalizador celular, con beneficiosos efectos en la artritis reumatoide, desempeña un papel importante en la metilación de las células del sistema nervioso (Arouri y Mouritsen, 2013; Sevastou *et al.*, 2013).

La síntesis de la SM puede tener lugar tanto en el aparato de Golgi como en la membrana plasmática, estando catalizada por la esfingomielina sintetasa I y la esfingomielina sintetasa II, respectivamente. Utilizando como sustratos PC y

ceramida, dichas enzimas producen SM y DAG. La ruta que implica la PE, transcurre en el retículo endoplasmático y consiste en transformar la ceramida en una ceramida fosforiletanolamina tras adquirir este grupo de una PE metilada. Estas SM se encuentran ancladas, junto a la PC, en la cara más externa de las membranas celulares, siendo particularmente abundantes en las vainas de mielina donde mantienen la estabilidad y la resistencia química. Por otro lado, realizan funciones reguladoras puesto que interaccionan con proteínas muy específicas y actúan de receptor para la internalización de la transferrina, responsable de la entrada de hierro en las células.

Sin embargo, la característica más peculiar de las SM es su capacidad para generar junto al CHOL unas estructuras denominadas "rafts" tanto en las membranas celulares como en la superficie de las lipoproteínas. Investigaciones recientes sugieren que el metabolismo del CHOL y el de la SM están muy integrados e incluso se ha descrito a la SM como la responsable de la distribución del CHOL en las células (Aureli *et al.*, 2011; Merrill, 2011).

Mientras que en la composición de la SM encontramos FA saturados (SFA) de larga cadena (>16 átomos de carbono), los FA de los fosfolípidos constituyentes de la membrana celular son en mayor medida el C16:0, el C18:0, el C18:1, el C18:2, el C20:4 y el C20:6. Se ha descrito una mayor presencia de FA insaturados (UFA) en la posición *sn*-2 del glicerol, mientras que en la posición *sn*-1 se encuentran mayoritariamente SFA como el C16:0 o el C18:0.

1.3. Contenido en alimentos

Aunque no se han establecido recomendaciones de consumo diario de PL, se estima que la cantidad de PC, el PL mayoritario en los alimentos, ingerida habitualmente varía entre 2 y 8 gramos/día, lo que representa entre el 1 y 10% de la grasa diaria consumida (Cohn *et al.*, 2010). Esta cantidad de PL es incorporada principalmente a través de alimentos como yema de huevo, órganos y carnes magras, pescados y mariscos, granos de cereales y algunas semillas oleosas como la soja. Aunque estos alimentos aportan mayoritariamente PC, también podemos encontrar Liso-PC y SM en productos de origen animal, así como PE y PI en los de origen vegetal, siendo estos

deficientes en PI y PS, respectivamente (Cohn *et al.*, 2010). Otros productos de origen vegetal como frutas, verduras o tubérculos son muy pobres o carentes en PL (Weihrauch y Son, 1983). En soja, un 4% del peso total de grasa corresponde a PL, de los cuales mas de la mitad (55%), corresponde a PC, seguido de PE (26%) y PI (18%) (Wang *et al.*, 1997). Otro producto de origen vegetal que posee un contenido elevado de PL es el cacahuete con 1-2% sobre la grasa total, donde la PC, la PE y el PI representa un 57%, 11% y 32%, respectivamente sobre el total de PL (Weihrauch y Son, 1983).

No obstante, son los alimentos de origen animal los que presentan mayores posibilidades como fuentes de PL. En esta línea, el alimento más extensamente estudiado ha sido la yema de huevo, que contiene un 10% de PL en peso, siendo la PC el compuesto mayoritario (60-70%), seguido por PE, SM y liso-PL con un 15-26%, 2.5-5% y 6-8%, respectivamente (Waldemar, 2002). Entre las carnes de consumo habitual, la ternera contiene aproximadamente un 14 % de PL sobre el total de grasa (<1% de PL del peso total) que se distribuyen entre PE, PC y SM con valores alrededor de 31%, 62% y 7%, respectivamente, mientras que PI y PS son inexistentes. Sin embargo, la pechuga de pollo presenta un 14% de PS sobre el total de PL (<1% en peso) (Weihrauch y Son, 1983; Cohn *et al.*, 2010) (Tabla 1). Pero son las vísceras de animales, como el hígado y el cerebro (porcino y bovino), las fuentes más ricas en PL y en cuya composición se hallan presentes todos los PL. Sin embargo, el consumo de estos productos se halla muy restringido y regulado por constituir una posible fuente de transmisión de encefalopatía espongiforme. Los productos de origen marino como el calamar o el krill poseen igualmente un elevado contenido en PL, con más del 50% sobre el total de grasa, siendo también en este caso la PC el compuesto mayoritario (87.5%), seguido de PE (5.8%), SM (3.4%) y PI (2.2%) tal y como describen Kullenberg *et al.* (2012) y Castro-Gómez *et al.* (2015). En los citados estudios destaca la elevada cantidad de ácidos grasos poliinsaturados (PUFA) de larga cadena omega 3 (LCPUFA n-3), como el eicosapentanoico (EPA) y el docosahexanoico (DHA), esterificados al PL, en el caso del aceite de krill (KO), lo que parece otorgar a dichos FA una mayor biodisponibilidad que cuando se encuentran esterificados en TAG (Ulven *et al.*, 2011).

La leche y productos lácteos constituyen la fuente alimentaria que proporciona de una forma segura todas las especies de PL aunque su contenido no sea muy elevado, ya que unicamente suponen entre el 0,3-1% del total de grasa de producto. Sin embargo, algunos subproductos lácteos como la mazada y en especial la membrana del glóbulo graso de la leche (MFGM) contienen PL en concentraciones significativamente importantes. En esta última alcanza una concentración de PL hasta de un 40% en peso sobre el total de grasa, donde la PE representa aproximadamente el 30%, el PI un 7%, la PS un 5%, la PC un 31% y la SM un 20% sobre el total de PL (Fong *et al.*, 2007; Rodríguez-Alcalá y Fontecha, 2010). En la actualidad, debido a la tendencia del consumo de productos lácteos con bajo contenido en grasa, la ingesta de PL a través la dieta está disminuyendo. No obstante, dado que la lecitina de soja es añadida de manera habitual como emulsionante en alimentos procesados, el balance en el consumo total de PL, y en particular de PC, aumenta de manera significativa (Cohn *et al.*, 2010).

Tabla 1. Tabla de composición de fosfo- y esfingolípidos en alimentos

	Lípidos totales (g/100 g)	**mg/100g**						
		Total PL	**PC**	**PE**	**PI**	**PS**	**SM**	**lysoPC**
Yema de huevo	31.8	10306	6771	1917	64	-	486	419
Hígado de cerdo	3.7	2901	1688	618	209	38	131	61
Hígado de pollo	5.6	2542	1120	829	-	146	291	-
Soja	20.8	2308	917	536	287	-	-	-
Calamar	1.7	1098	777	114	-	83	102	-
Pechuga de pollo	1.1	782	391	187	-	100	56	-
Ternera	4.1	660	407	207	-	-	46	-
Cacahuete	48.5	620	270	50	150	-	-	
Espinaca	0.3	157	37	36	11	-	-	-
Patata	0.2	76	38	22	12	1	-	-
Zanahoria	0.3	55	23	15	5	3	-	-
Manzana	0.1	40	21	10	6	1	-	-
Leche de vaca	3.7	34	12	10	2	1	9	-

PE: fosfatidiletanomlamina, PI: fosfatidilinositol, PS: fosfatidilserina, PC: fosfatidilcolina, SM: esfingomielina, lysoPC: lisofosfatidilcolina, PL: lípidos polares

Fuente: Cohn *et al.* (2010)

1.4. Absorción intestinal

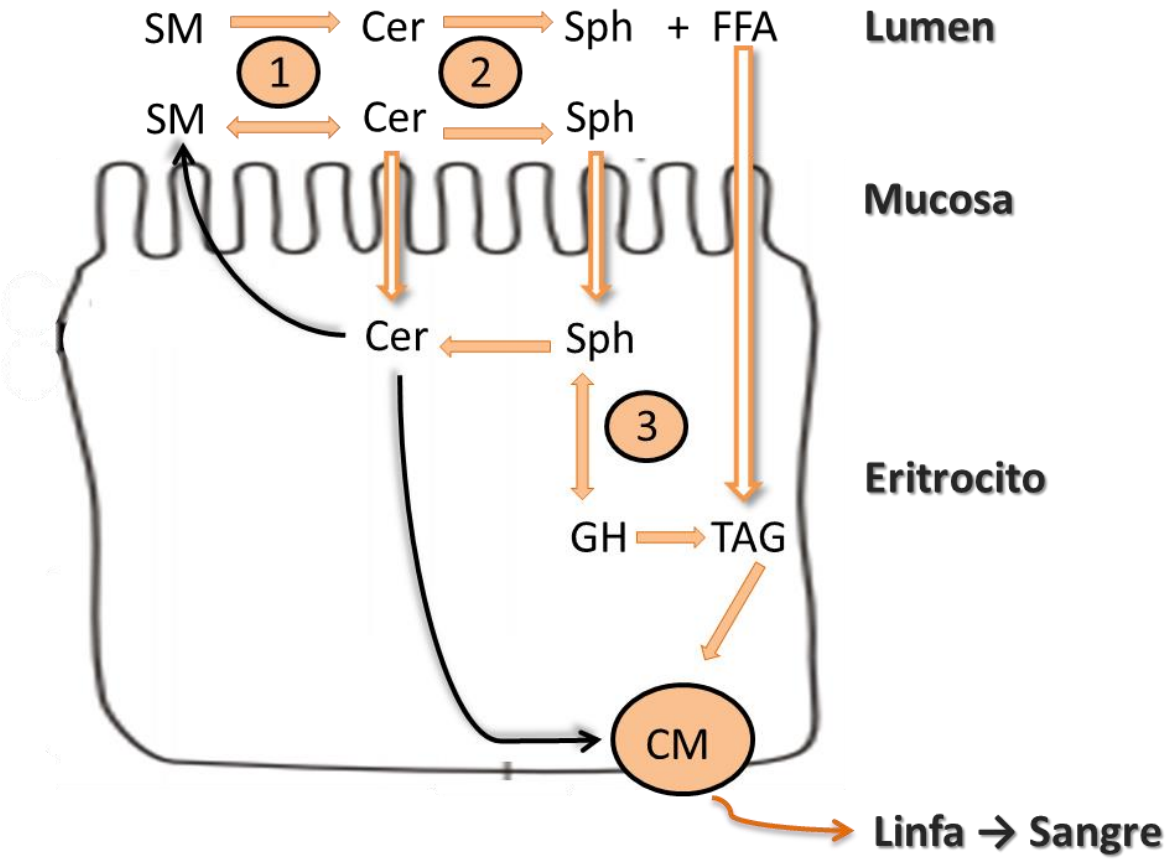
Aunque los PL de la dieta no se ven afectados por la acción de las lipasas lingual y gástrica, en el lumen intestinal la fosfolipasa pancreática actúa sobre la posición *sn*-2 del PL liberando de esta manera el FA y la correspondiente lisoforma. Dichos compuestos pueden pasar al interior del enterocito donde el FA es re-esterificado en la lisoforma para formar un nuevo PL que bien puede incorporarse a la parte externa del quilomicrón (CM), o bien se esterifica a un glicerolípido para formar TAG que se localizarán en el núcleo del CM (Nilsson y Duan, 2006; Cohn *et al.*, 2010). Una vez que el CM se ha formado, es liberado a la linfa para rápidamente incorporarse al torrente sanguíneo donde permanece hasta 5 horas después de la ingestión. El porcentaje de absorción de los PL se estima en más de un 90% (Zierenberg y Grundy, 1982; Agren *et al.*, 2006). Sin embargo, no todos los PL son hidrolizados, y parte de ellos se incorporan a las lipoproteínas de alta densidad (HDL), alcanzan el hígado, y es aquí donde los PL, al igual que los TAG, son hidrolizados y re-esterificados en forma de nuevos TAG o PL. Posteriormente pasan a formar parte de componentes endógenos como las VLDL que pasarán a plasma para ser hidrolizadas y absorbidas por las células de igual manera que los CM (Tall *et al.*, 1983). Resulta interesante destacar que un déficit de fosfolipasa no parece limitar la absorción de PL debido a la presencia de mecanismos compensatorios (Richmond *et al.*, 2001). Por otro lado, es conocido que el 20% de PL son absorbidos pasivamente en el lumen intestinal por los enterocitos debido a su pequeño tamaño, para ser incorporados a los CM y pasar al torrente sanguíneo (Zierenberg y Grundy, 1982). Tras ser hidrolizados, los FA de los CM, pasarán a formar parte de los PL de las membranas celulares de los diferentes tejidos alterando su composición y en consecuencia su funcionalidad y fluidez (Taylor *et al.*, 2010).

La absorción de la SM se produce por la acción de la esfingomielasa alcalina y de la ceramidasa, presentes en las mucosas intestinales antes de su entrada al enterocito. La primera enzima hidroliza la SM hasta ceramida pudiendo bien incorporarse al enterocito o bien ser hidrolizada de nuevo por la ceramidasa hasta esfingosina y el FA, que seguidamente pasarán al enterocito. Una vez en el enterocito, la ceramida pasa a formar parte del CM o de la membrana celular

y se reestructura en forma de SM. Sin embargo, la esfingosina libre, o bien es reestructurada a SM o es excretada casi enteramente en las heces. La acción de la esfingosina 1 fosfato le permitiría, al igual que al FA liberado, formar parte de un TAG e incorporarse al núcleo del CM. (Duan y Nilsson, 2009). Ver el esquema de absorción de la SM representado en la Figura 4.

La absorción de los PL no es sólo importante por la transferencia de nuevos FA a la membrana celular, sino que los PL biliares, así como los hidrolizados de la dieta, son de vital importancia para la solubilización y absorción de nutrientes (Iqbal y Hussain, 2009; Cohn *et al.*, 2010).

Figura 4. Esquema de la absorción de la esfingomielina por el enterocito antes de pasar a sangre



SM: esfingomielina; Cer: ceramida; Sph: esfingosina; FFA: ácidos grasos libres; TAG: triacilglicerido; CM: quilimicron; GH: grupo hidrocarbonado. Los número redondeados son las enzimas que se encuentran implicadas. 1: esfingomielasa alcalina; 2:ceramidasa; 3: esfingosina 1 fosfato

1.5. Efectos beneficiosos sobre:

En los últimos años, son numerosos los estudios que han dirigido sus investigaciones a la determinación tanto *in vitro* como *in vivo* de los efectos de los PL de la dieta sobre la salud. Los efectos atribuidos a estos PL han sido evaluados de manera exhaustiva en artículos y en numerosos reviews publicados en prestigiosas revistas (Modrak *et al.*, 2006; Dewettinck *et al.*, 2008; Cohn *et al.*, 2010; Vanderghem *et al.*, 2010; El-Loly, 2011; Kuchta *et al.*, 2012; Kullenberg *et al.*, 2012; Contarini y Povolo, 2013).

Aunque son muchas las enfermedades en las que se han descrito potenciales efectos beneficiosos relacionados con PL, dada su especial incidencia, dichos estudios se han centrado principalmente en algunos tipos de cáncer y en enfermedades neurodegenerativas.

1.5.1. Cáncer

Dentro de los distintos tipos de cáncer, el de colon quizá sea al que se han dirigido la mayoría de los estudios relacionando el empleo de PL en el tratamiento de la enfermedad. Así, Dial *et al.* (2006) observaron que al tratar células de cáncer de colon SW-480, con PC aislada de la soja en combinación con un fármaco antinflamatorio no esteroideo, se inhibía potencialmente la síntesis de 2-dimetilacetales (DMA) y en consecuencia se veía reducido el crecimiento celular. Cabe señalar que los DMA C18:0 son representativos de los plasmalógenos, un tipo de fosfolípidos-éter abundantes en las membranas celulares (Braverman y Moser, 2012). Fukunaga et al. (2008), tras la administración de PL de origen marino, ricos en LCPUFA n-3, detectaron actividad antiproliferativa en líneas celulares de cáncer de colon Caco-2, así como un incremento de la apoptosis en células tumorales en ensayos con ratas. El efecto de PL de origen marino, como los presentes en el KO, podría ser atribuido no tanto a los PL como a la actividad de los LCPUFA n-3 de cadena muy larga.

Sin embargo, Hossain *et al.* (2009) demostraron que el efecto de estos LCPUFA n-3 esenciales cuando estaban unidos a las moléculas de PL era

significativamente mayor que cuando formaban parte de TAG, puesto que de esta forma la biodisponibilidad del FA por parte de los tejidos, sobre todo en cerebro, era mucho mayor (Wijendran *et al.*, 2002; Di Marzo *et al.*, 2010). Otro estudio en ratas Fischer-344 con criptas aberrantes (cáncer de colon) llevado a cabo por Snow *et al.* (2010) demostró que el uso de MFGM (rica en PL y proteínas bioactivas) reducía significativamente la incidencia de dicho cáncer, sugiriendo de esta manera que la MFGM podría ser un potencial nutracéutico o ingrediente bioactivo (Visioli y Strata, 2014). Sin embargo, como establece un reciente estudio (Zanabria *et al.*, 2014), cuando se han sometido a un calentamiento excesivo, las MFGM pueden verse afectadas disminuyendo o anulando su bioactividad.

La SM es uno de los compuestos más estudiados en relación consu potencial efecto sobre células cancerígenas, dada su actividad como mensajero en el control del crecimiento, diferenciación y apoptosis en líneas celulares humanas. Por esa razón, son varios los estudios que se han centrado en estos compuestos y han demostrado que podría llegar a convertirse en un posible tratamiento para el cáncer de colon (Kuchta *et al.*, 2012). Concretamente se ha señalado que la SM aislada de grasa láctea reduce el número de focos de criptas de foci, exhibiendo un efecto preventivo del cáncer de colon inducido con 1, 2-dimetildracina en ratones ICR y ratones CF1 (Dillehay *et al.*, 1994; Zhang *et al.*, 2008). Asímismo se ha demostrado que cuando se utiliza un aislado de SM láctea como tratamiento, se produce la transformación de adenocarcinomas en adenomas más benignos (Schmelz *et al.*, 1996). Por otro lado, Lemonnier *et al.* (2003) en un estudio con ratones CF1 alimentados con SM, antes y después del comienzo del tumor, detectaron una reducción del desarrollo tumoral, otorgando de esta manera un efecto tanto quimioprotector como quimioterapéutico a la SM. Los estudios en modelos murinos llevados a cabo por Berra *et al.* (2002), Schmelz *et al.* (2004) y Zhang *et al. (2008)*, también demostraron el drástico efecto protector que ejerce la SM de la dieta sobre la formación de cáncer de colon. Un posible mecanismo de acción de los esfingolípidos en la supresión de la carcinogénesis colónica es que la presencia de metabolitos tales como esfingosina, esfingosina-fosfato y ceramida parece inducir la apoptosis celular.

En cuanto a las células cancerosas de próstata o de mama, se ha observado que sus membranas son especialmente ricas en rafts de CHOL y que el control de la densidad de estos rafts modifica la funcionalidad de la célula cancerosa así como su comportamiento frente a la metástasis (Li *et al.*, 2006). Por otro lado, en un reciente estudio Abd El Baky *et al.* (2014) detectaron actividad anticancerígena *in vitro*, en las células de cáncer de mama MCF-7, después del tratamiento con PL extraídos de diferentes microalgas marinas. Doria *et al.* (2013), tras realizar un análisis lipidómico de los PL en células de cáncer de mama (MCF10A, T47-D y MDA-MB-231), observaron cambios en la composición de los FA durante el desarrollo de la enfermedad. Esto sugiere la posibilidad de utilizar los FA de PL como biomarcadores de la enfermedad, así como para el desarrollo de una terapia para ciertos tipos de cáncer. Del mismo modo, Patel *et al.* (2014), aunque no evaluaron los efectos de la administración de PL sobre células de cáncer de próstata, observan que éstos juegan un papel importante en el desarrollo de la enfermedad, detectándose 3 tipos de PC cuya presencia en plasma actuaría como biomarcador de la enfermedad.

El cáncer de hígado es otro de los cánceres sobre el que los PL parecen ejercer un efecto beneficioso. En experimentos *in vitro* se ha observado una inhibición del crecimiento de las células de cáncer hepático humano Hep-3B, Hep-G2, HuH-7 y Alexander, cuando son cultivadas en presencia de PC de soja y de yema de huevo, siendo dicho efecto potenciado por la presencia de menaquinona 4 (vitamina K2). Estos resultados fueron confirmados por experimentos *in vivo* en ratas Sprangue-Dawley, observándose que el tratamiento intragástrico con PC reducía los tumores hepáticos inducidos (Sakakima *et al.*, 2007; Sakakima *et al.*, 2009). Adb El Baky *et al.* (2014) también detectaron actividad IC_{50} *in vitro* en la célula cancerígena hepática humana Hep G2 tras la aplicación de PL aislados de diferentes microalgas marinas.

El crecimiento de células tumorales pancreáticas también se ve afectado por la presencia de esfingolípidos. La línea celular AsPc1 redujo su crecimiento en presencia de SM de yema de huevo debido a que este PL potencia el efecto de la sustancia quimioterápica 5-fluorouracil (Modrak *et al.*, 2004). En general, se ha demostrado que el uso de los esfingolípidos y especialmente las ceramidas en ensayos clínicos, favorecen la reducción del crecimiento tumoral al ejercer

un efecto complementario al tratamiento con quimioterapia (Modrak *et al.*, 2000; Modrak *et al.*, 2002). Estos compuestos aparecen de forma natural en plasma cuando se llevan a cabo tratamientos de radio- y quimioterapia potenciando su efecto y además se comportan como mensajeros secundarios críticos en la apoptosis.

Del mismo modo, se ha evaluado el uso de ceramidas sintéticas sobre células de cáncer de ovario SKOV3 resistentes a múltiples fármacos. Van Vlerken *et al.* (2007) observaron que el uso de ceramidas junto con el quimioterápico Paclitaxel conseguía restaurar la señalización de la apoptosis en estas células cancerígenas .

También se ha descrito que en ratas con cáncer gástrico, PC, PE, SM y Ly-PC reducirían la adhesión de células tumorales, impidiendo de esta manera su migración a otros tejidos y mejorando la supervivencia (Jansen *et al.*, 2004).

Las lisoformas como la Liso-PC, han mostrado actividad anticancerígena al disminuir la metástasis. Esto se debe a que la actividad de la fosfolipasa pancreática se ve aumentada en presencia de células tumorales muy agresivas. En estas condiciones, la lisoforma hace que la célula cancerígena pierda las propiedades de adhesión a otras células y plaquetas. Se ha demostrado que el pretratamiento *in vivo* con liso-PC en ratones y ratas (VCAM-1 Fc, CD49d y P-selectin Fc) con células de melanoma, impidió la posterior metástasis al pulmón (Jantscheff *et al.*, 2011).

1.5.2. Desarrollo cognitivo y enfermedades neurodegenerativas

Tal y como se ha comentado anteriormente, los efectos de los PL de la dieta sobre la actividad y el desarrollo cognitivo han tenido un gran impacto, debido a sus potenciales beneficios en una sociedad como la actual, con elevadas cifras de población envejecida. Durante el envejecimiento se puede observar una disminución constante del nivel de DHA en el sistema nervioso, el cual, se halla directamente relacionado con la demencia senil o enfermedad de Alzheimer (Jicha y Markesbery, 2010). En un estudio reciente, Konagai *et al.* (2013) evaluaron el efecto de la ingesta de LCPUFA n-3 sobre la salud cognitiva de 45 ancianos sanos (61-72 años) durante 12 semanas. Dichos autores descubrieron

un efecto positivo sobre la salud cognitiva, que fue mayor cuando los LCPUFA n-3 procedían de KO que cuando provenían de aceite de sardina. La diferencia radica en el hecho de que en el KO la mayor parte de los LCPUFA n-3 se localiza en los PL (sobre todo PC) en vez de en los TAG, como ocurre en el caso de los aceites de pescado. Lagarde *et al.* (2001) obtuvieron resultados similares, al observar un incremento de DHA en cerebro de rata cuando éste era vehiculizado por PC. Nagata *et al.* (2011), detectaron una mejoría en el aprendizaje y en habilidades de la memoria de ratas, así como una disminución del deterioro cognitivo leve (DCL) en humanos, cuando se les administraba PC. Igualmente, en un estudio clínico se observó una mejoría de la memoria en estudiantes de colegio tras suplementar su dieta con PC (Ladd *et al.*, 1993). Estos resultados sugieren, que los PL, especialmente aquellos que contienen LCPUFA n-3, podrían ser considerados como compuestos potenciales para la mejora del desarrollo cognitivo y prevención de DCL.

Por otro lado, la PC ha sido ampliamente estudiada dado el importante papel que ejerce en el desarrollo del cerebro (Zeisel, 2004), aumentando de hecho sus requerimientos durante el embarazo y la lactancia. Se ha demostrado que una dieta suplementada en colina desde la infancia puede ocasionar cambios en la función cerebral a largo plazo que conducen a la mejora de procesos cognitivos en la edad adulta (Meck y Williams, 1999). Sin embargo, la PC no sólo se ha utilizado en el tratamiento de enfermedades cognitivas como Alzheimer, o en el desarrollo cerebral temprano sino que también se ha observado que la ingesta de PL de soja (25% PC) mejora el movimiento de personas que padecen Parkinson (Tweedy y Garcia, 1982). Del mismo modo, otros estudios corroboran que la PC también actúa como antioxidante de LCPUFA n-3 en las membranas de las células cerebrales de ratas a las que se les administra alcohol para provocarles estrés oxidativo (Jayaraman *et al.*, 2008).

La PS parece ser uno de los PL con mayor importancia en la actividad cognitiva. Este PL era obtenido fundamentalmente a partir del córtex bovino, sin embargo en la actualidad dicho procedimiento no está permitido legalmente debido a la incidencia de encefalopatía espongiforme bovina. Algunos autores han conseguido obtener PS a partir de la modificación de la PC al sustituir enzimáticamente el grupo colina por serina (Kullenberg *et al.*, 2012). Se ha

descrito que la PS permite mejorar alteraciones relacionadas con la edad en modelos animales, por lo que podría también tener un impacto positivo en humanos en aspectos como la memoria, el lenguaje, el aprendizaje y la concentración (Pepeu *et al.*, 1996; Pepeu, 2003). El trabajo de Mi *et al.* (2013), demuestra que el empleo del nutraceútico Fortasyn Connect (Souvenaid), cuya composición consiste en un complejo de EPA y DHA, fosfolípidos, colina, UMP, vitaminas E, C, B12, B6, B9 y Se, reduce los síntomas de Alzheimer en ratones adultos, mejorando la respuesta de la región M1 del receptor de acetilcolina. Además, se ha observado una mejoría de la memoria en pacientes con Alzheimer en grado moderado, lo que podría ser explicado por el papel clave que juegan los PL y en particular la PS, en la función y en la homeostasis de las membranas celulares neuronales. Igualmente se ha descrito que pacientes con pérdida de memoria mejoran significativamente tras la suplementación con PS (Crook *et al.*, 1991).

Por otra parte, además de las funciones de mejora del desarrollo cognitivo, a la PS se le han atribuido efectos positivos sobre el control del estrés en personas jóvenes (Benton *et al.*, 2001). Otro estudio relacionado con el estrés fue el realizado por Hellhammer *et al.* (2010) en adultos sanos de mediana edad a los que durante 3 semanas se les administraron, 13.5 gramos/día de PL, especialmente PS y SM, aislados de un concentrado de PL leche (12% PL). Respecto al grupo tratado con el placebo, estos individuos mostraron tiempos de reacción más cortos en las tareas de memoria, una mejor función cognitiva, mejor humor y una mayor capacidad para manejar situaciones de estrés agudo o crónico. En animales sometidos a tratamiento con PS y PC, McDaniel *et al.* (2003), encontraron que la PS disminuía muchas de las alteraciones neurológicas relacionadas con el envejecimiento, además de restaurar la memoria en diferentes tareas. Cuando estos mismos estudios fueron realizados en humanos, se logró una leve mejora de la memoria en pacientes con DCL, sin embargo, en aquellos que padecían enfermedades degenerativas graves no se detectó mejoría alguna. El mecanismo de acción de algunos de estos compuestos parece relacionarse con la potenciación de la circulación sanguínea y del metabolismo cerebral de adultos que sufren problemas de memoria asociados con una deficiente vascularización del sistema nervioso. Además, en trabajos realizados en pacientes con enfermedad de Alzheimer,

estos compuestos muestran mejoras generalizadas, pero rara vez ventajas significativas (Uauy y Dangour, 2006).

Respecto a los estudios que analizan el efecto de los PL procedentes del consumo de productos lácteos, Crichton *et al.* (2012) concluyeron que en edades adultas un vaso de leche al día tiene un impacto positivo al mejorar significativamente el rendimiento del funcionamiento cognitivo en pruebas de funciones ejecutivas y de evaluación de memoria. Recientemente, Park y Fulgoni (2013) observaron una mejora de la función cognitiva global en adultos y ancianos que consumían productos lácteos frente a aquellos que no lo hacían. Por otro lado, se ha comprobado que la suplementación de fórmulas infantiles para lactantes con MFGM conduce a una mejora de las funciones cognitivas del niño (Timby *et al.*, 2014), así como que la ingesta de una leche suplementada con SM láctea promueve un mejor desarrollo neuro-comportamental en lactantes de bajo peso (Tanaka *et al.*, 2013).

1.5.3. Enfermedades hepáticas

En un estudio realizado con babuinos, Lieber *et al.* (1990) comprobaron que al administrarles simultaneamente un concentrado de PL de soja y una cantidad de alcohol (que suponía el 50% de la energía diaria), el incremento del contenido de lípidos en plasma que se producía era similar al del grupo control. Sin embargo, detectaron diferencias significativas en cuanto al grado de desarrollo de cirrosis respecto a los animales no tratados, concluyéndose que los PL de soja eran capaces de prevenir el daño hepático causado por fibrosis. En un trabajo similar (Navder *et al.*, 1997) con ratas que consumían alcohol y a las que se les suplementó con PC enriquecidas con LCPUFA, se concluyó que la administración de este PL prevenía la discapacidad respiratoria mitocondrial y atenuaba significativamente el incremento de TAG y ésteres de CHOL (CE), mientras que en los animales control no hubo efectos. Cuando este experimento fue llevado a cabo en humanos, se produjo una disminución de las enfermedades hepáticas en consumidores de alcohol tratados con PL. Estos resultados permiten concluir que la PC es un potencial agente protector frente a la enfermedad hepática inducida por alcohol, mientras que la PE, los ácidos

grasos libre (FFA) y la colina, no tuvieron efectos significativos. Gundermann *et al.* (2011), obtuvieron resultados similares sobre la capacidad preventiva, antifibrogénica y mejoradora del tratamiento tras el consumo de un concentrado de PC de soja. Se ha descrito que la PC evita la acumulación de TAG y CE que se liberan en el hígado con el consumo de alcohol, disminuyendo de esa manera el daño hepático. Los estudios realizados por Mak *et al.* (2003), Xu *et al.* (2005) y Cohn *et al.* (2008) corroboraron que se producía una disminución de la oxidación celular tanto *in vitro* como *in vivo* cuando se utilizaba PC en el tratamiento. Buang *et al.* (2005) sugirieron que para que estos efectos hepáticos se produzcan, es necesaria la presencia de la molécula de PL, independientemente de los FA presentes.

Son también varios los estudios que demuestran la disminución de los síntomas en enfermedad hepática no desarrollada por el consumo de alcohol, así como la mejora de su funcionalidad por el efecto de la PC. Así, Demirbilek *et al.* (2006), observaron efectos beneficiosos de la PC en casos de daño hepático provocado por isquemia debido a la actividad antioxidante y antiinflamatoria de este PL.

Las enfermedades hepáticas causadas por virus, como la hepatitis C, también se ven mejoradas por la incorporación de PL en la dieta y en consecuencia se aprecia una recuperación de la actividad enzimática (Singal *et al.*, 2011) así como una mejora en pacientes con fibrosis (Lieber CS, 2005). Sin embargo, la ingesta de PC no resultó eficaz contra la hepatitis tipo B cuando se administró sinérgicamente con la terapia estándar (Niederau *et al.*, 1998). El efecto de la PC sobre los dos tipos de hepatitis fue diferente probablemente debido a que el daño provocado a las células hepáticas varía según el tipo virus.

Cabe destacar que los efectos sobre enfermedades hepáticas, fueron especialmente beneficiosos cuando la fuente de PL era de origen lácteo. Esto hace pensar que podría producirse una acción sinérgica combinada de todos los PL (no sólo de la PC), junto a otros compuestos con efecto positivo sobre este tipo de dolencias. Así, en un estudio llevado a cabo en ratas a las que se les administraba una dieta rica en grasa láctea, Wat *et al.(2009)* detectaron una mejora no solo en la hepatomegalia y en la esteatosis hepática, sino también en el perfil lipídico en sangre puesto que se reducía la hiperlipidemia.

1.5.4. Hiperlipidemia y riesgo cardiovascular

Los PL de soja han sido ampliamente estudiados para evaluar su potencial efecto beneficioso sobre la salud humana. Aunque su administración, según algunos autores, no causa ningún efecto a nivel de lípidos séricos (Oosthuizen *et al.*, 1998), otros estudios indican que provoca una mejora generalizada en el perfil lipídico de la sangre, pudiéndose observar una bajada de TAG, del CHOL total y del CHOL LDL, así como un aumento del CHOL HDL en pacientes con hiperlipidemia (Wójcicki *et al.*, 1995; Cohn *et al.*, 2008; Wat *et al.*, 2009), con diabetes (Kirsten *et al.*, 1994; Ristić Medić *et al.*, 2006; Singh *et al.*, 2008), con hipercolesterolemia (Hori *et al.*, 2001) y con enfermedades coronarias (Pandey y Sparks, 2008). Además, también parecen disminuir la agregación plaquetaria, lo cual resulta beneficioso para la prevención de enfermedades como la aterosclerosis. En el caso de pacientes con hipercolesterolemia y obesidad, a los que se les administró un concentrado de PL soja, no sólo se observó una disminución del CHOL total y del CHOL LDL, sino que también disminuyeron los fibrinógenos, los cuales participan en el desarrollo o aparición de enfermedades arterioscleróticas (Eshigina *et al.*, 2007). Por otro lado, este mismo concentrado de PL aumentó la concentración APO A y disminuyó las de APO A2 y de APO E, todos ellos importantes factores relacionados con una mejora de la ateroesclerosis. Estos resultados parecen sugerir que dichos PL son capaces de estimular el transporte inverso en el metabolismo del CHOL (Pandey y Sparks, 2008).

En cuanto a los lípidos lácteos, la administración de una dieta rica en mazada a ratones jóvenes dio como resultado una mejora del perfil metabólico de aquellos lípidos plasmáticos relacionados con enfermedades cardiovasculares (CVD) (Oosting *et al.*, 2012). Del mismo modo, también se observó una bajada del CHOL en plasma de humanos que consumían mazada, posiblemente debido al efecto inhibitorio de la absorción intestinal del CHOL, cuyo mecanismo aún se desconoce (Conway *et al.*, 2013). Estos mismos autores en un estudio reciente sugieren que la suplementación con MFGM de mazada en humanos con tensión normal durante un corto tiempo provoca una mejora de los niveles de CHOL y TAG en plasma (Conway *et al.*, 2014). Por otro lado, ya se había descrito que la SM de origen lácteo parece tener un efecto positivo en

la disminución de los niveles de CHOL (Jiang *et al.*, 2001; Noh y Koo, 2004), aunque en un estudio donde se incorporaba el nutraceútico comercial Lacprodan PL-20, con un contenido 2-3 veces mayor en SM que una dieta control, no mostró diferencias significativas en el nivel de lípidos plasmáticos ni en el de lipoproteínas (Ohlsson *et al.*, 2009).

En cuanto a los PL de origen marino, la administración de un KO a personas con hipertrigliceridemia provocó una mejora en los marcadores de CVD, al producirse una disminución de CHOL total, CHOL LDL, TAG y un aumento de CHOL HDL (Bunea *et al.*, 2004; Berge *et al.*, 2014). Con el fin de dilucidar si estos efectos positivos estaban relacionados con el contenido de PL ricos en LCPUFA n-3, como el EPA y el DHA, presentes en el KO, se administró aceite de pescado con el mismo contenido en estos LCPUFA n-3 pero en TAG. Los resultados demostraron que mientras que el consumo de KO provocaba todos los efectos previamente citados en los niveles de lípidos plasmáticos, la administración de aceite de pescado sólo conseguía disminuir los niveles de TAG. Esto hace pensar que no sólo es necesario el consumo de LCPUFA n-3, sino que su presencia en los PL confiere a dichos FA una mayor biodisponibilidad y por lo tanto una mayor funcionalidad al aceite (Ulven *et al.*, 2011).

Los estudios de revisión de Cohn *et al.* (2010) y Kullenberg *et al.* (2012) describen algunos de los posibles mecanismos metabólicos por los que los PL ejercen efectos tan positivos sobre los lípidos plasmáticos y en consecuencia un potencial beneficio en la CVD o en el síndrome metabólico. No obstante, ambos trabajos concluyen en la necesidad de investigar más profundamente estos mecanismos para poder confirmar y interpretar dichos efectos.

1.5.5. Procesos inflamatorios

Muchos de los estudios que relacionan el consumo de PL con la atenuación de los procesos inflamatorios se han llevado a cabo empleando soja o bien un concentrado de PL de soja. El administración de PL, y en particular de la PC, parece resultar efectiva en la reducción del desarrollo de artritis reumatoide (Erős *et al.*, 2009a; Hartmann *et al.*, 2009), en casos de pleuresía (Erős *et al.*,

2009b) y en procesos de adhesión endotelial, favoreciendo una mejor migración leucocitaria desde el vaso al foco inflamatorio (Erős *et al.*, 2009a). De acuerdo con los estudios de Treede *et al.* (2007) y de Taylor *et al.* (2010), parece ser que la PC ejerce un efecto inhibitorio sobre la producción del factor de necrosis tumoral (TNF) alpha y TNF beta, relacionadas con un proceso inflamatorio que puede desencadenar en una caquexia o estado de extrema desnutrición. Al evitarse la producción de dichas citoquinas mediante la presencia de PC, el proceso inflamatorio se ve ralentizado o detenido y en consecuencia la pérdida de peso es menor y el tratamiento resulta más efectivo. Por otro lado, se ha demostrado que la administración de PC tiene efectos positivos sobre la colitis, ya que restaura a nivel gástrico el contenido de este PL que se pierde durante el padecimiento de dolencias gástricas relacionadas con la inflamación (Ehehalt *et al.*, 2010; Schneider *et al.*, 2010; Stremmel *et al.*, 2010; Karner *et al.*, 2014).

Respecto a los antiinflamatorios no esteroideos, estos ven aumentada significativamente su acción cuando son administrados conjuntamente con PC (Lichtenberger *et al.*, 2009), reduciendo al mismo tiempo los daños gástricos provocados por la administración de dichos fármacos. La PC también regula de forma indirecta la producción del jugo gástrico al aumentar la síntesis de prostaglandinas (PGE), relacionadas con la disminución de la producción de ácidos gástricos (Leyck *et al.*, 1985).

El consumo de PL de origen marino como los del KO, parece modular la inflamación por TNF alpha en ratones con más eficacia que cuando los LCPUFA n-3 se administran mediante aceite de pescado (Vigerust *et al.*, 2013). Existe un mecanismo metabólico que relaciona los LCPUFA n-3 con la inhibición de PGE de serie 2 de carácter pro-inflamatorio (Tou *et al.*, 2007), siendo dicho efecto antiinflamatorio más evidente cuando los n-3 se encuentran incorporados a los PL. También se ha descrito que el consumo de KO reduce la sintomatología asociada a la artritis y al reumatismo (Deutsch, 2007; Ierna *et al.*, 2010). Otra de las sintomatologías que se ven mejoradas con el consumo del KO es el síndrome premenstrual, no apreciándose dicho efecto con el consumo de aceite de pescado (Sampalis *et al.*, 2003) lo que sugiere de nuevo la importancia del PL asociado al LCPUFA n-3. Innis (2007) describe una mejora de los síntomas emocionales asociados al síndrome premenstrual tras

el consumo del KO, probablemente debido al efecto que el PC-DHA ejerce sobre las funciones cerebrales.

Sin embargo, la PC no parece ser el único PL con actividad antinflamatoria ya que las esfingosinas, principal componente de las SM, poseen una gran actividad como señalizadores de la inflamación intestinal (Cowart, 2009), aliviando entre otras dolencias un gran número de tipos de colitis (Duan, 2005). De hecho, existen varios trabajos que relacionan la supresión de colitis o enfermedad de Crohn en ratas o ratones con la inhibición de la esfingosina kinasa (Maines *et al.*, 2008; Maines *et al.*, 2010). Un trabajo reciente (Chatterton *et al.* (2013) atribuye al consumo de lácteos un incremento de la actividad antinflamatoria en el intestino de niños recién nacidos, debido principalmente a los efectos potenciales de las proteínas de la MFGM.

1.5.6. Inmunología

La funcionalidad de los linfocitos parece estar en parte vinculada a la cantidad de PL que se hallan presentes en su membrana. Se ha descrito que con el paso del tiempo, dichos PL se van perdiendo, lo que se ha puesto de manifiesto tras observarse un aumento constante del ratio de CHOL/PL. Así, Maczek et al. (1998), lograron restaurar la funcionalidad inmunológica de los linfocitos en un estudio llevado a cabo en ratones, al conseguir aumentar la cantidad de PL de la membrana de dichas células. Miranda *et al.* (2008), también observaron un aumento en la actividad fagocítica de los macrófagos de ratas diabéticas cuando se les suplementaba con un concentrado de PC de soja. Igualmente, en personas jóvenes, se detectó un aumento en el número de leucocitos y en la actividad fagocítica de los macrófagos cuando se les administraba PC de soja (Jannace *et al.*, 1992). Además, parece comprobado que esta actividad radica en la presencia de PL, ya que cuando se les administraba un aceite con la misma composición de FA pero unidos a TAG, no se producían los mismos beneficios inmunológicos. Estos resultados parecen confirmarse en el trabajo de Robertson *et al.* (2014) donde se demuestra que en ratas alimentadas con KO se produce un incremento del número de linfocitos mayor que en aquellas que ingieren aceite de pescado.

Veereman-Wauters *et al.* (2012) observaron que la administración de una formula enriquecida en MFGM a niños en edad preescolar, además de ser bien tolerada y segura, disminuía el número de episodios febriles cortos y mejoraba la regulación conductual. No existen muchos más trabajos que analicen el posible efecto inmunológico de los productos lácteos, sin embargo, se ha publicado que la presencia de esfingosina, presente en la SM de la MFGM, resulta clave para el transito y funcionamiento normal de los linfocitos (Mandala *et al.*, 2002; Duan y Nilsson, 2009). Además, El Alwani *et al.* (2006) concluyen que los esfingolípidos puede actuar bien como reguladores o bien como activadores del sistema inmune e inflamatorio.

1.5.7. Infecciones

Desde hace más de una década, existen estudios que demuestran que la administración gástrica enteral de PC y PI en ratas, ejerce un efecto protector frente a la translocación de bacterias entéricas y la disminución de la mucosa intestinal (Wang *et al.*, 1994), además de reducir la capacidad infectiva de los virus *in vitro* (Sinibaldi *et al.*, 1992).

Speelmans *et al.* (2004), patentaron la aplicación de la lecitina de soja en la prevención y/o tratamiento de la sepsis bacteriana, mientras que Gundermann *et al.* (2011), observaron que los PL de soja parecen mejorar la respuesta al tratamiento de la hepatitis vírica. Por otro lado, una patente de Touitou (2012) también atribuye a los PL de soja actividad antiviral, antimicrobiana y antifúngica en el tratamiento de desinfección de superficies sanitarias.

Por otro lado, recientemente Abd El Baky *et al.* (2014), han observado que los PL extraídos de diferentes tipos de especies de microalgas marinas, presentaban actividad antibacteriana frente a *Escherichia coli* y *Bacillus subtilis*, y antifúngica frente a los mohos *Aspergillus niger* y *Candida albicans*.

Asimismo, el KO parece inducir una mayor resistencia a virus tanto en plantas como en animales y además previene el desarrollo de infecciones microbianas (Chirkov, 2002). Sin embargo, parece que tales actividades podrían ser atribuidas en mayor medida a la acción de los quitosanos presentes en el aceite y no tanto a los PL. En el estudio de Sprong *et al.* (1998), diseñado para evaluar

el efecto antibacteriano de los PL lácteos, se demostró que el grupo de ratas al que se administraba mazada rica en PL mejoró su capacidad defensiva frente *Listeria monocytogenes*, puesto que los PL prevenían la adhesión de la bacteria a la mucosa intestinal. Otro trabajo del mismo grupo (Sprong *et al.*, 2001) concluyó que las SM y lisoesfingolípidos de grasa láctea eran potentes agentes antibacterianos puesto que mejoraban la resistencia contra patógenos intestinales (*Escherichia coli, Salmonella enteritidis, Campylobacter jejuni, Listeria monocytogenes*) fundamentalmente por la presencia de FA de cadena media C10:0 y C12:0 en los lisoderivados. Por otro lado, Haug *et al.* (2007), observaron que los gangliósidos presentes en la MFGM, pueden actuar como receptores de toxinas provocando su inactivación y previniendo de esta manera el desarrollo de enfermedades digestivas.

1.5.8. Ejercicio físico

No hay demasiados estudios que relacionen la funcionalidad de los PL de la dieta con el ejercicio físico, sin embargo, cabe mencionar la potencial participación de la PS en la mejora del rendimiento físico. Dos ensayos controlados con placebo investigaron el efecto de una dosis diaria de 750 mg de PS procedente de soja durante 10 días. En el primer estudio, no se encontró una mejora frente a la respuesta de cortisol, sensación de dolor, marcadores de daño muscular y la peroxidación posterior al agotamiento en carrera. Sin embargo, tras la suplementación, el tiempo necesario para que se produzca el agotamiento en carrera tiende a incrementarse (Kingsley *et al.*, 2005). Los mismos autores (Kingsley *et al.*, 2006), investigaron las propiedades ergogénicas de la PS, concluyendo que la suplementación provocaba un aumento significativo en el tiempo requerido para alcanzar el agotamiento al 85% de la velocidad máxima de oxígeno (VO_2max). Monteleone *et al.* (1990) detectaron que el consumo de PS antes de un ejercicio en bicicleta provocaba una respuesta directa de dos hormonas, el cortisol y la adrenocorticotropina, cuyos niveles disminuían lo que resulta beneficioso frente el estrés físico. El estudio realizado por Jäger *et al.* (2007), corrobora los beneficios de la suplementación con PS sobre el ejercicio físico. Por un lado, se observó que se

producía un menor deterioro fisiológico inducido por ejercicios muy intensos, y por otro que la presencia del suplemento, aceleraba la recuperación, prevenía el dolor muscular y mejoraba el bienestar del deportista. Como en los trabajos anteriores, tras la suplementación con PS también se observó una variación en la producción de cortisol, lo que desencadena una protección de las membranas musculares frente a las fosfolipasas que aparecen tras un estrés muscular. El mecanismo de acción parece relacionar el consumo de PS con una mayor entrada de glucosa en la célula muscular lo que supone una mejora en la recuperación de glucógeno (Jäger *et al.*, 2007; Starks *et al.*, 2008).

Por otro lado, es sabido que durante el ejercicio, el contenido de colina en plasma disminuye de una manera significativa por lo que podría recuperarse mediante la suplementación de colina y PC. Aunque la mejora del rendimiento deportivo tras la suplementación no está consensuada en todos los estudios que han abordado este tema, parece que cuando esta suplementación es previa al ejercicio, podría prevenir la disminución de los niveles de colina y por tanto, mejorar el rendimiento en ciclistas y corredores de larga distancia (Jäger *et al.*, 2007).

2. Fosfo- y esfingolípidos lácteos

Los fosfo- y esfingolípidos lácteos se encuentran formando parte de una compleja mezcla de diferentes compuestos lipídicos, muchos de los cuales también presentan efectos beneficiosos sobre la salud (Fontecha *et al.*, 2011).

La grasa de la leche es el único alimento que muestra un perfil completo de PL (incluye todos los fosfolípidos y la esfingomielina) y además seguro desde el punto de vista sanitario, aunque su contenido en PL no supera el 1% sobre el total de grasa. Sin embargo, existen subproductos derivados de la producción de la mantequilla que presentan una elevada concentración de MFGM y de PL.

Por otro lado, también se han desarrollado estrategias para la mejora del perfil de estos PL de MFGM, sin embargo la información al respecto es escasa.

Además, dados los efectos beneficiosos asociados a la MFGM, los PL y algunos FA, el uso de metodologías de aislamiento y análisis que permitan determinar la composición de estos, representa un aspecto relevante en la actualidad.

2.1. Grasa láctea

La leche es un alimento complejo formado por una plétora de componentes, que se considera esencial para satisfacer los requerimientos nutricionales en la primera etapa de la vida y que en base al elevado valor biológico y alta densidad de sus nutrientes, tiene un papel fundamental en la dieta equilibrada del adolescente y del adulto.

En la actualidad, las recomendaciones dietéticas reconocen que la leche y los productos lácteos son una excelente fuente de nutrientes esenciales (ej: calcio, potasio, magnesio, zinc, riboflavina, vitamina A, folato, vitamina D y proteínas de elevada calidad nutricional), así como un vehículo ideal de componentes bioactivos que pueden aportar beneficios para la salud humana (Collomb et al., 2006; Hur et al., 2007). No obstante, se insiste en la recomendación de un consumo preferente de productos lácteos desnatados o con reducido contenido en grasa debido a su contenido en SFA y CHOL. Sin embargo, durante los últimos años se han realizado investigaciones que han dado lugar a un número creciente de publicaciones, encaminadas a reconsiderar la significativa actividad biológica de los FA presentes en la leche, en relación con la salud humana (German y Dillard, 2006; Steijns, 2008; Parodi, 2009). En consecuencia, actualmente estamos asistiendo a un proceso de revalorización de la imagen de la grasa láctea, detectándose un creciente interés en todos aquellos aspectos que se refieren a los lípidos lácteos como fuente de ingredientes bioactivos y funcionales cuyo consumo aporta beneficios para el mantenimiento de la salud y la prevención de enfermedades crónicas en humanos (Juárez y Fontecha, 2009; Fontecha, 2010)

La grasa de leche se presenta en forma de glóbulos rodeados de una membrana compuesta por PL y proteínas, en cuyo núcleo están los componentes mayoritarios, principalmente TAG (97-98%) (Tabla 2). Además, la grasa láctea presenta otros lípidos simples (DAG, monoglicéridos (MAG), CE y ceras), lípidos complejos (mayoritariamente fosfolípidos), CHOL, antioxidantes (especialmente tocoferoles), carotenoides y escualeno (Jensen, 2002; Calvo *et al.*, 2014).

Tabla 2. Intervalos de composición en clases lipídicas de la leche de vaca. (Rango mínimo y máximo en % de grasa)

	% mínimo	**% máximo**
Triglicéridos	97.0	98.0
Diglicéridos	0.3	0.6
Monoglicéridos	0.2	0.4
Ácidos grasos libres	0.1	0.4
Fosfo- y esfingolípidos	0.2	1.0
Colesterol	0.3	0.4
Otros	Trazas	

Datos: Calvo et al (2014)

Durante décadas, las investigaciones realizadas en grasa láctea tienen relación principalmente con su contenido en FA, por ser estos los componentes más abundantes de la fracción lipídica. No obstante, son también los que presentan una mayor variabilidad, dado que el perfil de FA puede sufrir importantes variaciones como consecuencia de la dieta del ganado.

De los más de 400 FA diferentes (de 4 a 26 número de átomos de carbono (CN)) de la grasa láctea, sólo un número próximo a 30 se hallan presentes en

una proporción superior al 0,1%, mientras que del resto sólo se encuentran trazas.

La Tabla 3 recoge los principales FA presentes en las leches de vaca, oveja y cabra. Como se ha indicado anteriormente, la grasa láctea contiene un elevado contenido de SFA, del orden del 60-70%, un 20-25% corresponden a FA monoinsaturados (MUFA), con un 20-22% de ácido oleico (C18:1 cis 9) y un 1-4% a FA trans (TFA), y 3-5% de PUFA, en cuya fracción están incluidos el ácido linoleico (C18:2 cis 9, cis 12), el α-linolénico (C18:3 cis 9, cis 12, cis 15) y el ácido linoleico conjugado (CLA). En la leche de las especies más difundidas para consumo existen diferencias notables en cuanto al contenido de algunos FA. Tal es el caso de los ácidos caprílico y cáprico, que influyen en el sabor de productos elaborados, como el queso, y cuya concentración puede ser de 2-3 veces mayor en las leches de oveja y cabra, que en leche de vaca. (Juárez y Fontecha, 2009; Fontecha, 2010; Juárez Iglesias, 2015)

Tabla 3. Composición media de los ácidos grasos mayoritarios en leche de vaca, oveja y cabra (% del total de ácidos grasos)

Ácido graso	Vaca (a)	Oveja (b)	Cabra (c)
C4:0	3,13	3,51	2,18
C6:0	1,94	2,90	2,39
C8:0	1,17	2,64	2,73
C10:0	2,48	7,82	9,97
C12:0	2,99	4,38	4,99
C14:0	10,38	10,43	9,81
C14:1 *cis*-9	1,08	0,28	0,18
C15:0 *iso*	0,29	0,34	0,13
C15:0 *anteiso*	0,50	0,47	0,21
C15:0	1,05	0,99	0,71
C16:0 *iso*	0,22	0,21	0,24
C16:0	28,51	25,93	28,23
C16:1 *cis*-9	1,73	1,03	1,59
C17:0 *iso*	0,55	0,53	0,35
C17:0 *anteiso*	0,52	0,30	0,42
C17:0	0,73	0,63	0,72
C18:0	10,51	9,57	8,88
C18:1 *cis*-9	20,50	18,20	19,29
C18:1 *trans* (total)	4,25	2,90	2,12
C18:2 *cis*-9 *cis*-12	3,13	2,33	3,19
C18:2 otros	1,03	0,88	0,70
C18:3 *cis*-9 *cis*-12 *cis*-15	0,59	0,63	0,42
Ácido linoleico conjugado	1,03	0,74	0,70
Total Saturados	64,97	70,65	71,96
Total Monoinsaturados	27,56	22,41	23,18
Total Poliinsaturados	5,78	4,58	5,01

Fuente: Alonso *et al.* (1999) (c), Jensen (2002) (a), Goudjil *et al.* (2004) (b) y Moate *et al.* (2007) (b)

2.1.1 Lípidos lácteos y salud

Estudios de meta-análisis (Elwood et al., 2010) indican que el consumo de leche y productos lácteos tiene una incidencia positiva en la salud al disminuir el riesgo sobre las CVD y en lo que a la grasa láctea respecta, no existe ninguna evidencia científica clara que demuestre que su consumo moderado tenga incidencia negativa sobre las CVD (Steijns, 2008; O'Keeffe y St-Onge, 2013; Ravnskov, 2014). Además, gracias a las herramientas de investigación disponibles, en las últimas décadas se ha documentado la presencia en grasa láctea de distintos compuestos lipídicos que ejercen una importante actividad biológica. Entre los componentes lipídicos y FA bioactivos presentes cabe destacar el ácido butírico, el CLA, los constituyentes de la MFGM como fosfo- y esfingolípidos, etc. Estos compuestos ofrecen una potencial aplicación comercial en el desarrollo de alimentos funcionales, encaminados a la promoción de la salud humana y/o en la prevención de enfermedades (Juárez y Fontecha, 2009).

Ácidos grasos saturados

Conviene indicar que a pesar del elevado contenido en SFA de la grasa láctea, solo la fracción correspondiente a los ácidos laúrico (C12), mirístico (C14) y palmítico (C16), podría considerarse desfavorable para la salud, si se produce un consumo excesivo (Legrand, 2008). El ácido esteárico (C18) es considerado neutro desde la perspectiva de la salud humana, aunque sin duda es tan efectivo para reducir el CHOL plasmático como el ácido oleico. La exclusiva presencia en grasa láctea de FA de cadena corta (SCFA), butírico (C4), caproico (C6) y de cadena media, caprílico (C8) y cáprico (C10), no ejerce efecto sobre los niveles del CHOL en sangre (Parodi, 2004). El ácido butírico, ha sido descrito como un agente antitumoral por inhibir el crecimiento y la diferenciación de células tumorales de próstata, mama y colon, así como por favorecer su apoptosis en animales de experimentación (Hassig *et al.*, 1997; German, 1999). El ácido butírico parece además actuar de forma sinérgica con otros componentes de la dieta como resveratrol y retinol o fármacos específicos utilizados para el tratamiento de la hipercolesterolemia, por lo que no serían

necesarias concentraciones plasmáticas muy elevadas para proporcionar un efecto beneficioso. Por otro lado, para los ácidos C6, C8 y C10 se han descrito actividades antibacterianas y antivíricas tanto en ensayos *in vitro* como en animales de experimentación (Hilmarsson *et al.*, 2006). Además, la presencia de estos SCFA y media, favorece el punto de fusión más bajo a la grasa láctea, lo que la confiere diferentes propiedades químicas y físicas frente a otras grasas animales saturadas, afectando de manera positiva su digestibilidad y favoreciendo su biodisponibilidad (Rodríguez-Alcalá *et al.*, 2010).

Ácidos grasos monoinsaturados

Dentro de los MUFA, son numerosos los estudios que investigan el ácido oleico y los beneficios que ejerce sobre la salud. A este FA se le han atribuido efectos como modulador de funciones fisiológicas, en cáncer, sobre los niveles de CHOL plasmático o en enfermedades autoinmunes, inflamatorias y CVD (Sales-Campos *et al.*, 2013).

La presencia de TFA en grasa láctea y su relación con la salud cardiovascular es un tema que genera gran controversia. Si bien es cierto que se ha demostrado claramente que los TFA de origen industrial obtenidos a partir de procesos de hidrogenación parcial de aceites vegetales, provocan un aumento del riesgo de enfermedad coronaria y cerebral al originar un incremento en la relación colesterol LDL/HDL y de la lipoproteína A (Mozaffarian *et al.*, 2009; Brouwer *et al.*, 2010), la cuestión sobre si los TFA presentes en la leche reducen tales efectos adversos no está clara (Wang *et al.*, 2012). Actualmente se admite que las consecuencias negativas derivadas de la ingesta de TFA se inician mediante cambios en el perfil de las lipoproteínas séricas, aunque también se pueden ver afectadas la respuesta inflamatoria y la función endotelial. El riesgo asociado al consumo de TFA parece depender de los alimentos que forman la dieta y de su contenido en TFA. Así, aunque los perfiles de TFA de la grasa láctea y de los aceites vegetales parcialmente hidrogenados guardan considerables similitudes, sin embargo difieren en las proporciones en las que se hallan presentes los distintos isómeros *trans* (Calvo *et al.*, 2014). La alimentación del ganado es el factor que más afecta al contenido de TFA en la grasa láctea (entre 2-6 % del total de FA), siendo el

ácido vacénico (VA) (C18:1 *trans* 11), el isómero cuantitativamente más importante constituyendo del 50 al 60 % del total de TFA. En relación con este tema, son numerosos los estudios publicados recientemente que sugieren que el consumo moderado de TFA por la ingesta de productos lácteos, podría no contribuir a aumentar los riesgos cardiovasculares (Chardigny *et al.*, 2008; Bendsen *et al.*, 2011; Gebauer *et al.*, 2011), y que en particular el VA ejercería efectos beneficiosos en el metabolismo de lípidos y dislipemia (Field *et al.*, 2009) y de protección frente a aterosclerosis en modelos animales (Gayet-Boyer *et al.*, 2011). Estas posibles contradicciones podrían ser debidas al doble papel que juega el VA en el metabolismo, ya que es a la vez un TFA y precursor del ácido ruménico (RA, C18:2 *cis* 9, *trans*11), el isómero mayoritario del CLA. Actualmente se reconoce que aproximadamente el 90 % del RA de la leche se produce de forma endógena implicando la actividad de la enzima delta-9-desaturasa a partir del VA (Kay *et al.*, 2004), pero también se ha confirmado esa bioconversión a CLA, metabólicamente activo, en tejidos animales y humanos (Jutzeler van Wijlen y Colombani, 2010).

Ácidos grasos poliinsaturados

A pesar de ser los FA que se presentan en menor concentración en leche, estos compuestos destacan por su potente bioactividad. Algunos PUFA como el C18:2 *cis* 9, *cis* 12 y el C18:3 *cis* 9, *cis* 12, *cis* 15 , no son sólo FA esenciales para la síntesis de PUFA de mayor longitud (≥20 átomos de carbono), sino que promueven beneficios sobre CVD (Fontecha *et al.*, 2011). Por otro lado, dentro del grupo de los isómeros del CLA (0.7-1.2%, Tabla 3), el compuesto más abundante es el RA (75-90% del total del CLA) (Collomb *et al.*, 2006). Son numerosos los efectos positivos atribuidos a este isómero *in vitro* y en animales, destacando entre ellos la actividad anticancerígena, la mejora de la aterosclerosis, la diabetes, la hipertensión, la mineralización y las funciones inmunológicas (Ryder *et al.*, 2001; Gaullier *et al.*, 2004; Brownbill *et al.*, 2005; Song *et al.*, 2005; Soel *et al.*, 2007; Mitchell y McLeod, 2008; Zhao *et al.*, 2009; Rodríguez-Alcalá *et al.*, 2010). Además, otros isómeros que se presentan en menor concentración en la grasa de leche también podrían contribuir a los efectos observados (Pariza *et al.*, 2001).

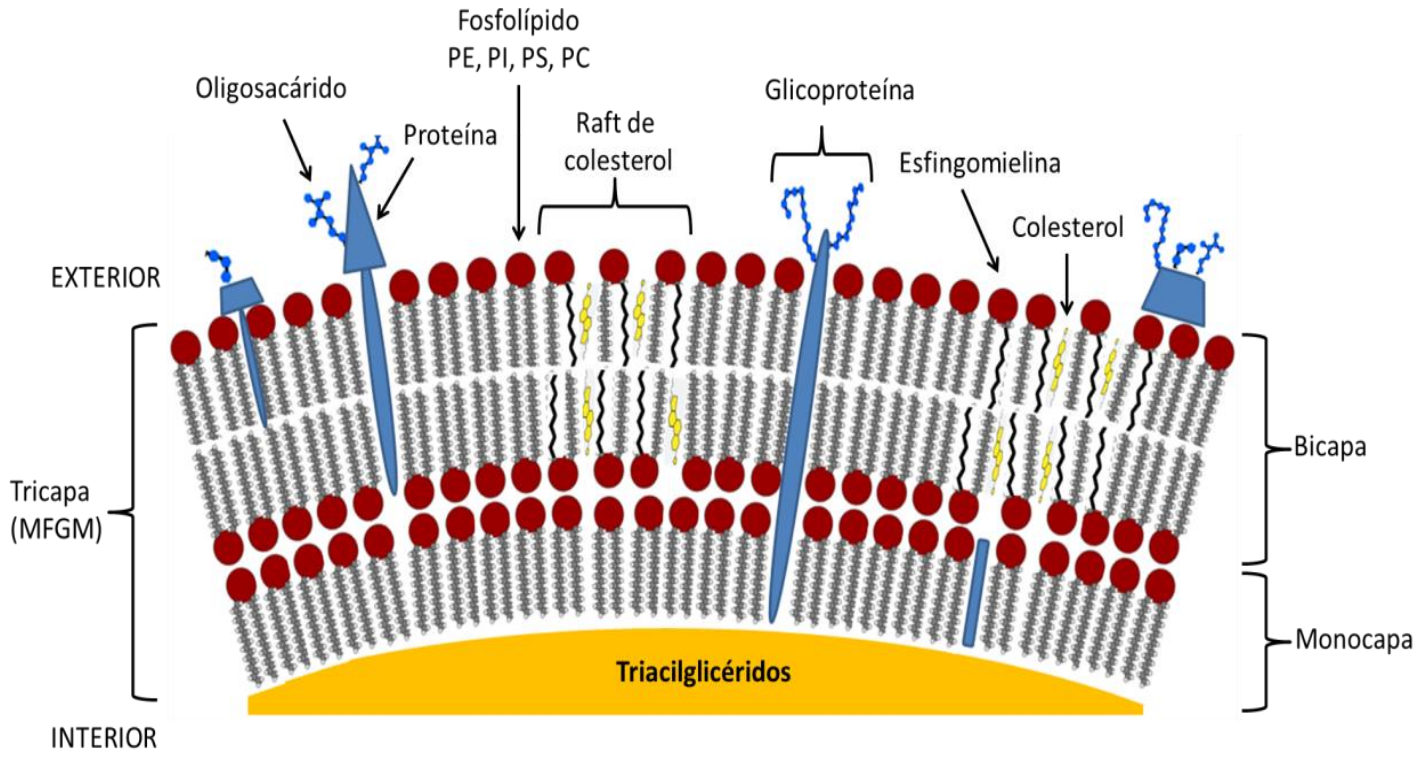
2.2. Membrana del glóbulo graso lácteo (MFGM)

Durante los últimos años, la MFGM ha despertado un creciente interés en comunidad científica debido a sus actividades biológicas potencialmente beneficiosas para la salud humana y que han llevado a considerarla un potencial nutracéutico (Spitsberg, 2005; Hintze *et al.*, 2011).

La MFGM envuelve a los lípidos neutros, mayoritariamente TAG, permiténdoles permanecer en emulsión y protegidos frente a los procesos lipolíticos (Mather y Keenan, 1998; Danthine *et al.*, 2000). La MFGM es una tricapa lipidíca única en la naturaleza donde una monocapa interna, rica en proteínas y adquirida del retículo endoplásmico de la célula mamaria, es a su vez rodeada por la bicapa proveniente de la membrana celular cuando el glóbulo está siendo excretado (Evers *et al.*, 2008). La MFGM está compuesta mayoritariamente por un 70% de proteínas (asociadas a la membrana y enzimas) y un 25% de PL (fosfo- y esfingolípidos) (Keenan y Patton, 1995; El-Loly, 2011). Otros compuestos como CHOL, FFA, glicéridos totales o parciales, glicoproteínas y glicolípidos (cerebrósidos y gangliósidos) están también formando parte de la membrana (Singh, 2006). La Figura 5 muestra de forma esquemática un posible modelo de la estructura de la MFGM.

Al igual que ocurre en las membranas celulares, la distribución de cada uno de los PL en la MFGM es diferente. Mientras que la monocapa (proveniente del retículo endoplásmico) está mayoritariamente formada por PC y PE (van Meer *et al.*, 2008), la parte de la bicapa más cercana a ésta contiene mayoritariamente PE, PI y PS. Sin embargo, el lado más externo es especialmente rico en PC, encontrándose además en ella los rafts de CHOL adquiridos de la membrana de la célula mamaria (Lopez *et al.*, 2011).

Figura 5. Representación esquemática de la estructura de la membrana del glóbulo graso de la leche (MFGM)



PE: fosfatidiletanomlamina, PI: fosfatidilinositol, PS: fosfatidilserina, PC: fosfatidilcolina, SM: esfingomielina.

Dadas las propiedades de la superficie activa de la MFGM, ésta puede desempeñar un papel en el transporte y en la transferencia de nutrientes liposolubles a lo largo del tracto gastrointestinal (Liu *et al.*, 1995).

2.2.1. Composición lipídica de la MFGM

Casi la totalidad de los lípidos que se encuentran en la MFGM son PL, sin embargo en la práctica, cuando la grasa de la MFGM es extraída sólo alcanzan un porcentaje en peso del ~41% sobre el total de lípidos de la MFGM. Esto se debe a que los TAG forman una fina película a lo largo de la capa interna de la MFGM que le hacen alcanzar una concentración del ~56% sobre el total de los

lípidos de la MFGM (Patton y Keenan, 1975; Vanderghem et al., 2010). Otras clases lipídicas como DAG pueden llegar a representar el 2.1%, sin embargo los MAG y los FFA están por debajo del 1% (0.4 y 0.9 % respectivamente) (Fong et al., 2007). La concentración de cada uno de los fosfo- y esfingolípidos de la MFGM puede variar entre estudios debido a factores como el tipo de animal, la alimentación o el método de análisis. Sin embargo, el contenido total de PL de la MFGM representa entre el 0.2% y el 2% p/p de la grasa total de la leche e incluye a la PC (35 %), la PE (30%), la SM (25%), el PI (5%), y la PS (3%) (Rombaut y Dewettinck, 2006; Argov et al., 2008; Rodríguez-Alcalá y Fontecha, 2010; Castro-Gomez et al., 2014) (Tabla 4).

Tabla 4. Distribución de fosfolípidos y esfingomielina (% sobre PL totales) en leche de vaca.

Referencia	**PE**	**PI**	**PS**	**PC**	**SM**
Fagan and Wijesundera (2004)	38.6	-	-	32.2	29.2
Avalli and Contarini (2005)	32.3	9.3	10.5	27.3	20.5
Rombaut *et al.* (2006)	33.2	5.2	9.3	27.4	25.1
Rombaut *et al.* (2007)	46.4	5.3	7.4	21.1	19.8
Fong et al (2007)	32.6	7.6	5.3	33.2	21.3
Fauquant *et al.* (2007)	36.4	7.6	6.5	32.1	17.3
Lopez *et al.* (2008)	26.8	13.6	16.1	22.0	21.6
Sánchez-Juanes *et al.* (2009b)	28.5	14.1		32.7	23.0
Rodríguez-Alcalá and Fontecha (2010)	38.5	6.5	7.7	25.9	21.4
Gallier *et al.* (2010)	26.4	3.4	2.0	42.8	25.5
Le *et al.* (2011)	36.9	6.1	6.3	27.0	23.7
Garcia *et al.* (2012)	33.8	3.9	10.6	30.5	21.2
Castro-Gomez *et al.* (2014)	42.0	3.9	3.4	29.3	21.0

PE: fosfatidiletanomlamina, PI: fosfatidilinositol, PS: fosfatidilserina, PC: fosfatidilcolina, SM: esfingomielina

Entre las actividades biológicas descritas para los PL presentes en la MFGM, destacan su carácter antioxidante (Frede *et al.*, 1990; Saito y Ishihara, 1997), sus propiedades antimicrobianas y antivirales (Van Hooijdonk *et al.*, 2000), así como el efecto protector frente a las úlceras gástricas (Kivinen *et al.*, 1992) y su importante papel como agentes activos frente a patógenos gastrointestinales (Sánchez-Juanes *et al.*, 2009b).

2.2.2. Mazada como fuente de MFGM y lípidos polares

Como se ha indicado, la concentración de PL en la leche alcanza un máximo del ~1% sobre el total de grasa. Sin embargo, hay subproductos lácteos donde la concentración de PL puede llegar a alcanzar entre un 20 y un 30% sobre el total de lípidos (Rombaut *et al.*, 2006; Argov *et al.*, 2008; Rodríguez-Alcalá y Fontecha, 2010)

La mazada o suero de mantequilla, es un subproducto de color amarillo de muy bajo coste en el mercado, obtenido durante el proceso de fabricación de mantequilla. Está constituido por la fase acuosa de la nata, que se libera durante el proceso de batido cuando los granos de mantequilla se están formando. La producción nacional de suero de mantequilla en polvo se estima en 15.000 Tm/año (MAPA, 2009). En los últimos años, la mazada está despertando un creciente interés, más que por sus propiedades tecnológicas, por el alto contenido en PL de elevada actividad biológica (33%-45% de PL sobre la grasa total) presentes en la MFGM. Entre los subproductos lácteos con importante presencia de PL, destaca también el suero procedente de la elaboración de grasa anhidra de leche, denominado aceite de mantequilla, con un ~28% de PL sobre la grasa total. Además, la leche desnatada también alcanza un contenido de PL del ~11% (Rombaut *et al.*, 2005; Rombaut y Dewettinck, 2006; Castro-Gomez *et al.*, 2014).

2.2.3. Efecto de la dieta del rumiante sobre los lípidos polares de la MFGM

Entre las estrategias tecnológicas disponibles actualmente, las más efectivas para aumentar la concentración de UFA en grasa de la leche son: a) el fraccionamiento en seco de la grasa mediante cristalización y posterior separación con membranas; b) los métodos químicos, tales como la interesterificación enzimática (lipidos estructurados) y su posterior fraccionamiento; c) la selección genética del rumiante, con elevada actividad delta-9 desaturasa y d) la suplementación de la dieta del rumiante con aceites de semillas ricos en PUFA.

La incidencia de la dieta del rumiante sobre la disminución del contenido en SFA de la grasa de leche y el aumento en UFA, incluido el CLA y su precursor fisiológico VA, ha sido probablemente el factor más estudiado en estos últimos años. En la mayoría de los estudios realizados se ha observado un aumento del contenido en CLA y VA al emplear dietas reforzadas con fuentes lipídicas ricas en UFA. Respecto a los suplementos de origen vegetal, una comparación entre diferentes tipos de aceites sugiere que aquellos con mayores contenidos de los ácidos linoleico y α-linolénico (como los procedentes de semillas de soja, algodón, girasol, lino, cártamo y colza) resultan ser los más idóneos para incrementar el nivel del CLA en leche bovina (Khanal, 2004). La suplementación con aceites ricos en MUFA y ácidos di-insaturados, ha incidido de forma favorable en el perfil de FA, causando un descenso en los niveles SFA superior al 35% y aumentos de hasta cuatro veces en los niveles de CLA y VA (Gómez-Cortés *et al.*, 2008; Luna *et al.*, 2008). En ocasiones se producen aumentos importantes del ácido C18:1 *trans*10, sobre el que se han descrito posibles efectos negativos en cuanto a su actividad biológica (Anadón *et al.*, 2010). Sin embargo, la utilización de pasto verde o de semillas ricas en ácidos triinsaturados, como la semilla de lino, multiplicó por cuatro los niveles de CLA, VA y omega-3 (ácido α-linolénico), sin modificar sensiblemente los contenidos en otros TFA (Gómez-Cortés *et al.*, 2009). No obstante, la mayoría de los estudios que investigan el efecto de la dieta de los rumiantes en la composición grasa de la leche, se centran en el perfil de FA de la fracción de TAG y no se ha

prestado suficiente atención al posible efecto en la composición de los PL de la MFGM.

En la bibliografía reciente, solo se ha encontrado un estudio de leche de vacas alimentadas con una dieta rica en PUFA que hace referencia a la determinación de la composición de los lípidos de la MFGM (Lopez, *et al.*, 2008). En este trabajo, además de cambios en el perfil de FA con la disminución del contenido en SFA y el aumento de la fracción de UFA, se encontró un incremento de PL en la MFGM y en particular de la SM (> 18%), que se relacionó con un menor tamaño, aunque mayor número, de los glóbulos de grasa de la leche. No obstante, en este trabajo no se describe la composición en FA de los PL ni se concretan las posibles consecuencias nutricionales de estos cambios en la composición lipídica de la MFGM inducida por la dieta.

2.3. Aislamiento de la MFGM y análisis de lípidos polares lácteos

Dados los numerosos efectos biológicos asociados a la MFGM y a los PL, el aislamiento y análisis de éstos representa un aspecto prioritario en la actualidad.

2.3.1. Aislamiento de la MFGM

Los estudios que describen los procesos de aislamiento de fracciones enriquecidas en MFGM son muy escasos y los son mucho más aquellos que describen la composición en PL de las fracciones obtenidas. Corredig *et al.*, (2003) y Rombaut *et al.*,(2006), utilizan el citrato de sodio para provocar la disociación de las micelas de caseína presentes en una disolución de mazada comercial, y posteriormente, mediante procesos de micro- y ultrafiltración (MF y UF) tangencial, obtener una fracción concentrada de MFGM aunque con muy escaso rendimiento. Utilizando el mismo proceso, los resultados parecen mejorar si se aumenta la temperatura durante la filtración de mazada obtenida

a partir de leche fresca en lugar de reconstituida como describen Morin *et al.* (2004; 2007). En investigaciones más recientes del mismo grupo (Spence *et al.*, 2009), se ha desarrollado un procedimiento mejorado en el que utilizan procesos de MF acoplados a la extracción con fluidos supercríticos (SFE) para obtener fracciones enriquecidas en PL, utilizables como nuevos ingredientes de interés para la salud. No obstante, aunque logran enriquecer cinco veces la fracción de fosfo- y esfingolípidos y reducir el contenido de lípidos neutros, el rendimiento obtenido es limitado. Rombaut y Dewettinck (2007), aislaron una fracción enriquecida de PL a partir del suero de queso Emmental basándose en un método de Fauquant *et al.*, (1985). El proceso, denominado "agregación termocálcica", provoca la agregación de membranas mediante la adición de calcio durante un calentamiento moderado, evitando así la colmatación de las membranas de UF. Estos aislados, consisten principalmente en pequeños glóbulos, liposomas y partículas de MFGM que precipitan y pueden ser recuperados mediante centrifugación incrementando al menos 3 veces su contenido en PL. Este método, aunque desarrollado para la clarificación de suero de leche, podría ser de interés para su aplicación en mazadas y en suero de aceite de mantequilla debido a su alto contenido en PL de MFGM.

2.3.2. Aislamiento y fraccionamientos de lípidos polares de la MFGM

Como se ha indicado la composición en PL de la MFGM es muy compleja, lo que hace necesaria la aplicación de un conjunto de sistemas de extracción y técnicas de análisis cromatográficos para realizar su caracterización.

La utilización de una mezcla de cloroformo/metanol (2:1) en proporción 20:1 con la muestra, Folch *et al.*,(1957), es el técnica más efectiva para la extracción de PL de matrices complejas en comparación con otros métodos oficiales o ampliamente utilizados en la extracción de lípidos lácteos como el método de Rose-Gottlieb (ISO, 2001; Avalli y Contarini, 2005). Iverson *et al.*, (2001), ensayaron con éxito una modificación del método de Folch, que permite obtener el mismo rendimiento para muestras de bajo contenido graso (<3%),

reduciendo así la proporción de la misma mezcla de disolventes a 7.5:1 con la muestra. Cequier-Sanchez *et al.*, (2008), evaluaron la sustitución del cloroformo por diclorometano, lo que minimiza la toxicidad de las mezclas de disolventes empleadas sin afectar al proceso de extracción.

Cuando alguno de los compuestos lipídicos quiere ser aislado del resto de la grasa, algunas técnicas como la cromatografía en capa fina (TLC) o los cartuchos de separación en fase sólida (SPE) permiten el aislamiento de éstos con gran pureza (Caboni *et al.*, 1996; Avalli y Contarini, 2005; Sánchez-Juanes *et al.*, 2009a; Gallier *et al.*, 2010). Sin embargo, la cantidad de fracción aislada que se recoge es escasa. Para solventar esto problema, se han desarrollado otros métodos como la cromatografía flash (Castro-Gómez *et al.*, 2015) o la separación con SFE que permiten aislar mayor cantidad de fracción lipídica de interés (Spence *et al.*, 2009; Costa *et al.*, 2010)

2.3.3. Análisis de lípidos polares

Para la determinación de la composición lipídica de compuestos neutros y polares, tanto de muestras grasas completas como de fracciones de estas, existen varias metodologías que permiten su caracterización cualitativa y cuantitativa, así como su identificación molecular.

Rombaut *et al.* (2005) desarrollaron un método de cromatografía de líquidos de alta eficacia (HPLC) acoplado a un detector de dispersión de luz evaporativa (ELSD) en el que separó cada uno de los fosfo- y esfingolípidos de la muestra láctea mientras que los lípidos neutros eluían en un único pico. Trabajos posteriores llevados a cabo en nuestro laboratorio, consiguieron mejorar esta separación de PL, además de separar las clases lipídicas neutras de forma individual (Rodríguez-Alcalá y Fontecha, 2010; Castro-Gomez *et al.*, 2014). Otras técnicas de cromatografía líquida (LC) acoplados a detectores de masas (ionización electrospray (ESI)-MS, desorción/ionización láser asistida por matriz (MALDI)-ToF-MS) (Gallier *et al.*, 2010; Yan *et al.*, 2010; Calvano *et al.*, 2013; Zhou *et al.*, 2014) permitieron la determinación de especies de fosfo- y

esfingolípidos de productos lácteos. Con frecuencia los análisis LC usan columnas de sílica de fase normal o reversa lo que implica el uso de disolventes orgánicos para separar los PL, sin embargo la LC de interacción hidrofílica (HILIC), que usa columnas con fase estacionaria hidrofílica, posee la ventaja de usar disolventes miscibles con agua que podrían facilitar la separación de los PL en la columnas. Por último, son varios los trabajos que describen el empleo de resonancia magnética nuclear (NMR) para determinar las especies de fosfo- y esfongolípidos lácteos, identificando el isótopo marcado ^{31}P NMR de los PL (Murgia *et al.*, 2003; MacKenzie *et al.*, 2009; Garcia *et al.*, 2012)

Por otro lado, el estudio exhaustivo de la grasa y de sus fracciones implica el uso de métodos de derivatización que permitan conocer los FA que los constituyen. Para ello se requiere un conocimiento previo de la composición en clases lipídicas de la muestra a analizar con el fin de utilizar un método de derivatización adecuado o la utilización de un método que asegure la derivatización de todos los componentes. Los métodos de derivatización catalizados por compuestos básicos, como el oficial (ISO, 2002) que usa hidróxido potásico en metanol transesterifican tan sólo los glicerolípidos, incluyendo los fosfolípidos. Sin embargo, los llevados a cabo por ácidos, como ácido cloridrico, trifluoruro de boro o ácido sulfúrico, esterifican además los FFA y transmetilan los esfingolípidos (Carrapiso y García, 2000). Para solventar estas limitaciones, existen métodos de derivatización directa que no necesitan la extracción lipídica de la muestra, y que al combinar el uso de catalizadores básicos y ácidos, permiten conocer el perfil completo de FA de las muestras, tanto biológicas como de alimentos (Castro-Gómez *et al.*, 2014). Además, existen varios estudios que tras un aislamiento previo de los PL de la muestra, han dilucidado la composición total de los FA presentes en los fosfo- y esfingolípidos (Fong *et al.*, 2007; Hauff y Vetter, 2009; Sánchez-Juanes *et al.*, 2009a).

2. OBJETIVOS Y PLAN DE TRABAJO

Actualmente, los productos lácteos constituyen la fuente alimentaria más importante de PL, frente a otras fuentes de origen vegetal como la soja o animal como el huevo, que presentan un bajo contenido de algunos fosfolípidos y de SM. Asímismo, el empleo de cerebro y de médula de los rumiantes, aún siendo interesante por sus elevados contenidos de SM, se halla restringido legalmente para evitar la transmisión de priones.

En los últimos años, la mazada o suero de mantequilla, rica en fosfo- y esfingolípidos de la MFGM, ha despertado gran interés debido a que presenta actividades biológicas potencialmente beneficiosas para la salud humana. La MFGM es una tricapa lipídica que envuelve a la grasa de la leche. Su composición consiste en una mezcla compleja de glicoproteínas, glicerofosfolípidos, esfingolípidos (principalmente SM), glicolípidos (cerebrosidos y gangliosidos), CHOL, enzimas y algunos otros componentes minoritarios. Los fosfo- y esfingolípidos constituyen aproximadamente un tercio del peso de la MFGM.

Debido a su carácter lipofílico e hidrofílico, estos PL, se encuentran profundamente relacionados con el metabolismo celular y son considerados mensajeros secundarios, implicados en la transducción de señales transmembrana y en la regulación del crecimiento, la proliferación, la diferenciación y la apoptosis celular. También juegan un papel importante en la señalización neuronal, la coagulación de la sangre, la inmunidad y las respuestas inflamatorias, además de estar vinculados a enfermedades relacionadas con la edad. Todos estos datos permiten considerar a la MFGM como un potencial nutracéutico o ingrediente bioactivo.
No obstante, debido a la ausencia de estudios concluyentes, la mazada, en la que se concentra una gran parte de la MFGM presente en la grasa de leche, sigue siendo considerada un subproducto y es empleado industrialmente como emulsionante o en la elaboración de cosméticos.

En base a lo expuesto anteriormente, el **objetivo general** de la presente Tesis Doctoral ha sido, el desarrollo de estrategias que permitan mejorar el valor nutricional de la mazada mediante su enriquecimiento en lípidos polares de la MGFM (fuente de fosfo- y esfingolípidos), para su empleo como ingrediente bioactivo.

Para la consecución de dicho objetivo general, fue necesaria la realización de los siguientes **objetivos parciales**:

1. Desarrollo, optimización y validación de métodos analíticos y técnicas cromatográficas para el aislamiento de fracciones ricas en fosfo- y esfingolípidos y su posterior caracterización.

2. Estudio de leche enriquecida en fosfo- y esfingolípidos, procedente de rumiantes con dietas suplementadas con aceites ricos en ácidos grasos poliinsaturados.

3. Estudio de la actividad biológica y bioaccesibilidad de fracciones y extractos lipídicos ricos en fosfo- y esfingolípidos obtenidos de mazada mediante ensayos *in vitro* y estudios en humanos.

En definitiva, esta Tesis pretende aportar conocimientos sobre los fosfo- y esfingolípidos de la fracción de MFGM de mazada para su utilización como ingrediente funcional en el desarrollo de nuevos productos lácteos y fórmulas infantiles, lo que podría incrementar el valor añadido de un subproducto lácteo cuyo empleo se encuentra en la actualidad desaprovechado.

El objetivo final, sería por lo tanto la obtención de productos lácteos que aporten un valor añadido para la salud humana.

3. RESULTADOS Y DISCUSIÓN

• Estado de arte de la investigación en fosfo- y esfingolípidos de la dieta en la salud humana.

• Relevance of dietary glycerophospholipids and sphingolipids to human health (Review).

Elsevier Editorial System(tm) for Prostaglandins, Leukotrienes & Essential Fatty Acids
Manuscript Draft

Manuscript Number:

Title: Relevance of dietary glycerophospholipids and sphingolipids to human health

Article Type: Review Article (REV)

Keywords: phospholipids; sphingolipids; phosphatidylcholine; milk fat globule membranes; human health; atherosclerosis; neurodegeneration

Corresponding Author: Dr. JAVIER FONTECHA, Ph.D.

Corresponding Author's Institution: CIAL (CSIC)

First Author: Pilar Castro-Gómez

Order of Authors: Pilar Castro-Gómez; Alba M García-Serrano; Francesco Visioli, PhD; JAVIER FONTECHA, Ph.D.

Abstract

Glycerophospholipids and sphingolipids participate in a variety of indispensable metabolic, neurological, and intracellular signaling processes. In this didactic paper we review the biological roles of phospholipids and try to unravel the precise nature of their putative healthful activities. We conclude that the biological actions of phospholipids activities potentially be nutraceutically exploited in the adjunct therapy of widely diffused pathologies such as neurodegeneration or the metabolic syndrome. As phospholipids can be recovered from inexpensive sources such as food processing by-products, ad-hoc investigation is warranted.

Keywords: phospholipids; sphingolipids; sphingosine; phosphatidylcholine; milk fat globule membranes; atherosclerosis; neurodegeneration

HIGHLIGHTS

- Phospholipids are indispensable to human metabolism.
- Their consumption is decreasing due to the use of “low fat” products.
- Soy lecithin is an emulsifier and provides mainly phosphatidylcholine.
- Phospholipids can be recovered from inexpensive sources and used as nutraceuticals.

Relevance of dietary glycerophospholipids and sphingolipids to human health

P. Castro-Gómez[a*], A. Garcia-Serrano[a*], F. Visioli[b,c], J. Fontecha [a,*]

[a]Department of Bioactivity and Food Analysis, Group of Lipids, Instituto de Investigación en Ciencias de la Alimentación (CIAL CSIC-UAM), Nicolás Cabrera, 9, Universidad Autónoma de Madrid, 28049, Madrid, Spain;

[b]Department of Molecular Medicine, University of Padova, Italy

[c]IMDEA-Food, CEI UAM + CSIC, Madrid, Spain.

*The first two authors contributed equally to this work.

*Corresponding author. Tel.: 034 91 0017935 fax: 034 91 0017905.

E-mail address: j.fontecha@csic.es (J. Fontecha).

Abstract

Glycerophospholipids and sphingolipids participate in a variety of indispensable metabolic, neurological, and intracellular signaling processes. In this didactic paper we review the biological roles of phospholipids and try to unravel the precise nature of their putative healthful activities. We conclude that the biological actions of phospholipids activities potentially be nutraceutically exploited in the adjunct therapy of widely diffused pathologies such as neurodegeneration or the metabolic syndrome. As phospholipids can be recovered from inexpensive sources such as food processing by-products, ad-hoc investigation is warranted.

Keywords: phospholipids; sphingolipids; sphingosine; phosphatidylcholine; milk fat globule membranes; atherosclerosis; neurodegeneration

1. Introduction

Glycerophospholipids and sphingolipids are polar lipids (PLs) ubiquitous to all tissues because they are essential components of cell membranes. The most important ones among the former are phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC); among the latter, sphingomyelin is the most biologically important one (Figure1). In this didactic paper we review the biological roles of PLs and try to discriminate the exact and precise nature of their putative healthful activities.

The biological importance of PLs derives from their amphiphilic qualities, in that the hydrophilic head and the hydrophobic tail create a bi-layer that makes it possible to build cells' and organelles' membranes. Of note, in addition to their structural roles, cell membranes exert important physiological roles, due to the proteins interjected among PLs and to the roles of PLs themselves as precursors of lipid mediators. Therefore, PLs participate in a variety of indispensable metabolic, neurological, and intracellular signaling processes [1], such as development, necrosis and apoptosis, transport, DNA replication, neuronal signaling, or secretion, to name a few [2]. In addition, cell membranes need other compounds such as protein- or lipid-associated carbohydrates (glycoproteins and glycolipids) and cholesterol (CHOL) to function correctly.

2. Glycerophospholipids and sphingolipids. Biosynthesis, chemical structure, and functions

Glycerophospholipids share a common structure consisting of two fatty acid (FA) molecules esterified in the *sn*-1 y *sn*-2 positions of the glycerol moiety. This portion of the molecule contributes to its hydrophobicity. However, the *sn*-3

position features a phosphate group that contributes hydrophilicity. Four such groups have been identified: ethanolamine, inositol, serine, and choline [3] (Figure 1). Glycerophospholipid biosynthesis starts with the enzyme glycerol-3-phosphate acyltransferase (located in the external leaflet of the mitochondrial membrane and of the endoplasmic reticulum) linking a fatty acid-CoA (often a saturated one) to the *sn*-1 position of glycerol-3-phosphate, to generate lysophosphatidic acid. The subsequent formation of phosphatidic acid (PA) in the endoplasmic reticulum requires an acylglycerol-3-acyltransferase, which esterifies another fatty acid CoA (often an unsaturated one) to the *sn*-2 position of glycerol. PA then becomes the substrate of two important metabolic enzymatic pathways. The first one is governed by a cytosolic phosphatidic acid phosphatase and takes place in the endoplasmic reticulum's membrane and yields diacylglycerols (DAG) by removing the phosphate group from the *sn*-3 position of PA. By esterifying another fatty acid in this position, triacylglycerols (TAG) are formed and become the main energetic substrate in the body.

Another route that originates from PA is the synthesis of PC and PE in the cytosol following the addition of choline or ethanolamine, respectively [4] (Figure 2). These phospholipids exercise important cellular functions. PC is the most abundant phospholipid and is preferentially located in the external leaflet of the cellular membrane, where it is critical to its integrity and function. Systemically, PC plays an essential role in the hepatic secretion of VLDL, therefore governing the distribution of CHOL and lipids to organs and tissues. This phospholipid also contributes to the formation of micelles in the intestinal lumen, via its role in biliary salts, in turn favoring the absorption of lipo-soluble molecules.

The second most abundant PL is PE, which accounts for ~20-30% of total membrane PLs. As opposed to PC, PE is most concentrated (up to 80%) in the internal membrane leaflet and is also plentiful in mitochondria, where it plays crucial roles in growing and stability. Other actions carried out by PE include its being precursor of glycosylphosphatidylinositol and its facilitation of protein binding to the membrane. This last activity can be explained by the small size of PE, which favors membrane stability [3].

The biosynthesis of PS differs between prokaryotic and eukaryotic cells. Whereas the former utilize the cytidine diphosphate diacylglycerol (CDP-DAG) route identical to that of PI (see later), the latter require the presence of PE and PC. As for PE, there are two metabolic pathways for PS synthesis, which use differential substrates and enzymes. PS synthesis takes place in the ER, where PC releases choline in the presence of PS synthase I and acquires serine. The final products are PS and choline. PE follows a similar pathway, by changing its ethanolamine head for serine, mediated by PS synthase II and originating PS and ethanolamine. This last reaction is unique in that it is reversible and, in the presence of the same ER enzyme, PS can release serine and replace it with ethanolamine, in turn re-creating PE and serine (Figure 3).

The cytosolic PS-to-PE interchange is not the only physiologically-relevant one: in mitochondria, a PS decarboxylase releases CO_2 from PS and originates PE. Of note, this PE can move to the ER to be turned into PS again. These reactions are sketched in Figure 3. PS accounts for 5-10% of all membrane PL and is mostly located in the inner leaflet; however PS in the outer leaflet plays crucial roles in cell signaling involved in coagulation and apoptosis, via its stimulation of protein kinase C [5]. Also, the anionic nature of PS head favors its

interaction with positively-charged proteins and facilitates contacts between proteins and receptors [6, 7].

PI is synthesized in the ER from CDP-DAG and inositol, mediated by CDP-DAG inositol phosphatidyl transferase. In short, an inositol molecule binds a CDP-DAG one and produces PI and cytidine monophosphate (CMP). The same pathway is used for the formation of other essential molecules such as cardiolipin [8]. PI accounts for ~10% of total inner leaflet PLs and yet it is indispensable to the membrane's structure and to several signaling pathways. Indeed, phospholipase A-mediated hydrolysis of PI liberates bioactive fatty acids (FAs) such as arachidonic acid. This is very important in inflammation, among other physiopathological processes [9].

When FAs are liberated from glycerophospholipids, the resulting lyso-PLs can carry out diverse biological activities that depend on their molecular structure. As an example, lyso-PE has manifold activities described in animals and in *in vitro* systems; lyso-PI [10] is being suggested as a biomarker in cancer patients; lyso-PS [11] acts in signal transduction of neutrophil activation and, apparently, has anti-inflammatory properties; and lyso-PC [12] might be beneficial is rheumatoid arthritis and plays important roles in DNA methylation in the central nervous system [13]

SM is a sphingolipid unique to animal cells: its plant analogue is ceramide phosphorylinositol [14]. SM's structure consists of a ceramide (FA linked to sphingosine with an amidic link) usually linked to phosphorylcholine in the *sn*-1 position, even though ethanolamine is not infrequent in that position (Figure 1). Its synthesis takes place in the Golgi apparatus and in the plasma membrane, catalized by sphingomyelin synthase I and sphingomyelin synthase II,

respectively. These enzymes produce SM and DAG by using PC and ceramide as substrates. The biosynthetic route takes place in the ER and consists in the conversion of ceramide to ceramide phosphorylethanolamine after the acquisition of this polar head from a methylated PE. These SM are most often found – together with PC – in the external leaflet of the cell membrane and in myelin sheaths, where they maintain stability and chemical resistance. SM also performs regulatory actions because it interacts with specific proteins and operates as receptor for the internalization of transferrin, thus facilitating the cellular uptake of iron. However, SM's main feature is its formation of rafts together with cholesterol, in both cell membranes and lipoproteins. Recent studies suggest that cholesterol and SM metabolisms are integrated; some authors reported that SM is responsible for the distribution of CHOL in the cells [15, 16].

Of note, SM largely features long (> 16 carbon atoms) saturated fatty acids (SFAs), while glycerophospholipids mostly contain medium-chain FAs (C16:0, C18:0, C18:1, C18:2, C20:4, and C20:6); their *sn*-2 position usually contains and unsaturated FAs while the *sn*-1 position often contains SFAs such as C16:0 or C18:0 or longer-chain fatty acids.

3. Phospho- and sphingolipids in food

Currently, the global consumption of PLs is decreasing due to the widespread use of "low fat" products. One notable, though "hidden" contributor is soy lecithin, which is increasingly used as emulsifier and provides noteworthy amounts of PC [17]. Even though there are no solid data on the daily consumption of PL, we can estimate 2 to 7 gr/d as the average intake, which

157 represents 1 to 10% of total fat [17]. Most of these PL come from egg yolk, meat,
158 fish and shellfish, grains, and some oily seeds such as soy. Even though these
159 foods mostly contribute PC, they also contain lyso-PC and SM (animal products)
160 as well as PE and PI (vegetal products) while they are scarce in PI and PS,
161 respectively [17]. Other vegetal products such as fruits, vegetables, or roots are
162 almost devoid of PLs [18]. Soy contains up to 4% of total fat as PLs, of which
163 ~55% is PC, ~26% is PE, and ~18% is PI [19]. Other vegetal products rich in
164 PLs are peanuts, whose percentage of PLs is ~1-2% of total fat, of which ~57%
165 is PC, and ~11% and ~31% are PE and PI, respectively [18].

166 Animal food is the most abundant in PLs. One notable example is egg
167 yolk, which contains ~10% in weight of PLs, of which PC is the foremost (~60 –
168 70%), followed by PE, SM, and lyso-PC (15-26%, 2.5-5%, and 6-8%,
169 respectively [20]). Among meats, beef contains ~1% of PLs, distributed among
170 PE, PC, and SM (31, 62, and 7 %, respectively), while it is devoid of PI and PS.
171 However, chicken breast contains ~4% of PL, of which 14% is PS [17, 18]). The
172 most abundant source of PLs is, however, offal such as bovine and porcine liver
173 and brain. Nevertheless, their dietary contribution to PLs intake is quantitatively
174 not very relevant.

175 Marine products such as squid and krill are also rich in PLs, which can
176 account for up to 50% of total fat. Their profile shows PC as the most abundant
177 PL (~87%), followed by PE (~6%), SM (~3.5%), and PI (~2%) [21, 22]. It is
178 noteworthy that eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are
179 very abundant in marine PLs; one notable example is that of krill oil, whose
180 bioavailability is being suggested to be higher than that of fish oil precisely
181 because of its relative higher proportion of PL [23].

Milk and dairy products contain around 0.3 – 1% of total fat as PLs. However, some milk by products such as buttermilk and, especially, its MFGM fraction contain up to 40% (in weight) of PL, of which 30% is PE, 7% is PI, 5% is PS, 31% is PC, and 20% is SM [24, 25].

4. Absorption of dietary phospho- and sphingolipids

Dietary PLs are not acted upon by lingual and gastric lipases, but the pancreatic one – in the intestinal lumen – does cleave FAs from their *sn*-2 position. The resulting lyso-PLs and FFAs enter the enterocyte, where FAs are re-esterified to form new PLs, which are then either incorporated into the outer layer of chylomicrons (CM) or are esterified into a glycerolipid to form TAG which migrates into CM nuclei [17, 26]. The newly-formed CM enter the lymph and the circulation, where they can be found approximately five hours after meals. The estimated percent absorption of PLs is >90% [27, 28]. Yet, not all PLs are hydrolyzed and part of them incorporate into HDL; in the liver, PLs and TAG are hydrolyzed and re-esterified to form new PLs and TAG which then are delivered to tissues as VLDL [29]. Interestingly, a deficit of phospholipase appears not limit PL absorption because of compensatory mechanisms [30]. Also, PLs (especially PC) can be excreted in the bile in quantities that might reach up to 2-4 fold those ingested with the diet. Approximately 20% of PLs are passively absorbed in the intestinal lumen due to their small molecular weight, to be incorporated into CM and enter the circulation [28]. Absorption is due to the actions of alkaline sphingomyelinase and ceramidase in the intestinal mucosa. The first enzyme hydrolyzes SM to ceramide, which can then be further hydrolyzed to sphingosine

206 and FA; all of them then enter the enterocyte. Once internalized, ceramide can
207 become part of CM or of cell membranes, restructuring itself in SM.
208 Following hydrolysis, FAs from CM are incorporated into cell membranes
209 in very selective manners [31, 32], thereby altering their fluidity and function [33].
210 Sphingosine 1 phosphate allows SM to become part of a TAG and enter into the
211 CM nucleus [34]. A schematic absorption route can be seen in Figure 4. In
212 addition to the transfer of FAs to cell membranes from PLs absorption, biliary
213 PLs are essential for the solubilization and absorption of water-insoluble
214 molecules such as CHOL [17, 35].
215

216 **5. Milk fat globule membranes**

217 PLs can also be found as scaffold of non-cellular structures such as
218 vesicles, organelles, and milk fat globules. In the latter case, the goal is to
219 protect fat (mostly TAG) from lipolysis and oxidation, in addition to providing
220 structural support [36, 37]. Milk fat globule membranes (MFGM) are unique lipid
221 trilayers, where an internal protein-rich monolayer that originates from the
222 endoplasmic reticulum (ER) is covered by the bilayer of the mammary cell
223 membrane, which is formed when the globule is secreted with the rest of milk
224 components [38]. Similar to cell membranes, MFGM also contains proteins
225 associated to the membrane, enzymes, CHOL, free fatty acids (FFA),
226 glycoproteins, and glycolipids among which the most prominent ones are
227 oligosaccharides, cerebrosides, and gangliosides [39]. It needs to be
228 underscored that MFGM often exhibits more PE, PI, and PS in the internal layer,
229 whereas PC is more abundant in the external one. The external layer also
230 features SM which, together with CHOL, form dynamic and structured

membrane rafts [40] (Figure 5). The PL composition of cow milk MFGM is shown in Table 1.

As mentioned, in addition to being important structural and functional components of cell membranes, PLs – namely, those derived from the diet - are being actively investigated for their role in human physiology and health [21, 41, 42]. One current major limitation is that dietary PLs are integral part of membranes and, therefore, it is unclear if their observed biological actions are exclusively due to PLs, protein, or a combination of both. This is, indeed, the case of MFGM.

6. Health effects of dietary phospho- and sphingolipids

Much *in vitro* and *in vivo* research is focusing on the health effects of PLs. Even though there is a wide array of diseases whose outcome can be potentially influenced by PLs intake modulation, most research is centered on some cancers and neurological pathologies. The effects of both dietary intakes and administration of pharmaceutical preparations have been extensively reviewed [17, 21, 41, 43-47].

6.1 Effects of PLs on cancer

The majority of “cancer and PLs” research concerns colon cancer. One example is that of Dial et al. [48], who supplemented colon SW-480 with soy PC and nonsteroidal anti-inflammatory drugs, inhibiting the synthesis of 2-dymethilacetales (DMA) and limiting cell proliferation. Of note, C18:0 DMA are representative of plasmalogens, a type of PL abundant in membranes [49].

Another study carried out in Caco-2 colon cancer cells reported anti-proliferative activities of omega 3-rich PLs (of marine origin); *in vivo* studies confirmed these data [50]. Interesting comparative studies attributed these effects to PLs rather than to the same FAs incorporated into TAG Hossain et al. [51], probably because of the higher bioavailability of the former [52, 53]. A study in Fischer-344 rats with aberrant crypts demonstrated that administration of MFGM (rich in PLs and bioactive proteins) significantly reduced the development of colon cancer [54], hinting to the use of MFGM as potential nutraceuticals or medical foods [55]. In this regard, it should be kept in mind that MFGM are susceptible to heat [56] and should be formulated accordingly.

SM is one of the best-studied compounds in chemoprevention, ought to its roles as messenger in the development, growth, differentiation, and apoptosis of human cells. Therefore, several studies suggested its use in adjunct colon cancer treatment [46]. In synthesis, SM isolated from milk fat reduced the number of cryptic foci and prevented 1,2-dimethylhydrazine-induced colon cancer in ICR rats and CF1 mice [57, 58]. Milk-derived SM also transformed adenocarcinomas to more benign adenomas [59] and exhibited chemopreventive and chemotherapic effects in CF1 mice Lemonnier et al. [60]. Similar murine studies reached the same conclusions Berra et al. [61], Schmelz et al. [62] y Zhang et al. [63]. Mechanistically, the chemopreventive effects of SM appear to be due to its principal metabolites, i.e. sphingosin, sphingosin-phosphate, and ceramide, all of which induce apoptosis [64].

Prostate and breast cancer cells are particularly rich in lipid rafts, whose density modifies cellular functionality and the evolution of metastases [65]. Indeed, a recent study by Abd El Baky et al. [66] reported in vitro anti-cancer

activities in breast cancer MCF-7 cells treated with algal PL. Accordingly, Doria et al. [67] carried out a lipidomic study of several breast cancer cell lines (MCF10A, T47-D, and MDA-MB-231) during cancer development. The potential repercussion of these data is the use of PLs' FAs as biomarkers of breast cancer and as potential targets for treatment, pending appropriate investigations. This is in accordance with Patel et al. [68], who reported three types of PC in prostate cancer cells as biomarkers of progression.

Liver cancer is also interesting in terms of PL and health. Of note, the liver readily incorporates omega 3 FAs that inhibit lipogenesis [31]. In vitro studies reported growth inhibition of the human cancer cell lines Hep-3B, Hep-G2, HuH-7, and Alexander following addition of soy and egg yolk PC; these effects are potentiated by menaquinone 4 (vitamin K_2). These data have been confirmed in hepatic cancer-induced Sprague-Dawley rats administered with PC [69, 70]. In vitro, Adb El Baky et al. [66] reported inhibition of HepG2 growth when treated with algal PLs.

Pancreatic cancer cells are also affected by sphingolipids. As an example, AsPc-1 cells growth is retarded when they are supplemented with egg yolk SM, which increases the activity of 5-fluorouracil [71]. In clinical settings, the use of sphingolipids and, especially, of ceramides has been shown to decrease tumor growth when given in combination with chemotherapy [72, 73], possibly because of their relevant role in apoptosis. From a pharmacological viewpoint, synthetic ceramides have been tested in drug-resistant ovary cancer cells (SKOV3). Van Vlerken et al. [74] reported that the concomitant use of these ceramides and paclitaxel restored apoptosis pathways in such cancer cells.

PC, PE, SM, and lyso-PC have also been tested in rat models of gastric cancer, where they reduced adhesion and migration to other tissues, thereby potentially affecting survival [75].

Lysoforms, e.g. lyso-PC have also been shown to possess chemopreventive and anti-metastatic properties. This is due to the observed increased pancreatic phospholipase activity in the presence of aggressive cancer cells, when the lysoform makes it difficult to cancer cells to adhere to other cells such as platelets. In fact, pre-treatment of rats and mice (VCAM-1 Fc, CD49d y P-selectin Fc) with lyso-PC prevents the formation of pulmonary metastases [76].

6.2 Effects of PL on neurological disorders and cognitive impairment

As mentioned, the effects of diet on cognitive development and impairment are heavily researched because of the rampant incidence of age-associated neurological diseases [77]. The concentrations of DHA in the CNS decrease with age and this phenomenon is directly correlated with senile dementia or Alzheimer's disease [78]. A recent study by Konagai et al. [79] evaluated the effects of dietary n-3 FAs on cognitive function in a cohort of 45 aged (61-71 yo) healthy subjects, followed for 12 weeks. The results showed improvement of cognitive function: more so when krill oil was given instead if sardine oil (reinforcing the notion that PL-rich krill oil leads to better incorporation of omega 3 into membranes than TAG-rich fish oils, in agreement with Lagarde et al. [80]). In terms of function, the administration of PC to rats and humans improves learning, memory, and cognition, respectively Nagata et al. [81]. These nootropic effects have also been recorded after the administration of PC to

college students [82]. In summary, PLs – namely those rich in omega 3 fatty acids – might provide useful nutraceutical tools for cognitive development and prevention of dementia. Indeed, PC has been studied for its critical roles in brain development [83] and increased requirements during pregnancy and lactation have been suggested. Fittingly, a choline-supplemented diet started early on might produce long-term improvements in cognitive function [84]. In addition to cognitive decline and Alzheimer's disease, PC (as 25% component of soy PLs) has also been successfully employed in Parkinson's patients [85]. Mechanistically, it has been shown that PC acts as antioxidant in the brains of alcohol-treated rats [86]. Indeed, accumulated evidence points to the antioxidant activities of PUFAs, namely omega 3 fatty acids [87].

In terms of cognitive function, PS appears to be of primary importance. Until recent, this PL was extracted from bovine brain, which is no longer possible due to spongiform encephalopathy concerns. Some authors employ PS enzymatically synthesized from PC (by polar head exchange) [21]. In animals, PS augments some age-related impairments and has been suggested as potential nutraceutical for human use, notably to improve memory, speech, learning, and concentrations [88, 89]. One current nutraceutical application is Souvenaid®, which has been tested in an Alzheimer mice model where it increases the M1 region of the acetylcholine receptor [90]. These data have been confirmed in humans [91]. Potential explanations point to the pivotal role played by PS in the neuronal membrane, which can also explain its protective actions toward memory loss [92].

In addition to its roles in cognitive function, PS is being attributed with salubrious roles in juvenile stress control [93]. One such study has been

355 undertaken by Hellhammer et al. [94], who recruited 46 healthy volunteers
356 (mean age: 41.5 years) and gave them 13.5 g/d of PLs (especially PS and SM)
357 isolated from a milk 12% PL paste, for three weeks. The results were shorter
358 reaction times and better cognitive function, mood, and ability to handle stress in
359 subjects under chronic or acute stress, as compared with placebo. In animals,
360 McDaniel et al. [95] treated rats with PS and PC and reported that PS
361 ameliorates several of the age-related neuronal alterations as well as functional
362 memory.

363 Similar studies performed in humans recorded modest and non-significant
364 improvements of mild cognitive impairment patients and no improvement of
365 severely impaired ones. Among the potential mechanisms of action,
366 amelioration of vasomotion (which is impaired in the elderly because of altered
367 sphingomyelinase and ceramide-activated phosphatase 2A [96] has been
368 suggested as the predominant one. Overall, human studies in AD patients
369 showed modest albeit non-significant improvements [97].

370 Studies focusing of dairy products-derived PLs show that consumption
371 (by adults) of one glass of milk per day does improve cognitive function and
372 memory Crichton et al. [98]. More recently, Park and Fulgoni [99] observed
373 better global cognitive function in adults and aged individuals who consumed
374 milk goods as compared with those who abstained from such products. Also,
375 supplementation of infant formula with MFGM improved cognitive function in
376 infants [100]; similar effects on neurobehavioral development of very low birth
377 weight infants during infancy have been reported after the consumption of SM-
378 fortified milk [101].

379 *6.3 Hepathopathies*

In a study performed in baboons, Lieber et al. [102] observed that PC-rich soy PLs' administration together with alcohol (50% of daily energy) increased plasma lipids to the same extent of alcohol-only treated animals; however, significant effects on cirrhosis development were noted, which lead to the conclusion that soy PLs reduce hepatic fibrosis. Similar experiments have been carried out in rats, where PC prevented mitochondrial uncoupling and significantly reduced the alcohol-induced increase in plasma TAG and cholesterol esters (CE) [103]. Human studies finally confirmed the hepatoprotective properties of soy PC, whereas PE, FFA, and choline appear to be ineffective Gundermann et al. [104]. Molecular studies showed that PC lowers the production of alcohol-induced TAG and CE and augments cellular redox status (observed in vitro and in vivo) Mak et al. [105] , Xu et al. [106] y Cohn et al. [107]. In addition to alcoholic hepatitis, PC (regardless of the individual fatty acids it contains [108]) appears to be effective in other hepatic disorders such as those that follow ischemia [109].

Virus-induced hepatopathies such as hepatitis C can benefit from PC administration, namely in terms of recovered enzymatic activity [110], which translates into amelioration of fibrosis [111]. Yet, PC has been reported as ineffective in hepatitis B when administered with standard therapy [112], maybe because of the different etiological agents. It is noteworthy that the hypothesized hepatoprotective effects of PLs appear to be stronger when they are derived from milk products or by-products. It is conceivable that the administration of a complex PL mixture rather than isolated PC would exert stronger protective actions. Indeed, a study by Wat et al. [113] performed in rats administered a milk

404 fat-rich diet reported amelioration of hepatomegaly and steatosis, along with
405 better blood lipid profile and reduced hyperlipidemia.
406
407 *6.4 Lipid profile, cholesterol, and CV risk*

408 Soy PLs have been extensively studied in the area of human health. Although
409 some studies did not report any effect on serum lipids of soy PLs administration
410 [114], others suggest amelioration of overall blood lipid balance, in terms of lower
411 TAG, total and LDL-CHOL, and increased HDL-CHOL in patients with
412 hyperlipidemia [107, 115, 116], diabetes [117-119], hypercholesterolemia [120],
413 and coronary disorders [121]. One additional positive effect of soy PLs concerns
414 their inhibition of platelet aggregation: hypercholesterolemic and obese patients
415 administered a soy PL extract exhibited reduced total and LDL-CHOL, but also
416 fibrinogen [122]. The same PL extract increased ApoA and decreased $ApoA_2$
417 and ApoE, suggesting stimulation of reverse CHOL transport and anti-
418 atherosclerotic effects [121].

419 Concerning dairy lipids, the administration of a buttermilk-rich diet to young
420 rats ameliorated their lipid profile [123]. Likewise, lower plasma CHOL
421 concentrations have been observed in humans who ingested buttermilk, possibly
422 as a consequence of lower intestinal CHOL absorption [124]. A recent study by
423 the same authors reported that short-term administration of buttermilk MFGM
424 improved the plasma CHOL and TAG profile of normotensive individuals Conway
425 et al. [125]. Accordingly, SM of dairy origin lowered CHOL in two human studies
426 [126, 127], even though a trial of the nutraceutical Lacprodan® PL-20 (which
427 provided two-three fold the SM amount of a control diet) did not result in
428 significant differences in plasma lipids or lipoproteins [128].

Marine PLs have also been studied: the administration of krill oil to hypertriglyceridemic subjects lowers total and LDL-CHOL, TAG, and increased HDL-CHOL [129, 130]. To discriminate the effects of EPA- and DHA-rich krill oil PLs, fish oil with equivalent amounts of such fatty acids incorporated in TAG has been tested in a head-to-head trial. The only effect recorded was a diminution of TAG, which points to the higher bioavailability and, hence, efficacy of krill oil PLs [23]. Cohn et al. [17] and Kullenberg et al. [21] published comprehensive reviews on the metabolic mechanisms underlying PLs' cardioprotective properties; however, the same authors call for more, ad-hoc studies to finally confirm this notion.

6.5 Inflammation and related diseases

Most studies on the effects of PLs on inflammation have been carried out with either soy or soy-extracted PLs. The use of PLs (especially PC) reduced rheumatoid arthritis [131, 132], pleurisy (Eros et al., 2009), and leukocyte adhesion [131]. Treede et al. [133] and Taylor et al. [33] explained the anti-inflammatory effects of PC with inhibition of TNF_{alpha} and TNF_{beta}, whose reduced levels might also play positive roles on weight control. In addition, PC administration improves colitis mostly because it restores the appropriate gastro-intestinal concentrations of this PL, which decrease with inflammation [134-137]. PC also improves the effects of NSAIDs [138] and reduces their gastric side effects via PGE-mediated gastric juice production [139].

The intake of PLs of marine origin such as krill oil modulates TNF_{alpha} production in mice and appear to be more efficacious than fish oil [140]. Mechanistically, these actions might be explains by lower series-2 prostaglandin

453 production [141] and by the effectiveness of PLs as vehicles of omega 3 fatty
454 acids. Krill oil also reduces arthritis and rheumatic symptoms [142, 143]. In
455 women, krill oil (but not fish oil) has been shown to improve pre-menstrual
456 syndrome [144] and its associated psychological disturbances Innis. [145].

457 Of interest, PC is likely not the only anti-inflammatory PL: sphingosines
458 appear to be important regulators of intestinal inflammation [146] and ameliorate
459 colitis-associated pain [147]. These data fit with reports of anti-colitis and
460 Chron's-beneficial (in rats and mice) effects of the inhibition of sphingosine
461 kinase [148, 149]. Chatterton et al. [150] actually suggested that milk and,
462 specifically, its fat globule membrane proteins produce potent anti-inflammatory
463 activities in the newborn's intestine.

464 *6.6 Immunology*

465

466 Lymphocyte function appears to be related to the quali/quantitative profile
467 of their membrane PLs, which decreased with age as shown by the constant
468 increase of the CHOL/PL ratio. Makzek et al. [151] restored proper lymphocytic
469 immunological function (in rats) by increasing their PL content. More recently,
470 Miranda et al. [152] managed to increase the phagocytic potency of diabetic
471 rats' macrophages by administering soy PC. These results have been replicated
472 in young subjects [153]. The specificity of PL is shown by the fact that when the
473 same fatty acids are given as TAG, no such beneficial effects can be recorded:
474 krill oil, rather than fish oil, increases the production of murine lymphocytes [154].
475 In humans, Veereman-Wauters et al. [155] administered a MFGM preparation to
476 pre-school children and recorded lower febrile episodes. This is one of the few
477 studies focusing on dairy products and immunology. Yet, sphingosine (abundant

in MFGM) is crucial to the trafficking and functionality of lymphocytes [34, 156]. Indeed, El Alwani et al. [157] underscore that sphingolipids are regulators of immune cells and of the inflammasome. Further ad-hoc trials are warranted, given the importance that the immune system plays in several degenerative diseases.

6.7 Infections

Several studies showed that the administration – by gavage – of PC and PI to rats protects against bacterial translocation and the degeneration of the enteric mucosa [158], in addition to conferring – in vitro – resistance versus BK virus [159]. Speelmans et al. [160] patented the use of soy lecithin for prevention and treatment of bacterial sepsis, while Gundermann et al. [104] observed that soy PLs improved the response to treatment of viral hepatitis. Another patent by Touitou [161] attributed soy PLs anti-viral, anti-bacterial, and anti-fungal properties when used as disinfectant in health and biological labs. More recently, Abd El Baky et al. [66] reported that PLs of algal origin exhibit anti-bacterial (versus *Escherichia coli y Bacillus subtili*) and anti-mold (versus *Aspergillus niger y Candida albicans*) properties.

Krill oil also induces plant resistance to virus infection in plants and animals, but its activities are likely due to its chitosan rather than PLs [162]. Sprong et al. [163] administered rats with PL-rich buttermilk or with defatted milk (as the control). The former exhibits better resistance versus *Listeria monocytogenes*, because PLs prevent its adhesion to the intestinal mucosa. The same group [164] later reported that milk fat SM and lyso-sphingolipids are potent anti-bacterial agents that increase resistance toward intestinal pathogens

503 (*Escherichia coli, Salmonella enteritidis, Campylobacter jejuni, Listeria*
504 *monocytogenes*) mostly due to the medium-chain (C10:0 and C12:0) fatty acids
505 of the lyso-derivatives. Finally, Haug et al. [165] observed that gangliosides –
506 which are components of MFGM – modify the gastrointestinal receptor for
507 microbial toxins, thereby partially preventing some digestive disorders.
508 Although more targeted studies are needed, the use of PLs from food
509 waste as antiseptic agents can be hypothesized, also in view of their low cost.
510
511 *6.8 Physical exercise*
512 Not many studies focused on dietary PLs and physical exercise. However,
513 as sources of FA, PLs might noticeably participate in physical activity and
514 exercise. Two placebo-controlled trials investigated the effects of 750 mg/d of
515 soy PS, given for 10 days. The first study reported no better cortisol
516 concentrations, feelings of pain, and markers of muscular damage and
517 peroxidation after an exhaustive run, even though the time-to-exhaustion
518 increased in the PL arm [166]. In another study, Kingsley et al. [167] studied the
519 ergogenic properties of PS and reported that its supplementation led to
520 significant increases in training time, reaching 85% of VO_{2max} before exhaustion
521 [168]. This study also reported direct effects on cortisol and adrenocorticotropin
522 when PS was taken before biking. Jäger et al. [169] also showed beneficial
523 effects of PS administration to athletes. On one hand, this study reported minor
524 physiological detrimental effects of intense physical activity. In addition, bikers
525 given PS witnessed lower muscular pain and faster recovery. This study too
526 reported positive cortisol effects, suggesting cell membrane protection. Accrued
527 evidence also indicates that PS increases glucose cellular uptake [169, 170]. In

addition, plasma concentrations of choline significantly decrease during exercise, which suggests its supplementation, possibly before rather than after performance [169].

7. Conclusions

The studies we reviewed in this paper underscore the importance of phospho- and sphingolipids in maintaining the integrity and functionality of cell membranes. Moreover, they appear to play important roles in human health; such activities could potentially be nutraceutically exploited in the adjunct therapy of widely diffused pathologies such as neurodegeneration or the metabolic syndrome. We want to underline that we still need more high-level clinical trials with, e.g. dose-response, incorporation into cell membranes and/or circulating lipids, specificity of the effects, and hard end-points to determine whether PLs could really be useful in the co-treatment of, e.g. neurodegeneration and associated dementia. However, given that several biologically active PLs can be recovered from inexpensive sources such as food processing by-products, ad-hoc investigation is warranted.

Acknowledgments

This work has been financially supported by the Spanish Ministry of Science and Innovation AGL 2011-26713. European FEDER Funds REF??. ALIBIRD-CM S2013/ABU-2728 de la Comunidad de Madrid.

References

[1] Z. Guo, A.F. Vikbjerg, X. Xu, Enzymatic modification of phospholipids for functional applications and human nutrition, Biotechnol. Adv. 23 (2005) 203-259.

[2] B.J. Pettus, A. Bielawska, P. Subramanian, et al., Ceramide 1-Phosphate Is a Direct Activator of Cytosolic Phospholipase A2, J. Biol. Chem. 279 (2004) 11320-11326.

[3] D.E. Vance, J.E. Vance, Chapter 8 - Phospholipid biosynthesis in eukaryotes, in: D.E.V.E. Vance (Ed.) Biochem. Lipids, Lipoproteins Membr. (5th Ed.), Elsevier, San Diego, 2008, pp. 213-VI.

[4] F. Baenke, B. Peck, H. Miess, A. Schulze, Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development, Dis. Models & Mech. 6 (2013) 1353-1363.

[5] R.A. Schlegel, P. Williamson, Phosphatidylserine, a death knell, Cell Death Differ. 8 (2001) 551-563.

[6] P.A. Leventis, S. Grinstein, The Distribution and Function of Phosphatidylserine in Cellular Membranes, Annu. Rev. Biophys. 39 (2010) 407-427.

[7] R. Mozzi, S. Buratta, G. Goracci, Metabolism and Functions of Phosphatidylserine in Mammalian Brain, Neurochem. Res. 28 (2003) 195-214.

[8] M.E. Gardocki, N. Jani, J.M. Lopes, Phosphatidylinositol biosynthesis: Biochemistry and regulation, Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1735 (2005) 89-100.

577 [9] K. D'Souza, R.M. Epand, Enrichment of phosphatidylinositols with specific
578 acyl chains, Biochim. Biophys. Acta, Biomembr. 1838 (2014) 1501-1508.
579 [10] A. Yamashita, S. Oka, T. Tanikawa, Y. Hayashi, Y. Nemoto-Sasaki, T.
580 Sugiura, The actions and metabolism of lysophosphatidylinositol, an
581 endogenous agonist for GPR55, Prostaglandins Other Lipid Mediators 107
582 (2013) 103-116.
583 [11] S.C. Frasch, D.L. Bratton, Emerging roles for lysophosphatidylserine in
584 resolution of inflammation, Prog. Lipid Res 51 (2012) 199-207.
585 [12] I. Sevastou, E. Kaffe, M.-A. Mouratis, V. Aidinis, Lysoglycerophospholipids
586 in chronic inflammatory disorders: The PLA2/LPC and ATX/LPA axes,
587 Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1831 (2013) 42-60.
588 [13] A. Arouri, O.G. Mouritsen, Membrane-perturbing effect of fatty acids and
589 lysolipids, Prog. Lipid Res. 52 (2013) 130-140.
590 [14] C. Bure, J.L. Cacas, S. Mongrand, J.M. Schmitter, Characterization of
591 glycosyl inositol phosphoryl ceramides from plants and fungi by mass
592 spectrometry, Anal. Bioanal. Chem. 406 (2014) 995-1010.
593 [15] A.H. Merrill, Sphingolipid and Glycosphingolipid Metabolic Pathways in the
594 Era of Sphingolipidomics, Chem. Rev. 111 (2011) 6387-6422.
595 [16] M. Aureli, N. Loberto, V. Chigorno, A. Prinetti, S. Sonnino, Remodeling of
596 Sphingolipids by Plasma Membrane Associated Enzymes, Neurochem. Res.
597 36 (2011) 1636-1644.
598 [17] J. Cohn, A. Kamili, E. Wat, R.W. Chung, S. Tandy, Dietary Phospholipids
599 and Intestinal Cholesterol Absorption, Nutrients 2 (2010) 116-127.
600 [18] J. Weihrauch, Y.-S. Son, Phospholipid content of foods, J. Am. Oil Chem.
601 Soc. 60 (1983) 1971-1978.

602 [19] T. Wang, E. Hammond, W. Fehr, Phospholipid fatty acid composition and
603 stereospecific distribution of soybeans with a wide range of fatty acid
604 composition, J. Am. Oi.l Chem. Soc. 74 (1997) 1587-1594.
605 [20] T. Waldemar, Egg Lipids, in: Chem. Funct. Prop. Food Lipids, CRC Press,
606 2002.
607 [21] D. Kullenberg, L.A. Taylor, M. Schneider, U. Massing, Health effects of
608 dietary phospholipids, Lipids Health Dis. 11 (2012) 3.
609 [22] M. Castro-Gómez, F. Holgado, L. Rodríguez-Alcalá, O. Montero, J.
610 Fontecha, Comprehensive Study of the Lipid Classes of Krill Oil by
611 Fractionation and Identification of Triacylglycerols, Diacylglycerols, and
612 Phospholipid Molecular Species by Using UPLC/QToF-MS, Food Anal.
613 Methods (2015) DOI 10.1007/s12161-015-0150-6.
614 [23] S. Ulven, B. Kirkhus, A. Lamglait, et al., Metabolic Effects of Krill Oil are
615 Essentially Similar to Those of Fish Oil but at Lower Dose of EPA and DHA,
616 in Healthy Volunteers, Lipids 46 (2011) 37-46.
617 [24] B.Y. Fong, C.S. Norris, A.K.H. MacGibbon, Protein and lipid composition of
618 bovine milk-fat-globule membrane, Int. Dairy J. 17 (2007) 275-288.
619 [25] L.M. Rodríguez-Alcalá, J. Fontecha, Major lipid classes separation of
620 buttermilk, and cows, goats and ewes milk by high performance liquid
621 chromatography with an evaporative light scattering detector focused on the
622 phospholipid fraction, J. Chromatogr. A. 1217 (2010) 3063-3066.
623 [26] A. Nilsson, R.D. Duan, Absorption and lipoprotein transport of
624 sphingomyelin, J. Lipid Res. 47 (2006) 154-171.
625 [27] J.J. Agren, M. Hallikainen, H. Vidgren, T.A. Miettinen, H. Gylling,
626 Postprandial lipemic response and lipoprotein composition in subjects with

627 low or high cholesterol absorption efficiency, Clin. Chim. Acta; Int. J. Clin.
628 Chem. 366 (2006) 309-315.
629 [28] O. Zierenberg, S. Grundy, Intestinal absorption of
630 polyenephosphatidylcholine in man, J. Lipid Res. 23 (1982) 1136-1142.
631 [29] A.R. Tall, C.B. Blum, S.M. Grundy, Incorporation of radioactive phospholipid
632 into subclasses of high-density lipoproteins, Am. J. Physiol. 244 (1983)
633 E513-516.
634 [30] B.L. Richmond, A.C. Boileau, S. Zheng, et al., Compensatory phospholipid
635 digestion is required for cholesterol absorption in pancreatic phospholipase
636 A2-deficient mice, Gastroenterol. 120 (2001) 1193-1202.
637 [31] A. Lamaziere, C. Wolf, U. Barbe, P. Bausero, F. Visioli, Lipidomics of
638 hepatic lipogenesis inhibition by omega 3 fatty acids, Prostaglandins Leukot.
639 Essent. Fatty Acids 88 (2013) 149-154.
640 [32] A. Lamaziere, D. Richard, U. Barbe, et al., Differential distribution of DHA-
641 phospholipids in rat brain after feeding: A lipidomic approach, Prostaglandins
642 Leukot. Essent. Fatty Acids 84 (2011) 7-11.
643 [33] L. Taylor, L. Pletschen, J. Arends, C. Unger, U. Massing, Marine
644 phospholipids—a promising new dietary approach to tumor-associated weight
645 loss, Support Care Cancer 18 (2010) 159-170.
646 [34] R.D. Duan, A. Nilsson, Metabolism of sphingolipids in the gut and its relation
647 to inflammation and cancer development, Prog. Lipid Res. 48 (2009) 62-72.
648 [35] J. Iqbal, M.M. Hussain, Intestinal lipid absorption, American journal of
649 physiology. Endocrinol. Metab. 296 (2009) E1183-1194.
650 [36] S. Danthine, C. Blecker, M. Paquot, N. Innocente, C. Deroanne, Progress in
651 milk fat globule membrane research: A review, Évolution des connaissances

652 sur la membrane du globule gras du lait : Synthèse bibliographique, 80
653 (2000) 209-222.
654 [37] I. Mather, T. Keenan, Origin and Secretion of Milk Lipids, J. Mammary
655 Gland. Biol. Neoplasia 3 (1998) 259-273.
656 [38] J.M. Evers, R.G. Haverkamp, S.E. Holroyd, G.B. Jameson, D.D.S.
657 Mackenzie, O.J. McCarthy, Heterogeneity of milk fat globule membrane
658 structure and composition as observed using fluorescence microscopy
659 techniques, Int. Dairy J. 18 (2008) 1081-1089.
660 [39] H. Singh, The milk fat globule membrane—A biophysical system for food
661 applications, Curr. Opin. Colloid Interface Sci. 11 (2006) 154-163.
662 [40] C. Lopez, M.-N. Madec, R. Jimenez-Flores, Lipid rafts in the bovine milk fat
663 globule membrane revealed by the lateral segregation of phospholipids and
664 heterogeneous distribution of glycoproteins, Food Chem, 120 (2011) 22-33.
665 [41] G. Contarini, M. Povolo, Phospholipids in milk fat: composition, biological
666 and technological significance, and analytical strategies, Int. J. Mol. Sci. 14
667 (2013) 2808-2831.
668 [42] V. Conway, S.F. Gauthier, Y. Pouliot, Buttermilk: Much more than a source
669 of milk phospholipids, Animal Frontiers 4 (2014) 44-51.
670 [43] K. Dewettinck, R. Rombaut, N. Thienpont, T.T. Le, K. Messens, J. Van
671 Camp, Nutritional and technological aspects of milk fat globule membrane
672 material, Int. Dairy J. 18 (2008) 436-457.
673 [44] M.M. El-Loly, Composition, properties and nutritional aspects of milk fat
674 globule membrane - a Review, Pol. J. Food Nutr. Sci. 61 (2011) 7-32.
675 [45] C. Vanderghem, P. Bodson, S. Danthine, M. Paquot, C. Deroanne, C.
676 Blecker, Milk fat globule membrane and buttermilks: From composition to

677 valorization, La membrane du globule gras du lait et les babeurres: De leur
678 composition à leur valorisation, 14 (2010) 485-500.
679 [46] A.M. Kuchta, P.M. Kelly, C. Stanton, R.A. Devery, Milk fat globule
680 membrane – a source of polar lipids for colon health? A Rev., Int. J. Dairy
681 Technol.65 (2012) 315-333.
682 [47] D.E. Modrak, D.V. Gold, D.M. Goldenberg, Sphingolipid targets in cancer
683 therapy, Mol. Cancer Ther. 5 (2006) 200-208.
684 [48] E.J. Dial, J.R. Doyen, L.M. Lichtenberger, Phosphatidylcholine-associated
685 nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit DNA synthesis and the
686 growth of colon cancer cells in vitro, Cancer Chemother. Pharmacol. 57
687 (2006) 295-300.
688 [49] N.E. Braverman, A.B. Moser, Functions of plasmalogen lipids in health and
689 disease, Biochim. Biophys. Acta, Mol. Basis Dis. 1822 (2012) 1442-1452.
690 [50] K. Fukunaga, Z. Hossain, K. Takahashi, Marine phosphatidylcholine
691 suppresses 1,2-dimethylhydrazine-induced colon carcinogenesis in rats by
692 inducing apoptosis, Nutr. Res. 28 (2008) 635-640.
693 [51] Z. Hossain, M. Hosokawa, K. Takahashi, Growth inhibition and induction of
694 apoptosis of colon cancer cell lines by applying marine phospholipid, Nutr.
695 Cancer 61 (2009) 123-130.
696 [52] V. Wijendran, M.C. Huang, G.Y. Diau, G. Boehm, P.W. Nathanielsz, J.T.
697 Brenna, Efficacy of dietary arachidonic acid provided as triglyceride or
698 phospholipid as substrates for brain arachidonic acid accretion in baboon
699 neonates, Pediatr. Res. 51 (2002) 265-272.
700 [53] V. Di Marzo, M. Griinari, G. Carta, etal., Dietary krill oil increases
701 docosahexaenoic acid and reduces 2-arachidonoylglycerol but not N-

acylethanolamine levels in the brain of obese Zucker rats, Int. Dairy J. 20 (2010) 231-235.

[54] D.R. Snow, R. Jimenez-Flores, R.E. Ward, etal., Dietary Milk Fat Globule Membrane Reduces the Incidence of Aberrant Crypt Foci in Fischer-344 Rats, J. Agric. Food Chem. 58 (2010) 2157-2163.

[55] F. Visioli, A. Strata, Milk, Dairy Products, and Their Functional Effects in Humans: A Narrative Review of Recent Evidence, Adv. Nutr.: Int. Rev. J. 5 (2014) 131-143.

[56] R. Zanabria, A. Tellez, M. Griffiths, M. Corredig, The antiproliferative properties of the milk fat globule membrane are affected by extensive heating, Dairy Sci. Technol. 94 (2014) 439-453.

[57] P. Zhang, B. Li, S. Gao, R.D. Duan, Dietary sphingomyelin inhibits colonic tumorigenesis with an up-regulation of alkaline sphingomyelinase expression in ICR mice, Anticancer Res. 28 (2008) 3631-3635.

[58] D.L. Dillehay, S.K. Webb, E.M. Schmelz, A.H. Merrill, Jr., Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice, J Nutr. 124 (1994) 615-620.

[59] E.M. Schmelz, D.L. Dillehay, S.K. Webb, A. Reiter, J. Adams, A.H. Merrill Jr, Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1, 2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis, Cancer Res. 56 (1996) 4936-4941.

[60] L.A. Lemonnier, D.L. Dillehay, M.J. Vespremi, J. Abrams, E. Brody, E.M. Schmelz, Sphingomyelin in the suppression of colon tumors: prevention versus intervention, Arch. Biochem. Biophys., 419 (2003) 129-138.

727 [61] B. Berra, I. Colombo, E. Sottocornola, A. Giacosa, Dietary sphingolipids in
728 colorectal cancer prevention, Eur. J. Cancer Prev. 11 (2002) 193-197.
729 [62] E.M. Schmelz, Sphingolipids in the chemoprevention of colon cancer, Front.
730 Biosci: J and Virtual Libr. 9 (2004) 2632.
731 [63] P. Zhang, B. Li, S. Gao, R.-D. Duan, Dietary sphingomyelin inhibits colonic
732 tumorigenesis with an up-regulation of alkaline sphingomyelinase expression
733 in ICR mice, Anticancer Res. 28 (2008) 3631-3635.
734 [64] G. Patwardhan, L. Beverly, L. Siskind, Sphingolipids and mitochondrial
735 apoptosis, J Bioenerg. Biomembr. (2015) 1-16.
736 [65] Y.C. Li, M.J. Park, S.-K. Ye, C.-W. Kim, Y.-N. Kim, Elevated Levels of
737 Cholesterol-Rich Lipid Rafts in Cancer Cells Are Correlated with Apoptosis
738 Sensitivity Induced by Cholesterol-Depleting Agents, Am. J. Pathol. 168
739 (2006) 1107-1118.
740 [66] H.H. Abd El Baky, F.K. El Baz, G.S. El Baroty, M.S. Asker, E.A. Ibrahim,
741 Phospholipids of some marine macroalgae: Identification, antivirus,
742 anticancer and antimicrobial bioactivities, Pharma Chem. 6 (2014) 370-382.
743 [67] M.L. Doria, C.Z. Cotrim, C. Simoes, B. Macedo, P. Domingues, M.R.
744 Domingues, L.A. Helguero, Lipidomic analysis of phospholipids from human
745 mammary epithelial and breast cancer cell lines, J. Cell. Physiol. 228 (2013)
746 457-468.
747 [68] N. Patel, R. Vogel, K. Chandra-Kuntal, W. Glasgow, U. Kelavkar, A novel
748 three serum phospholipid panel differentiates normal individuals from those
749 with prostate cancer, PLoS One 9 (2014) e88841.

[69] Y. Sakakima, A. Hayakawa, T. Nagasaka, A. Nakao, Prevention of hepatocarcinogenesis with phosphatidylcholine and menaquinone-4: In vitro and in vivo experiments, J. Hepatol. 47 (2007) 83-92.

[70] Y. Sakakima, A. Hayakawa, A. Nakao, Phosphatidylcholine induces growth inhibition of hepatic cancer by apoptosis via death ligands, Hepato-Gastroenterology 56 (2009) 481-484.

[71] D.E. Modrak, T.M. Cardillo, G.A. Newsome, D.M. Goldenberg, D.V. Gold, Synergistic Interaction between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer, Cancer Res. 64 (2004) 8405-8410.

[72] D.E. Modrak, W. Lew, D.M. Goldenberg, R. Blumenthal, Sphingomyelin potentiates chemotherapy of human cancer xenografts, Biochem. Biophys. Res. Commun. 268 (2000) 603-606.

[73] D.E. Modrak, M.D. Rodriguez, D.M. Goldenberg, W. Lew, R.D. Blumenthal, Sphingomyelin enhances chemotherapy efficacy and increases apoptosis in human colonic tumor xenografts, Int. J. Oncol. 20 (2002) 379-384.

[74] L.E. van Vlerken, Z. Duan, M.V. Seiden, M.M. Amiji, Modulation of intracellular ceramide using polymeric nanoparticles to overcome multidrug resistance in cancer, Cancer Res. 67 (2007) 4843-4850.

[75] M. Jansen, K.-H. Treutner, B. Schmitz, J. Otto, P. Jansen, S. Neuss, V. Schumpelick, Phospholipids reduce gastric cancer cell adhesion to extracellular matrix in vitro, BMC Gastroenterol. 4 (2004) 33.

[76] P. Jantscheff, M. Schlesinger, J. Fritzsche, etal., Lysophosphatidylcholine pretreatment reduces VLA-4 and P-Selectin-mediated b16.f10 melanoma cell

774 adhesion in vitro and inhibits metastasis-like lung invasion in vivo, Mol.
775 Cancer Ther. 10 (2011) 186-197.
776 [77] C.J.L. Murray, K.F. Ortblad, C. Guinovart, el al., Global, regional, and
777 national incidence and mortality for HIV, tuberculosis, and malaria during
778 1990–2013: a systematic analysis for the Global Burden of Disease Study
779 2013, Lancet 384 (2014) 1005-1070.
780 [78] G.A. Jicha, W.R. Markesbery, Omega-3 fatty acids: potential role in the
781 management of early Alzheimer's disease, Clin. Interv. Aging 5 (2010) 45-61
782 [79] C. Konagai, K. Yanagimoto, K. Hayamizu, L. Han, T. Tsuji, Y. Koga, Effects
783 of krill oil containing n-3 polyunsaturated fatty acids in phospholipid form on
784 human brain function: a randomized controlled trial in healthy elderly
785 volunteers, Clin. Interventions Aging 8 (2013) 1247-1257.
786 [80] M. Lagarde, N. Bernoud, N. Brossard, et al., Lysophosphatidylcholine as a
787 preferred carrier form of docosahexaenoic acid to the brain, J. Mol. Neurosci.
788 : MN, 16 (2001) 201-221.
789 [81] T. Nagata, T. Yaguchi, T. Nishizaki, DL- and PO-phosphatidylcholines as a
790 promising learning and memory enhancer, Lipids Health Dis. 10 (2011) 25.
791 [82] S.L. Ladd, S.A. Sommer, S. LaBerge, W. Toscano, Effect of
792 phosphatidylcholine on explicit memory, Clin. Neuropharmacol. 16 (1993)
793 540-549.
794 [83] S.H. Zeisel, Nutritional importance of choline for brain development, J. Am.
795 Coll. Nutr. 23 (2004) 621S-626S.
796 [84] W.H. Meck, C.L. Williams, Choline supplementation during prenatal
797 development reduces proactive interference in spatial memory, Dev. Brain
798 Res. 118 (1999) 51-59.

799 [85] J.R. Tweedy, C.A. Garcia, Lecithin treatment of cognitively impaired
800 Parkinson's patients, Eur. J. Clin. Invest. 12 (1982) 87-90.
801 [86] T. Jayaraman, S. Kannappan, M.K. Ravichandran, C.V. Anuradha, Impact of
802 Essentiale L on ethanol-induced changes in rat brain and erythrocytes,
803 Singapore Med. J. 49 (2008) 320-327.
804 [87] E. Giordano, F. Visioli, Long-chain omega 3 fatty acids: molecular bases of
805 potential antioxidant actions, Prostaglandins Leukot. Essent. Fatty Acids 90
806 (2014) 1-4.
807 [88] G. Pepeu, I.M. Pepeu, L. Amaducci, A review of phosphatidylserine
808 pharmacological and clinical effects. Is phosphatidylserine a drug for the
809 ageing brain?, Pharmacol. Res. 33 (1996) 73-80.
810 [89] G. Pepeu, Is There Evidence That Phospholipid Administration Is Beneficial
811 for Your Brain?, in: Nutr. Biochem. Phospholipids AOCS Publishing, 2003.
812 [90] W. Mi, N. van Wijk, M. Cansev, J.W. Sijben, P.J. Kamphuis, Nutritional
813 approaches in the risk reduction and management of Alzheimer's disease,
814 Nutrition (2013).
815 [91] C.W. Ritchie, J. Bajwa, G. Coleman, K. Hope, R.W. Jones, M. Lawton, M.
816 Marven, P. Passmore, Souvenaid(R): a new approach to management of
817 early Alzheimer's disease, J. Nutr., Health Aging 18 (2014) 291-299.
818 [92] T.H. Crook, J. Tinklenberg, J. Yesavage, W. Petrie, M.G. Nunzi, D.C.
819 Massari, Effects of phosphatidylserine in age-associated memory impairment,
820 Neurol. 41 (1991) 644-649.
821 [93] D. Benton, R.T. Donohoe, B. Sillance, S. Nabb, The influence of
822 phosphatidylserine supplementation on mood and heart rate when faced with
823 an acute stressor, Nutr. Neurosci. 4 (2001) 169-178.

824 [94] J. Hellhammer, A.R. Waladkhani, T. Hero, C. Buss, Effects of milk
825 phospholipid on memory and psychological stress response, Br. Food J. 112
826 (2010) 1124-1137.
827 [95] M.A. McDaniel, S.F. Maier, G.O. Einstein, "Brain-specific" nutrients: a
828 memory cure?, Nutrition 19 (2003) 957-975.
829 [96] A.R. Smith, F. Visioli, B. Frei, T.M. Hagen, Age-related changes in
830 endothelial nitric oxide synthase phosphorylation and nitric oxide dependent
831 vasodilation: evidence for a novel mechanism involving sphingomyelinase
832 and ceramide-activated phosphatase 2A, Aging cell 5 (2006) 391-400.
833 [97] R. Uauy, A.D. Dangour, Nutrition in brain development and aging: role of
834 essential fatty acids, Nutr. Rev. 64 (2006) S24-33; discussion S72-91.
835 [98] G. Crichton, P. C Howe, J. Buckley, A. Coates, K. Murphy, Dairy
836 consumption and cardiometabolic health: outcomes of a 12-month crossover
837 trial, Nutr. Metab. (Lond) 9 (2012) 1-11.
838 [99] K.M. Park, V.L. Fulgoni, 3rd, The association between dairy product
839 consumption and cognitive function in the National Health and Nutrition
840 Examination Survey, Br. J. Nutr. 109 (2013) 1135-1142.
841 [100] N. Timby, E. Domellöf, O. Hernell, B. Lönnerdal, M. Domellöf,
842 Neurodevelopment, nutrition, and growth until 12 mo of age in infants fed a
843 low-energy, low-protein formula supplemented with bovine milk fat globule
844 membranes: a randomized controlled trial, Am. J. Clin. Nutr. (2014).
845 [101] K. Tanaka, M. Hosozawa, N. Kudoetal., The pilot study: sphingomyelin-
846 fortified milk has a positive association with the neurobehavioural
847 development of very low birth weight infants during infancy, randomized
848 control trial, Brain & Dev. 35 (2013) 45-52.

849 [102] C.S. Lieber, L.M. Decarli, K.M. Mak, C.I. Kim, M.A. Leo, Attenuation of
850 alcohol-induced hepatic fibrosis by polyunsaturated lecithin, Hepatol. 12
851 (1990) 1390-1398.
852 [103] K.P. Navder, E. Baraona, C.S. Lieber, Polyenylphosphatidylcholine
853 attenuates alcohol-induced fatty liver and hyperlipemia in rats, J. Nutr. 127
854 (1997) 1800-1806.
855 [104] K.J. Gundermann, A. Kuenker, E. Kuntz, M. Drozdzik, Activity of essential
856 phospholipids (EPL) from soybean in liver diseases, Pharmacol. Rep.: PR 63
857 (2011) 643-659.
858 [105] K.M. Mak, K. Wen, C. Ren, C.S. Lieber, Dilinoleoylphosphatidylcholine
859 reproduces the antiapoptotic actions of polyenylphosphatidylcholine against
860 ethanol-induced hepatocyte apoptosis, Alcohol.: Clin. Exp. Res. 27 (2003)
861 997-1005.
862 [106] Y. Xu, M.A. Leo, C.S. Lieber, DLPC attenuates alcohol-induced cytotoxicity
863 in HepG2 cells expressing CYP2E1, Alcohol Alcohol. (Oxford, U. K.) 40
864 (2005) 172-175.
865 [107] J.S. Cohn, E. Wat, A. Kamili, S. Tandy, Dietary phospholipids, hepatic lipid
866 metabolism and cardiovascular disease, Curr. Opin. Lipidol. 19 (2008) 257-
867 262.
868 [108] Y. Buang, Y.-M. Wang, J.-Y. Cha, K. Nagao, T. Yanagita, Dietary
869 phosphatidylcholine alleviates fatty liver induced by orotic acid, Nutr. 21
870 (2005) 867-873.
871 [109] S. Demirbilek, A. Karaman, K. Gurunluoglu, E. Tas, M. Akin, R.T. Aksoy, E.
872 Turkmen, M.N. Edali, A. Baykarabulut, Polyenylphosphatidylcholine

pretreatment protects rat liver from ischemia/reperfusion injury, Hepatol. Res :Off.l J. Japan Society Hepatol, 34 (2006) 84-91.

[110] A.K. Singal, S.C. Jampana, S.A. Weinman, Antioxidants as therapeutic agents for liver disease, Liver Int. 31 (2011) 1432-1448.

[111] A.B. Lieber CS, Bibi EJ, Polyenylphosphatidylcholine (PPC) is beneficial for the treatment of hepatitis C, Hepatol 42 (2005) 695A.

[112] C. Niederau, G. Strohmeyer, T. Heintges, K. Peter, E. Gopfert, Polyunsaturated phosphatidyl-choline and interferon alpha for treatment of chronic hepatitis B and C: a multi-center, randomized, double-blind, placebo-controlled trial. Leich Study Group, Hepato-gastroenterol, 45 (1998) 797-804.

[113] E. Wat, S. Tandy, E. Kapera, A. Kamili, R.W.S. Chung, A. Brown, M. Rowney, J.S. Cohn, Dietary phospholipid-rich dairy milk extract reduces hepatomegaly, hepatic steatosis and hyperlipidemia in mice fed a high-fat diet, Atherosclerosis 205 (2009) 144-150.

[114] W. Oosthuizen, H. Vorster, W. Vermaak, et al., Lecithin has no effect on serum lipoprotein, plasma fibrinogen and macro molecular protein complex levels in hyperlipidaemic men in a double-blind controlled study, Eur. J. Clin. Nutr. 52 (1998) 419.

[115] E. Wat, S. Tandy, E. Kapera, A. Kamili, R.W. Chung, A. Brown, M. Rowney, J.S. Cohn, Dietary phospholipid-rich dairy milk extract reduces hepatomegaly, hepatic steatosis and hyperlipidemia in mice fed a high-fat diet, Atherosclerosis 205 (2009) 144-150.

[116] J. Wójcicki, A. Pawlik, L. Samochowiec, M. Kaldo□ ska, Z. Myśliwiec, Clinical evaluation of lecithin as a lipid-lowering agentPhytother. Res. 9 (1995) 597-599.

[117] R. Kirsten, B. Heintz, K. Nelsonetal., Polyenylphosphatidylcholine improves the lipoprotein profile in diabetic patients, Int. J. Clin. Pharmacol. Ther. 32 (1994) 53-56.

[118] D. Ristić Medić, V. Ristić, A. Arsić etal., Effects of soybean D-LeciVita product on serum lipids and fatty acid composition in type 2 diabetic patients with hyperlipidemia, Nutr., Metab. Cardiovasc. Dis. 16 (2006) 395-404.

[119] R. Singh, F. de Meester, V. Mechirova, D. Pella, K. Otsuka, Fatty Acids in the Causation and Therapy of Metabolic Syndrome, in: F. De Meester, R. Watson (Eds.) Wild-Type Food in Health Promotion and Disease Prevention, Humana Press, 2008, pp. 263-284.

[120] G. Hori, M.-F. Wang, Y.-C. Chan, et al., Soy Protein Hydrolyzate with Bound Phospholipids Reduces Serum Cholesterol Levels in Hypercholesterolemic Adult Male Volunteers, Biosci., Biotechnol., Biochem. 65 (2001) 72-78.

[121] N.R. Pandey, D.L. Sparks, Phospholipids as cardiovascular therapeutics, Curr. Opin. Invest. Drugs (London, England : 2000), 9 (2008) 281-285.

[122] S. Eshigina, M.M. Gapparov, G. Mal'tsev, S.N. Kulakov, [Influence of dietary therapy containing sunflower oil fortified with phospholipids on the lipid metabolism in patients with hypertension and obesity], Voprosy pitaniia, 76 (2007) 58-62.

919 [123] A. Oosting, D. Kegler, H.J. Wopereis, et al., Size and phospholipid coating
920 of lipid droplets in the diet of young mice modify body fat accumulation in
921 adulthood, Pediatr. Res. 72 (2012) 362-369.
922 [124] V. Conway, P. Couture, C. Richard, S.F. Gauthier, Y. Pouliot, B. Lamarche,
923 Impact of buttermilk consumption on plasma lipids and surrogate markers of
924 cholesterol homeostasis in men and women, Nutr., Metab. Cardiovasc. Dis.
925 23 (2013) 1255-1262.
926 [125] V. Conway, P. Couture, S. Gauthier, Y. Pouliot, B. Lamarche, Effect of
927 buttermilk consumption on blood pressure in moderately
928 hypercholesterolemic men and women, Nutric. 30 (2014) 116-119.
929 [126] Y. Jiang, S.K. Noh, S.I. Koo, Egg phosphatidylcholine decreases the
930 lymphatic absorption of cholesterol in rats, J. Nutric. 131 (2001) 2358-2363.
931 [127] S.K. Noh, S.I. Koo, Milk sphingomyelin is more effective than egg
932 sphingomyelin in inhibiting intestinal absorption of cholesterol and fat in rats,
933 J. Nutric. 134 (2004) 2611-2616.
934 [1287] L. Ohlsson, H. Burling, A. Nilsson, Long term effects on human plasma
935 lipoproteins of a formulation enriched in butter milk polar lipid, Lipids Health
936 Dis. 8 (2009) 44.
937 [129] K. Berge, K. Musa-Veloso, M. Harwood, N. Hoem, L. Burri, Krill oil
938 supplementation lowers serum triglycerides without increasing low-density
939 lipoprotein cholesterol in adults with borderline high or high triglyceride levels,
940 Nutr. Res. 34 (2014) 126-133.
941 [130] R. Bunea, K. El Farrah, L. Deutsch, Evaluation of the effects of Neptune
942 Krill Oil on the clinical course of hyperlipidemia, Altern.
943 Med. Rev. : J. Clin. Ther. 9 (2004) 420-428.

944 [131] G. Erős, S. Ibrahim, N. Siebert, M. Boros, B. Vollmar, Oral
945 phosphatidylcholine pretreatment alleviates the signs of experimental
946 rheumatoid arthritis, Arthritis Res. Ther. 11 (2009) R43.
947 [132] P. Hartmann, A. Szabó, G. Erős, et al., Anti-inflammatory effects of
948 phosphatidylcholine in neutrophil leukocyte-dependent acute arthritis in rats,
949 Eur. J. Pharmacol.. 622 (2009) 58-64.
950 [133] I. Treede, A. Braun, R. Sparla, et al., Anti-inflammatory effects of
951 phosphatidylcholine, J. Biol. Chem. 282 (2007) 27155-27164.
952 [134] W. Stremmel, A. Hanemann, R. Ehehalt, M. Karner, A. Braun,
953 Phosphatidylcholine (lecithin) and the mucus layer: Evidence of therapeutic
954 efficacy in ulcerative colitis?, Dig. Dis. (Basel, Switzerland), 28 (2010) 490-
955 496.
956 [135] R. Ehehalt, A. Braun, M. Karner, J. Fullekrug, W. Stremmel,
957 Phosphatidylcholine as a constituent in the colonic mucosal barrier--
958 physiological and clinical relevance, Biochim. Biophys. Acta 1801 (2010) 983-
959 993.
960 [136] M. Karner, A. Kocjan, J. Stein, et al., First Multicenter Study of Modified
961 Release Phosphatidylcholine LT-02in Ulcerative Colitis: A Randomized,
962 Placebo-Controlled Trial in Mesalazine-Refractory Courses, Am. J.
963 Gastroenterol. 109 (2014) 1041-1051.
964 [137] H. Schneider, A. Braun, J. Füllekrug, W. Stremmel, R. Ehehalt, Lipid Based
965 Therapy for Ulcerative Colitis—Modulation of Intestinal Mucus Membrane
966 Phospholipids as a Tool to Influence Inflammation, Int. J. Mol. Sci. 11 (2010)
967 4149-4164.

968 [138] L. Lichtenberger, J.J. Romero, E.J. Dial, Gastrointestinal safety and
969 therapeutic efficacy of parenterally administered phosphatidylcholine-
970 associated indomethacin in rodent model systems, Br. J. Pharmacol. 157
971 (2009) 252-257.
972 [139] S. Leyck, N. Dereu, E. Etschenberg, et al., Improvement of the gastric
973 tolerance of non-steroidal anti-inflammatory drugs by polyene
974 phosphatidylcholine (Phospholipon 100), Eur. J. Pharmacol. 117 (1985) 35-
975 42.
976 [140] N. Vigerust, B. Bjørndal, P. Bohov, T. Brattelid, A. Svardal, R. Berge, Krill
977 oil versus fish oil in modulation of inflammation and lipid metabolism in mice
978 transgenic for TNF-α, Eur. J. Nutr. 52 (2013) 1315-1325.
979 [141] J.C. Tou, J. Jaczynski, Y.-C. Chen, Krill for Human Consumption:
980 Nutritional Value and Potential Health Benefits, Nutr. Rev. 65 (2007) 63-77.
981 [142] L. Deutsch, Evaluation of the Effect of Neptune Krill Oil on Chronic
982 Inflammation and Arthritic Symptoms, J. Am. Coll. Nutr. 26 (2007) 39-48.
983 [143] M. Ierna, A. Kerr, H. Scales, K. Berge, M. Griinari, Supplementation of diet
984 with krill oil protects against experimental rheumatoid arthritis, BMC
985 Musculoskelet Disord. 11 (2010) 1-11.
986 [144] F. Sampalis, R. Bunea, M.F. Pelland, O. Kowalski, N. Duguet, S. Dupuis,
987 Evaluation of the effects of Neptune Krill Oil on the management of
988 premenstrual syndrome and dysmenorrhea, Altern. Med. Rev : J. Clin. Ther.
989 8 (2003) 171-179.
990 [145] S.M. Innis, Dietary (n-3) Fatty Acids and Brain Development, J. Nutr. 137
991 (2007) 855-859.

992 [146] L.A. Cowart, Sphingolipids: players in the pathology of metabolic disease,
993 rends Endocrinol. Metab. 20 (2009) 34-42.
994 [147] R.D. Duan, Anticancer compounds and sphingolipid metabolism in the
995 colon, In vivo (Athens, Greece), 19 (2005) 293-300.
996 [148] L. Maines, L. Fitzpatrick, C. Green, Y. Zhuang, C. Smith, Efficacy of a
997 novel sphingosine kinase inhibitor in experimental Crohn's disease,
998 Inflammopharmacol. 18 (2010) 73-85.
999 [149] L. Maines, L. Fitzpatrick, K. French, Y. Zhuang, Z. Xia, S. Keller, J. Upson,
1000 C. Smith, Suppression of Ulcerative Colitis in Mice by Orally Available
1001 Inhibitors of Sphingosine Kinase, Dig. Dis. Sci. 53 (2008) 997-1012.
1002 [150] D.E. Chatterton, D.N. Nguyen, S.B. Bering, P.T. Sangild, Anti-inflammatory
1003 mechanisms of bioactive milk proteins in the intestine of newborns, Int. J.
1004 Biochem. Cell Biol. 45 (2013) 1730-1747.
1005 [151] C. Maczek, G. Bock, G. Jurgens, D. Schonitzer, H. Dietrich, G. Wick,
1006 Environmental influence on age-related changes of human lymphocyte
1007 membrane viscosity using severe combined immunodeficiency mice as an in
1008 vivo model, Exp. Gerontol. 33 (1998) 485-498.
1009 [152] D.T. Miranda, V.G. Batista, F.C. Grando, et al., Soy lecithin
1010 supplementation alters macrophage phagocytosis and lymphocyte response
1011 to concanavalin A: a study in alloxan-induced diabetic rats, Cell Biochem.
1012 Funct. 26 (2008) 859-865.
1013 [153] P.W. Jannace, R.H. Lerman, J.I. Santos, J.J. Vitale, Effects of oral soy
1014 phosphatidylcholine on phagocytosis, arachidonate concentrations, and
1015 killing by human polymorphonuclear leukocytes, Am. J. Clin. Nutr. 56 (1992)
1016 599-603.

1017 [154] B. Robertson, L. Burri, K. Berge, Genotoxicity test and subchronic toxicity
1018 study with Superba™ krill oil in rats, Toxicol. Rep. 1 (2014) 764-776.
1019 [155] G. Veereman-Wauters, S. Staelens, R. Rombaut, et al., Milk fat globule
1020 membrane (INPULSE) enriched formula milk decreases febrile episodes and
1021 may improve behavioral regulation in young children, Nutr. 28 (2012) 749-
1022 752.
1023 [156] S. Mandala, R. Hajdu, J. Bergstrom, et al., Alteration of lymphocyte
1024 trafficking by sphingosine-1-phosphate receptor agonists, Science (New
1025 York, N.Y.), 296 (2002) 346-349.
1026 [157] M. El Alwani, B.X. Wu, L.M. Obeid, Y.A. Hannun, Bioactive sphingolipids in
1027 the modulation of the inflammatory response, Pharmacol. Ther. 112 (2006)
1028 171-183.
1029 [158] X.D. Wang, R. Andersson, V. Soltesz, W.Q. Wang, A. Ar'rajab, S.
1030 Bengmark, Phospholipids prevent enteric bacterial translocation in the early
1031 stage of experimental acute liver failure in the rat, Scand. J. Gastroenterol. 29
1032 (1994) 1117-1121.
1033 [159] L. Sinibaldi, P. Goldoni, V. Pietropaolo, L. Cattani, C. Peluso, C. Di
1034 Taranto, Role of phospholipids in BK virus infection and haemagglutination,
1035 Microbiol. 15 (1992) 337-344.
1036 [160] G. Speelmans, A.J.M. Vriesema, Z. Hofman, I. Kivit, R.J.J. Hageman,
1037 Enteral composition for the prevention and/or treatment of sepsis, in, Google
1038 Patents, 2004.
1039 [161] E. Touitou, Sanitizing compositions, in, Google Patents, 2012.
1040 [162] S.N. Chirkov, The Antiviral Activity of Chitosan (Review), Appl. Biochem.
1041 Microbiol. 38 (2002) 1-8.

1042 [163] C. Sprong, M. Hulstein, R. Van der Meer, Phospholipid-rich butter milk
1043 decreases the gastro-intestinal survival and translocation of listeria in rats,
1044 Gastroenterol. 114 (1998) A1090.
1045 [164] R.C. Sprong, M.F.E. Hulstein, R. Van der Meer, Bactericidal Activities of
1046 Milk Lipids, Antimicrob. Agents Chemother. 45 (2001) 1298-1301.
1047 [165] A. Haug, A.T. Hostmark, O.M. Harstad, Bovine milk in human nutrition–a
1048 review, Lipids Health Dis. 6 (2007) 1-16.
1049 [166] M.I. Kingsley, D. Wadsworth, L.P. Kilduff, J. McEneny, D. Benton, Effects
1050 of phosphatidylserine on oxidative stress following intermittent running, Med.
1051 Sci. Sports Exercise 37 (2005) 1300.
1052 [167] M.I. Kingsley, M. Miller, L.P. Kilduff, J. McEneny, D. Benton, Effects of
1053 phosphatidylserine on exercise capacity during cycling in active males, Med.
1054 Sci. Sports Exercise 38 (2006) 64.
1055 [168] P. Monteleone, L. Beinat, C. Tanzillo, M. Maj, D. Kemali, Effects of
1056 Phosphatidylserine on the Neuroendocrine Response to Physical Stress in
1057 Humans, Neuroendocrinol. 52 (1990) 243-248.
1058 [169] R. Jäger, M. Purpura, M. Kingsley, Phospholipids and sports performance,
1059 J. Int. Soc. Sports Nutr. 4 (2007) 5-5.
1060 [170] M.A. Starks, S.L. Starks, M. Kingsley, M. Purpura, R. Jäger, The effects of
1061 phosphatidylserine on endocrine response to moderate intensity exercise, J.
1062 Int. Soc. Sports Nutr. 5 (2008) 11-11.
1063 [171] P. Fagan, C. Wijesundera, Liquid chromatographic analysis of milk
1064 phospholipids with on-line pre-concentration, J. Chromatogr. A. 1054 (2004)
1065 241-249.

1066 [172] A. Avalli, G. Contarini, Determination of phospholipids in dairy products by
1067 SPE/HPLC/ELSD, J. Chromatogr. A. 1071 (2005) 185-190.
1068 [173] R. Rombaut, J.V. Camp, K. Dewettinck, Phospho- and sphingolipid
1069 distribution during processing of milk, butter and whey, J. Food. Technol. 41
1070 (2006) 435-443.
1071 [174] R. Rombaut, K. Dewettinck, J. Van Camp, Phospho- and sphingolipid
1072 content of selected dairy products as determined by HPLC coupled to an
1073 evaporative light scattering detector (HPLC-ELSD), J. Food Compos. Anal.
1074 20 (2007) 308-312.
1075 [175] C. Fauquant, V. Briard-Bion, N. Leconte, M. Guichardant, M.-C. Michalski,
1076 Membrane phospholipids and sterols in microfiltered milk fat globules, Eur. J.
1077 Lipid Sci. Technol. 109 (2007) 1167-1173.
1078 [176] C. Lopez, V. Briard-Bion, O. Menard, F. Rousseau, P. Pradel, J.M. Besle,
1079 Phospholipid, sphingolipid, and fatty acid compositions of the milk fat globule
1080 membrane are modified by diet, J. Agric. Food. Chem. 56 (2008) 5226-5236.
1081 [177] F. Sánchez-Juanes, J.M. Alonso, L. Zancada, P. Hueso,
1082 Glycosphingolipids from bovine milk and milk fat globule membranes: A
1083 comparative study. Adhesion to enterotoxigenic Escherichia coli strains, Biol.
1084 Chem. 390 (2009) 31-40.
1085 [178] S. Gallier, D. Gragson, C. Cabral, R. Jiménez-Flores, D.W. Everett,
1086 Composition and Fatty Acid Distribution of Bovine Milk Phospholipids from
1087 Processed Milk Products, J. Agric. Food Chem. 58 (2010) 10503-10511.
1088 [179] T.T. Le, J. Miocinovic, T.M. Nguyen, R. Rombaut, J. van Camp, K.
1089 Dewettinck, Improved solvent extraction procedure and high-performance
1090 liquid chromatography-evaporative light-scattering detector method for

analysis of polar lipids from dairy materials, J. Agric. Food Chem. 59 (2011) 10407-10413.

[180] C. Garcia, N.W. Lutz, S. Confort-Gouny, P.J. Cozzone, M. Armand, M. Bernard, Phospholipid fingerprints of milk from different mammalians determined by 31P NMR: towards specific interest in human health, Food Chem. 135 (2012) 1777-1783.

[181] M.P. Castro-Gomez, L.M. Rodriguez-Alcala, M.V. Calvo, et al., Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques, J. Dairy Sci, 97 (2014) 6719-6728.

1102 **Table 1**

1103 Phospho- and sphingolipid concentrations (% on total polar lipids) in cow milk.

Reference	PE	PI	PS	PC	SM
Fagan and Wijesundera [171]	38.6	-	-	32.2	29.2
Avalli and Contarini [172]	32.3	9.3	10.5	27.3	20.5
Rombaut et al. [173]	33.2	5.2	9.3	27.4	25.1
Rombaut et al. [174]	46.4	5.3	7.4	21.1	19.8
Fong et al [24]	32.6	7.6	5.3	33.2	21.3
Fauquant et al. [175]	36.4	7.6	6.5	32.1	17.3
Lopez et al. [176]	26.8	13.6	16.1	22.0	21.6
Sánchez-Juanes et al. [177]	28.5	14.1		32.7	23.0
Rodríguez-Alcalá and Fontecha [25]	38.5	6.5	7.7	25.9	21.4
Gallier et al. [178]	26.4	3.4	2.0	42.8	25.5
Le et al. [179]	36.9	6.1	6.3	27.0	23.7
Garcia et al. [180]	33.8	3.9	10.6	30.5	21.2
Castro-Gomez et al. [181]	42.0	3.9	3.4	29.3	21.0

1104 PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine,

1105 PC: phosphatidylcholine, SM: sphingomyelin

1106

Figure captions

Fig.1. Chemical structures of phospho- and sphingolipids.

Fig. 2. Biochemical pathways of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) synthesis.

Fig. 3. Phosphatidylserine's synthesis: metabolic routes.

Fig. 4. Sphingomyelin absorption

Fig. 5. A model for the structure of milk fat globule membranes (MFGM)

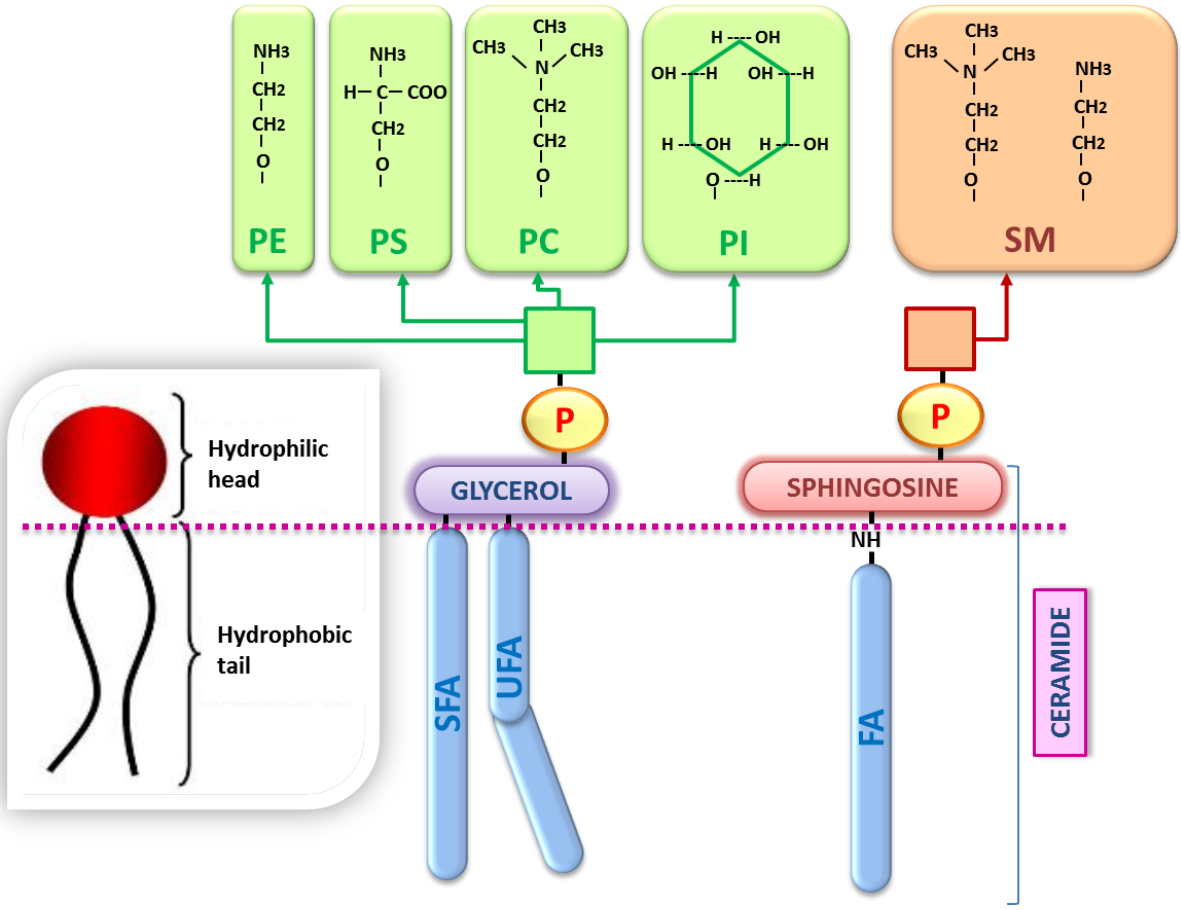


FIG.1.

SFA: saturated fatty acid; UFA: unsaturated fatty acid; FA: fatty acid; P: phosphate group; PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, PC: phosphatidylcholine, SM: sphingomyelin.

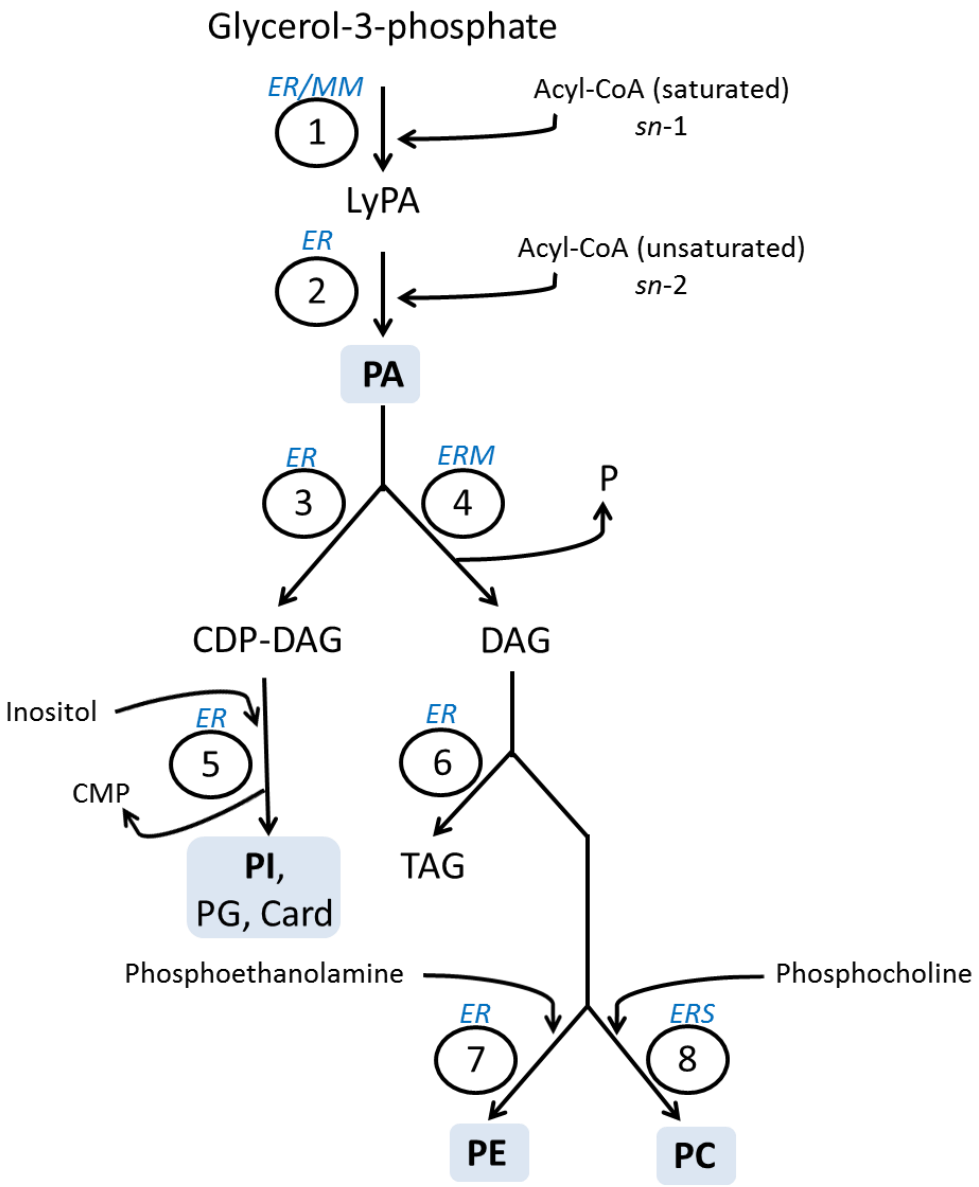


FIG.2

TAG: triacylglycerol; DAG: diacylglycerol; P: phosphate group; CMP: cytidine monophosphate; CDP-DAG: cytidine diphosphate diacylglicerol; LyPA: lysophosphatidic acid; PA: phosphatidic acid; PE: phosphatidylethanolamine, PI: phosphatidylinositol; PC: phosphatidylcholine, PG: phosphatidylglycerol; Card: cardiolipin. The numbers in circles are the enzymes envolved: 1: glycerol 3-phosphate acyltransferase; 2: acylglycerol-3-acyltransferase; 3: CDP-diacylglycerol synthase;4: phosphatidic acid phosphatase;5: CDP-diacylglycerol inositol phosphatidyltransferase;6: diacylglycerol acyltransferase;7:CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransfease ;8: CDP-choline: 1,2-diacylgylcerol cholinephosphotransferase. The italic letters on the circles are the place where the reaction are carried out: ER: endoplasmatic reticulum; ERM: endoplasmatic reticulum membrane; ERS: endoplasmatic reticulum surface; MM: mitochondria membrane

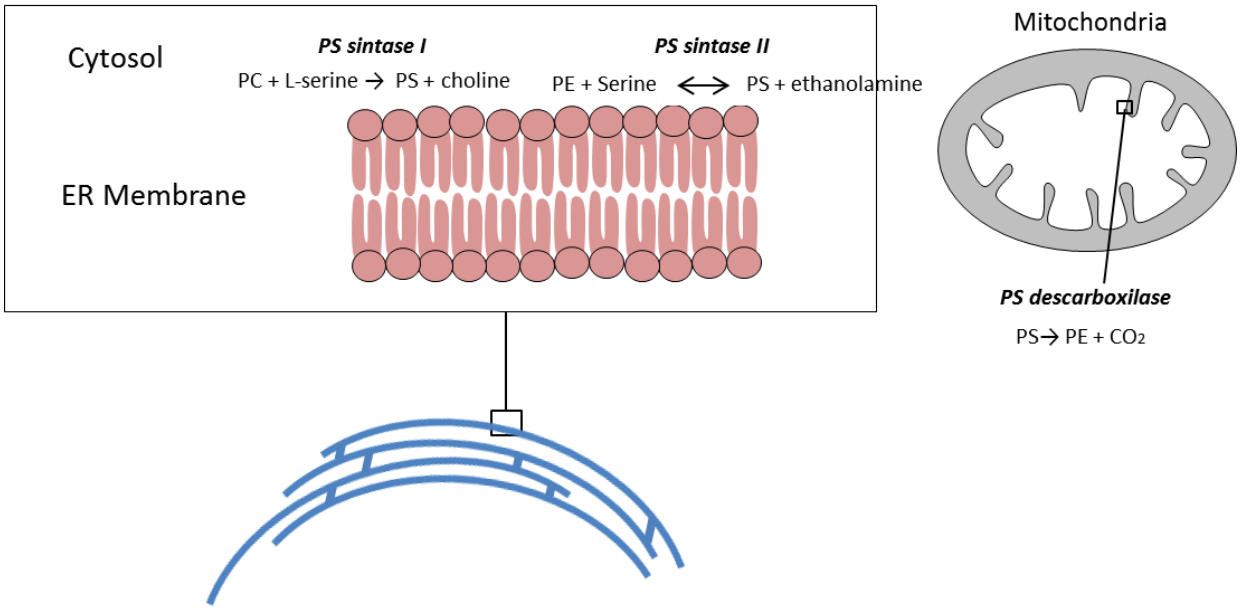


FIG.3

PE: phosphatidylethanolamine; PS: phosphatidylserine

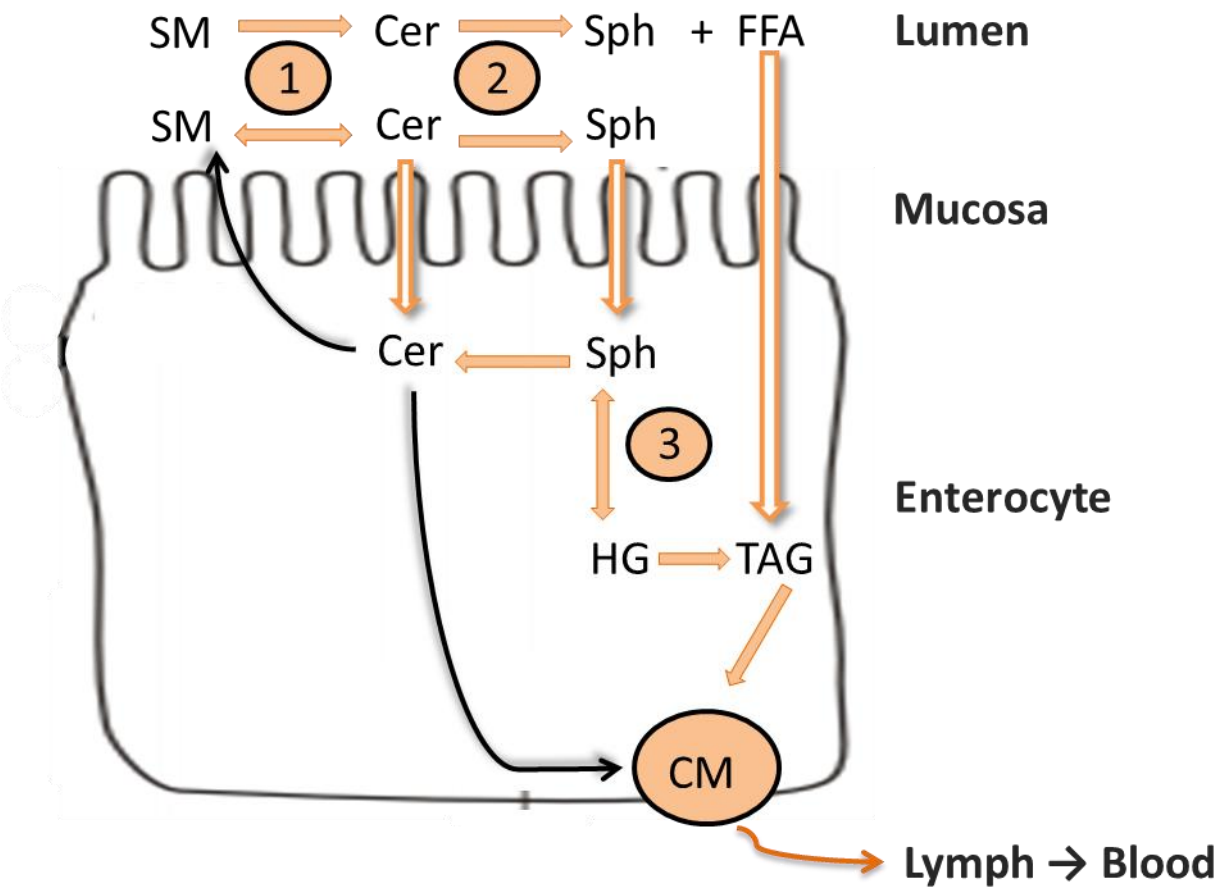


FIG.4

SM: sphingomyelin; Cer: ceramide; Sph: sphingosine; TAG: triacylglecerols; CM: chylomicron; HG: hydrocarbon group. The rounded numbers are the enzymes involved. 1: alkaline sphingomyelase; 2:ceramidase; 3: sphingosine 1 phosphate

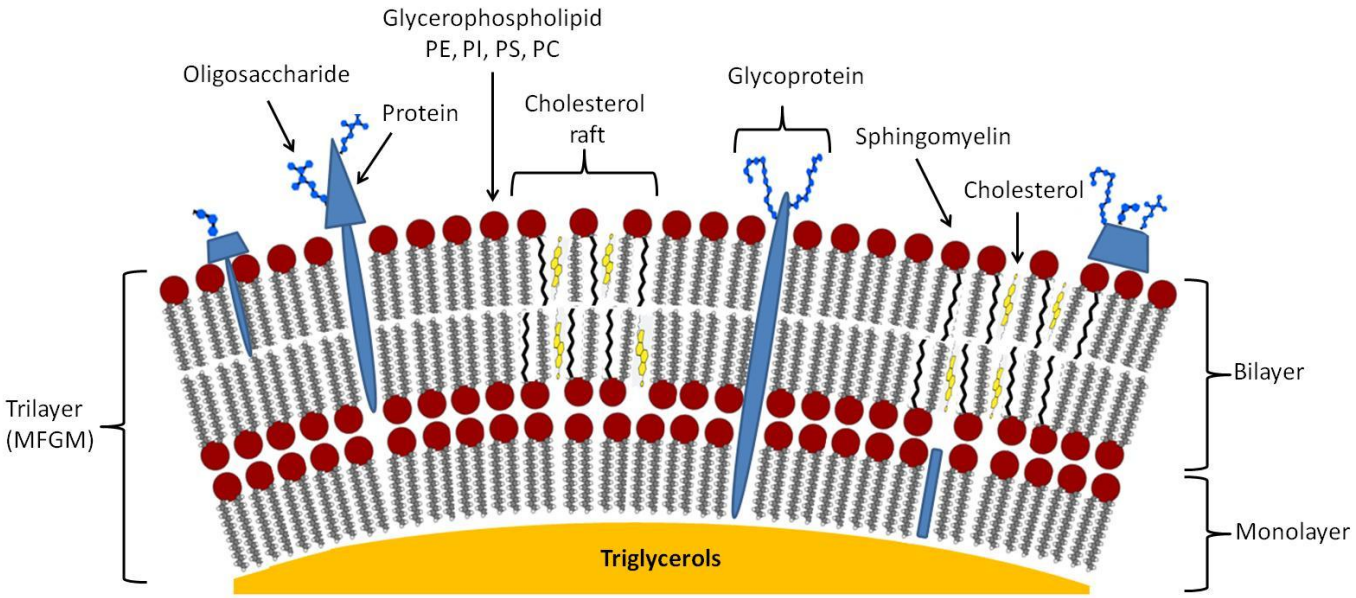


FIG.5

PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, PC: phosphatidylcholine.

1. Desarrollo, optimización y validación de métodos analíticos y técnicas cromatográficas para el aislamiento de fracciones ricas en fosfo- y esfingolípidos y su posterior caracterización.

1.1. Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques.

J. Dairy Sci. 97:6719–6728
http://dx.doi.org/10.3168/jds.2014-8128

Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using a pressurized liquid system and chromatographic techniques

M. P. Castro-Gómez,* L. M. Rodriguez-Alcalá,* M. V. Calvo,* J. Romero,† J. A. Mendiola,* E. Ibañez,* and J. Fontecha*[1]
*Bioactivity and Food Analysis Department, Instituto de Investigación en Ciencias de la Alimentación, Consejo Superior de Investigaciones Científicas (CIAL-CSIC), C/Nicolás Cabrera 9, 28049 Madrid, Spain
†Laboratorio Interprofesional Lácteo de Castilla la Mancha, Avda. Portugal 42, 45600 Talavera de la Reina, Toledo, Spain

ABSTRACT

Although milk polar lipids such as phospholipids and sphingolipids located in the milk fat globule membrane constitute 0.1 to 1% of the total milk fat, those lipid fractions are gaining increasing interest because of their potential beneficial effects on human health and technological properties. In this context, the accurate quantification of the milk polar lipids is crucial for comparison of different milk species, products, or dairy treatments. Although the official International Organization for Standardization-International Dairy Federation method for milk lipid extraction gives satisfactory results for neutral lipids, it has important disadvantages in terms of polar lipid losses. Other methods using mixtures of solvents such as chloroform:methanol are highly efficient for extracting polar lipids but are also associated with low sample throughput, long time, and large solvent consumption. As an alternative, we have optimized the milk fat extraction yield by using a pressurized liquid extraction (PLE) method at different temperatures and times in comparison with those traditional lipid extraction procedures using 2:1 chloroform:methanol as a mixture of solvents. Comparison of classical extraction methods with the developed PLE procedure were carried out using raw whole milk from different species (cows, ewes, and goats) and considering fat yield, fatty acid methyl ester composition, triacylglyceride species, cholesterol content, and lipid class compositions, with special attention to polar lipids such as phospholipids and sphingolipids. The developed PLE procedure was validated for milk fat extraction and the results show that this method performs a complete or close to complete extraction of all lipid classes and in less time than the official and Folch methods. In conclusion, the PLE method optimized in this study could be an alternative to carry out milk fat extraction as a routine method.

Received March 12, 2014.
Accepted July 24, 2014.
[1]Corresponding author: j.fontecha@csic.es

Key words: pressurized liquid extraction, milk lipid, fatty acid, phospholipid

INTRODUCTION

Milk lipid analysis is an important area of research and the field has experienced a new renaissance in the last decades. Although some concern exists about the high amount of saturated fat present in whole milk, the latest advances indicate the presence of bioactive FA, such as short-chain FA and CLA, and other minor components, such as polar lipids (phospholipids and sphingolipids), which may have favorable effects on human blood lipids and other cardiometabolic risk factors (Hilmarsson et al., 2006; Heinze and Actis, 2012; Küllenberg et al., 2012). Polar lipids in milk are the main constituents of the milk fat globule membrane, mainly constituted of phosphatidylcholine (**PC**), phosphatidylethanolamine (**PE**), phosphatidylinositol (**PI**), phosphatidylserine (**PS**) and sphingomyelin (**SM**; Singh, 2006). The interest in these molecules is high due to the potential positive effects on human health of dietary phospholipids (Küllenberg et al., 2012).

For an analysis of the total milk lipid composition, it is necessary to select the appropriate method of lipid extraction for preventing either the loss of some of these components or their chemical changes. The standard milk fat extraction methods, such as the Röse-Gottlieb (ISO, 2001), using a mixture of diethyl ether and *n*-pentane, as well as the method based on extraction with a mixture of hexane:isopropanol proposed by Hara and Radin (1978), give satisfactory results for neutral lipid extraction but they present important disadvantages due to losses of some phospholipids and sphingolipids (Feng et al., 2004; Avalli and Contarini, 2005). In addition, they are often performed manually, involving exhaustive and time-consuming steps and hazardous solvents at the large amounts required to remove the fat from the sample matrix. Moreover, these methods either are incompatible with the extraction of lipids with a wide range of hydrophobicity as phospholipids

or result in lower recoveries (Avalli and Contarini, 2005; Gallier et al., 2010).

One of the most commonly used methods for extracting and purifying lipids is the Folch procedure (Folch et al., 1957). Even though this method is highly efficient for extracting polar lipids, it is also associated with low sample throughput, long time, and large solvent consumption.

All these classical extraction schemes for fat extraction have meanwhile been outperformed by pressurized liquid extraction (**PLE**). Pressurized liquid extraction has developed into the most powerful extraction approach in routine analysis of lipids/FA in biological matrices as well as foods (Schäfer, 1998; Herrero et al., 2005; Señoráns and Luna, 2012). By means of a proper combination of temperature, pressure, time, and number of cycles of extraction, a reduction both in solvent consumption and in the extraction time per sample could be achieved, using the same mixture of solvents as in the traditional methods and offering as an additional advantage the possibility of process automatization (Conte et al., 1997; Macnaughton et al., 1997; Jansen et al., 2006). The aim of this study was to compare the classical extraction methods with a PLE procedure and to validate the procedure for milk fat extraction. Fat yield, FAME composition, triacylglyceride species, cholesterol (**CHOL**) content, and lipid class compositions, with special attention to polar lipids such as phospholipids and sphingolipids, were determined in raw whole cow, ewe, and goat milk.

MATERIALS AND METHODS

Samples

Raw whole milk from 3 different ruminant species (cows, ewes, and goats) was obtained from different farms of Castilla-La Mancha, Spain (10 samples for each species), and analyzed for composition in milk fat and protein by the Interprofessional Dairy Laboratory of Castilla-La Mancha (LILCAM, Castilla-La Mancha, Spain). One hundred milliliters of drawn milk was rapidly frozen and shipped to our laboratory in isothermal containers and then freeze-dried and stored at −35°C until use. A commercial powder skim milk with maximum 1% fat content [Corporación Alimentaria Peñasanta S.A. (CAPSA), Granda-Siero, Asturias, Spain] was used to optimize the lipid extraction conditions by the PLE method.

Reagents

All solvents (dichloromethane, chloroform, hexane, methanol, isooctane, and isopropanol) were HPLC grade and purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland; Labscan brand). Sodium carbonate and sea sand were obtained from Panreac Química S.A. (Barcelona, Spain). Formic acid (98%), trifluoroacetic acid, triethylamine (99.5%), trinonanoin, tritridecanoin, pelargonic acid (C9), tridecanoic acid (C13), myristic acid (C14), palmitic acid (C16), estearic acid (C18), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosahexapentaenoic acid (22:6), monostearin, diolein, PI, PS, PE, SM, PC, and *N*-oleoylethanolamine were purchased from Sigma (Bellefonte, PA). Reference butterfat BCR-164 and BCR-519 (EU Commission, Brussels, Belgium) were purchased from Fedelco Inc. (Madrid, Spain).

Fat Extraction

First, total milk fat amount was determined in the Interprofessional Dairy Laboratory (LILCAM) by either the Röse-Gottlieb method based on solvent extraction according to the official reference procedure (ISO, 2001) and by using an infrared spectrophotometer (MilkoScan; Foss Electric España S.A., Barcelona, Spain) as fat total content determination method.

Milk fat was extracted in our laboratory from each of the 30 stored freeze-dried milk samples (cow, ewe, and goat milk; n = 10) using the following 2 methods:

1) Folch method according to Iverson et al. (2001), modified as follows: from a well-mixed freeze-dried milk sample, a 2-g aliquot was placed in 50-mL centrifuge tubes with 1 mg of previously added trinonanoin as internal standard. Fifteen milliliters of a dichloromethane-methanol solution (2:1, vol/vol) was then added to each tube. The mixture was shaken mechanically for 30 min and centrifuged at 6,600 × *g* for 5 min at 4°C. As much of the upper organic solvent fraction as possible was carefully removed with a pipette. The sediment was washed with 12 mL of a dichloromethane-methanol solution (2:1, vol/vol) and, after shaking for 1 min, the sample was, again centrifuged at 6,600 × *g* for 5 min at 4°C. The removed organic solvent was combined with that previously collected and 3 mL of a 0.9% solution of sodium chloride was added and mixed mechanically for 1 min before the tubes were stored overnight at 4°C. Afterward, they were again centrifuged at 6,600 × *g* for 5 min at 4°C and the bottom dichloromethane layer was collected and filtered through a Whatman 1-phase separator filter paper (Whatman, Maidstone, UK) containing approximately 3 g of anhydrous sodium sulfate. Finally, the extract

was concentrated by removing dichloromethane in a rotatory evaporator and dried under a gentle stream of nitrogen. The extracted fat was weighted before and after in amber vials flushed with nitrogen and stored at −35°C until chromatographic analysis.

2) PLE method: extractions were carried out with an Accelerated Solid Extraction ASE-200 extractor (Dionex Corp., Sunnyvale, CA) using 2 g of freeze-dried milk sample that was well mixed with 2 g of sea sand and loaded into a stainless steel extraction cell covered with filters on both sides. For the maximum milk fat yield, the extraction included the use of dichloromethane-methanol solution (2:1, vol/vol) as solvent mixture and 10.3 MPa of pressure as fixed conditions. The extraction time assayed was either 1 or 2 static cycles of 5 min each and temperatures of 60, 80, or 100°C, using a commercial powder skim milk (see samples above) for optimization. The combined solvent extracts (approximately 11 mL from each cycle) were gently evaporated in a vacuum rotary evaporator (Strike 202 model; Steroglass S.r.l., Perugia, Italy) and the lipid extract was weighed and stored in amber vials, exposed to a stream of nitrogen, and frozen at −35°C until analysis.

FA Determination and Quantification

Fatty acid methyl esters were prepared by base-catalyzed methanolysis of the extracted FA fraction using 2 *N* KOH in methanol as described by the International Organization for Standardization (**ISO**; ISO, 2002). Fatty acid methyl esters were separated using a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2-μm film thickness; Agilent Technologies Inc., Palo Alto, CA) in an Agilent chromatograph (model 6890N; Agilent Technologies Inc.) equipped with a mass spectrometry detector. The column was temperature programmed as in Castro-Gómez et al. (2014) at 7°C/min to 170°C, held at 170°C for 55 min, and then temperature programmed at 10°C/min to 230°C and held at 230°C for 33 min. The injector temperature was set at 250°C. Helium was the carrier gas with a column inlet pressure of 206.9 kPa. The mass spectrometry detector conditions were as follows: transfer line temperature: 250°C, source temperature: 230°C, quad temperature: 150°C, electron impact ionization: 70eV, and the range from 50 to 500 m/z was scanned. For identification of the peaks, the National Institute of Standards and Technology (NIST, Gaithersburg, MD) library and mass spectra of the standards used in our laboratory were used. The injection volume was 1 μL and the split ratio used was 1:25. Response factors were calculated using an anhydrous milk fat (reference butterfat BCR-164) and tritridecanoin as internal standard (200 μL; 1.3 mg/mL) was used.

Triacylglycerides and CHOL Determination

Triacylglycerides (**TAG**) and CHOL analysis of milk fat was performed following Fontecha et al. (2005), on a Clarus 400 gas chromatograph (PerkinElmer Ltd., Beaconsfield, UK) equipped with an automatic split/splitless injector and a flame ionization detector. An Rtx-65TAG fused-silica capillary column (30 m × 0.25 mm i.d. × 0.1-μm film thickness; Restek Corp., Bellefonte, PA) was used. Experimental chromatographic conditions were carried out with a temperature program as follows: 120°C held for 30 s, 10°C/min to 220°C and held for 30 s, and 6°C/min to 350°C and held for 30 min. Injector and flame ionization detector temperatures were 355 and 370°C, respectively. Helium was used as carrier gas (172 kPa) and the injection volume was 0.5 μL of dilutions of milk fat (30 mg/mL) in hexane. For TAG and CHOL determination and quantification, the reference butterfat BCR-519 of known TAG and CHOL composition and glyceryl trinanoate as internal standard (100 μL; 1 mg/mL) were used.

Lipid Class Compositions by HPLC-Evaporative Light Scattering Detection

Separation of lipid classes was accomplished in an HPLC system (model 1260; Agilent Technologies Inc.) coupled with an evaporative light scattering detector (SEDEX 85 model; Sedere SAS, Alfortville Cedex, France) using prefiltered compressed air as the nebulizing gas at a pressure of 350 kPa at 60°C; the gain was set at 3. Two columns in series (250 × 4.5 mm Zorbax Rx-SIL column with 5-μm particle diameter; Agilent Technologies Inc.) and a precolumn with the same packing were used. Before analysis, samples were dissolved in CH_2Cl_2 (at 5 to 30 mg/mL) and 50 μL was injected after column equilibration at 40°C. The solvent gradient was as detailed in Rodríguez-Alcalá and Fontecha (2010), with slight modifications shown in Table 1.

Statistical Analysis

All results are expressed as means and standard deviations (n = 10 for each group). An exploratory analysis of data was performed to test normal distribution and homogeneity of variance. Thus, during the optimization of PLE conditions, the results where compared using the Kruskal-Wallis test.

Table 1. Solvent gradient required for the HPLC-evaporative light scattering detector elution of lipid classes and reactivation of the column

Time (min)	Solvent[1] (%)				Flow (mL/min)
	A	B	C	D	
0.00	0	0	100	0	1.4
5.00	0	0	100	0	1.4
5.10	5	0	95	0	0.5
9.50	9.2	0	90.8	0	0.5
13.50	85	0	15	0	0.5
19.49	100	0	0	0	0.5
19.50	100	0	0	0	1.0
25.00	75	25	0	0	1.0
35.00	0	100	0	0	1.0
45.50	0	0	0	100	1.0
46.00	0	0	0	100	1.0
46.50	0	0	100	0	1.0
47.00	0	0	100	0	1.4
53.00	0	0	100	0	1.4

[1]A = chloroform:methanol:water [87.5:12:0.5 (vol/vol/vol); 1 *M* formic acid; triethylamine; pH 3]; B = chloroform:methanol:water [28:60:12 (vol/vol/vol); 1 *M* formic acid; triethylamine; pH 3); C = isooctane:tetrahydrofuran [99:1 (vol/vol)]; D = 2-propanol.

The proposed PLE method was assayed on cow, ewe, and goat milk and results for lipid classes, FAME, TAG species, and CHOL contents were compared versus isolation using the Folch method. For such comparisons, the Mann-Whitney test was carried out.

All analyses were performed using the SPSS Statistics software (v19.0 for Windows; IBM Corp., Armonk, NY). The level of significance was fixed at $P < 0.05$.

RESULTS AND DISCUSSION

Yield of Milk Lipid Extraction

As is well known, the lipid extraction efficiency by PLE is dependent on the following factors: extraction time, extraction temperature, solvent composition, and number of extraction cycles. Extraction pressure usually does not have an important effect of the extraction yield, given it is kept high enough to maintain the solvent in the liquid state during the extraction; therefore, a pressure of 10.3 MPa was selected. In this study, a commercial skim milk powder with less than 1% fat content and the same sample spiked with 200 mg of anhydrous milk fat were used for optimization of the PLE conditions.

To maximize the yield of milk fat extraction, the effects of 2 factors: extraction temperature (at 60, 80, and 100°C) and extraction time (1, 2, or 3 cycles of 5 min each) were studied. The solvent selected was chloroform:methanol (2:1, vol/vol) because of its ability to extract total polar lipids.

The results of the effect of the extraction temperature are summarized in Table 2. The efficiency of milk fat extraction (% yield) from commercial skim milk powder and from the same milk spiked with 200 mg of milk fat was not significantly different among the temperatures tested, with values close to 100% in all cases. However, when the obtained lipid extract was analyzed to identify and quantify the lipid class contents, the total concentration of the polar lipid fraction at 100°C tended to decrease as the effect of temperature, especially due to the PE and SM, decreased. At the same time, a significant increase ($P < 0.05$) in phosphatidic acid concentration was observed and was related to the degradation of polar compounds. Other phospholipids, such as PI, PS, and PC, appeared to increase slightly but not significantly. Also, a light brown color of the lipid extracts was observed when the extraction temperature was 100°C, which was correlated with the progression of the Maillard reaction. The same trend was observed when the PLE method was used at 80°C, although at a lesser extent than at 100°C. Therefore, 60°C was the temperature selected to be used for the rest of the experiments. On the other hand, the extraction time was set at 2 cycles of 5 min each due to the fact that the extract weight was constant after 10 min.

To attain the maximum fat recovery, the PLE procedure was used applying the optimized parameters described above with 30 samples of freeze-dried raw whole milk from cows, ewes, and goats (n = 10 of each). The results were compared with the values obtained with 2 other extraction techniques: namely, the Röse-Gottlieb method [which is the official extraction procedure for milk fat (ISO, 2001)] and the Folch procedure, modified by Iverson et al. (2001). To accurately know how much fat was originally in the milk samples, infrared spectroscopy by MilkoScan analysis was also used as a quantitative fat-detection technique.

Table 2. Effect of the pressurized liquid extraction (PLE) temperature on the efficiency of milk fat extraction (% yield; mean ± SD) from commercial skim milk powder (SMP) and the same sample spiked with 200 mg of anhydrous milk fat (SSMP) and on the lipid class compositions

	Temperature (°C)			
Item[1]	60	80	100	*P*-value
Yield (%)				
SMP	98 ± 0.3	102 ± 0.7	99 ± 0.5	0.11
SSMP	98 ± 0.8	100 ± 0.5	101 ± 1.1	0.06
Lipid class (%)				
CE	0.17 ± 0.01	0.14 ± 0.02	0.14 ± 0.01	0.11
TAG	87.32 ± 0.49	88.59 ± 0.36	88.82 ± 0.81	0.06
DAG	1.24 ± 0.08	1.18 ± 0.01	1.09 ± 0.04	0.06
CHOL + FFA	1.51 ± 0.13	1.37 ± 0.03	1.23 ± 0.09	0.05
MAG	0.20 ± 0.01	0.24 ± 0.03	0.28 ± 0.03	0.05
GLUCER	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.20
LACCER	0.02 ± 0.01	0.03 ± 0.02	0.06 ± 0.02	0.25
PL	9.46 ± 0.64	8.36 ± 0.36	8.30 ± 0.76	0.11
Lipid compound (% of PL)				
PA	1.01 ± 0.21[a]	1.18 ± 0.10[a]	2.20 ± 0.64[b]	<0.05
PE	19.57 ± 2.31	17.88 ± 1.51	13.33 ± 4.82	0.33
PI	8.30 ± 0.53	8.65 ± 0.80	10.32 ± 1.40	0.19
PS	9.40 ± 1.61	8.88 ± 2.33	11.55 ± 0.79	0.11
PC	36.25 ± 0.46	39.73 ± 1.56	39.29 ± 2.61	0.12
SM	25.48 ± 1.68	23.67 ± 0.41	23.31 ± 2.16	0.43

[a,b]Means with different superscript letters within a same row are significantly different ($P < 0.05$).

[1]SMP = skim milk powder; SSMP = SMP spiked with 200 mg of anhydrous milk fat; CE = cholesterol esters; TAG = triacylglycerides; DAG = diacylglycerides; CHOL = cholesterol; MAG = monoglycerides; GLUCER = glucosylceramides; LACCER = lactosylceramides; PL = polar lipids; PA = phosphatidic acid; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylserine; SM = sphingomyelin.

Figure 1 shows that the detected amount of fat in cow milk by the infrared spectroscopy method was 29.5%, which matched exactly the amount of fat extracted by the ISO and PLE methods. For ewe and goat milk, the amount of fat extracted by the PLE method was slightly lower (but not significantly at $P < 0.05$) than that indicated by infrared spectroscopic determination and that obtained with the ISO method. However, the recovery of milk fat when the Folch method was used was significantly lower ($P > 0.05$). The Folch method was the least efficient method of all the tested procedures; this fact was particularly remarkable for cow milk, providing total milk fat yield that was less than half of the other methods (13.87 vs. 29%). These results are in agreement with other studies by Mulbry et al. (2009) and Boselli et al. (2001), who described higher fat extractions using the PLE procedure (50–75% higher) than using the Folch method for algae and egg noodles, respectively.

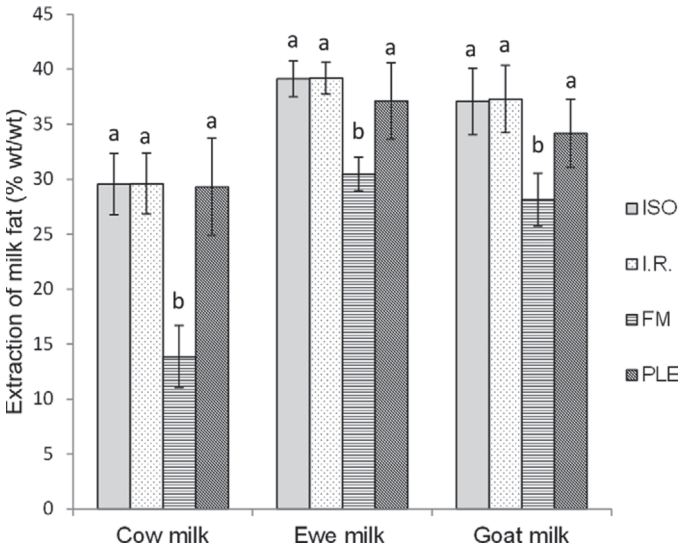


Figure 1. Yield of milk fat extracted (g of fat/100 g of milk powder) using different extraction procedures from cow, ewe, and goat milk powder. ISO = International Organization for Standardization Röse-Gottlieb method, which is the official extraction procedure for milk fat (ISO, 2001); I.R. = infrared spectroscopy method by MilkoScan Foss Electric España S.A., Barcelona, Spain) analysis; FM = Folch method (Folch et al., 1957), modified by Iverson et al. (2001); PLE = pressurized liquid extraction with optimized conditions. Different letters (a and b) show the significant differences between procedures ($P < 0.05$). Error bars represent SD values (n = 10).

Lipid Class Compositions

Comparison of the lipid class compositions of the milk fat from cows, ewes, and goats extracted by the PLE and Folch methods are shown in Table 3. Analyses were done by HPLC-evaporative light scattering detection as in Rodríguez-Alcalá and Fontecha (2010), with

Table 3. Lipid class analysis (mean ± SD) by HPLC-evaporative light scattering detection of the fat from cow, ewe, and goat milk powder extracted with the pressurized liquid extraction (PLE) and Folch (Folch et al., 1957) methods

	Cow milk		Ewe milk		Goat milk	
Lipid class[1] (%)	PLE	FM[2]	PLE	FM	PLE	FM
CE	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TAG	99.08 ± 0.20	99.04 ± 0.19	99.64 ± 0.18	99.53 ± 0.21	99.62 ± 0.06	99.60 ± 0.04
DAG	0.76 ± 0.21	0.80 ± 0.18	0.25 ± 0.13	0.30 ± 0.30	0.26 ± 0.04	0.25 ± 0.02
CHOL + FFA	0.09 ± 0.02	0.09 ± 0.03	0.07 ± 0.04	0.11 ± 0.11	0.08 ± 0.02	0.08 ± 0.01
MAG	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
GLUCER	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
LACCER	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PL	0.07 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.02	0.07 ± 0.01
g/100 g of PL						
PA	0.19 ± 0.18	<0.01	ND[3]	ND	ND	ND
PE	42.00 ± 4.60	46.73 ± 1.65	40.01 ± 3.57	43.04 ± 2.09	41.40 ± 2.43	46.08 ± 2.74
PI	3.98 ± 0.65[a]	0.10 ± 0.28[b]	4.43 ± 0.78[a]	1.53 ± 0.69[b]	5.88 ± 0.98[a]	2.21 ± 0.43[b]
PS	3.45 ± 0.57[a]	0.12 ± 0.34[b]	6.50 ± 1.08[a]	1.61 ± 0.76[b]	9.25 ± 3.73[a]	2.41 ± 0.78[b]
PC	29.30 ± 2.94	33.22 ± 1.88	26.43 ± 3.18	30.54 ± 2.45	27.37 ± 2.66	31.47 ± 1.02
SM	21.07 ± 4.45	19.82 ± 1.06	22.63 ± 4.10	22.28 ± 1.41	16.11 ± 2.25	17.84 ± 2.67

[a,b]Means with different superscript letters within a row and between extraction methods are significantly different ($P < 0.05$).

[1]CE = cholesterol esters; TAG = triacylglycerides; DAG = diacylglycerides; CHOL = cholesterol; MAG = monoglycerides; GLUCER = glucosylceramides; LACCER = lactosylceramides; PL = polar lipids; PA = phosphatidic acid; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylserine; SM = sphingomyelin.

[2]FM = Folch method modified by Iverson et al. (2001).

[3]ND = not detected.

some modifications described previously in the Materials and Methods section. This method permits not only the separation of lipid classes but also a further separation of phospholipids in the same run without prior lipid fractionation, thus allowing their qualitative and quantitative characterization.

The lipid extracts obtained by the ISO method were not analyzed because of its incomplete and minor ex-

Table 4. Comparison of FAME composition (%; mean ± SD) of milk fat obtained from cows, ewes, and goats determined by GC-MS and extracted with the pressurized liquid extraction (PLE) or Folch (Folch et al., 1957) method

	Cow milk		Ewe milk		Goat milk	
FAME (%)	PLE	FM[1]	PLE	FM	PLE	FM
C4:0	3.02 ± 0.25	3.20 ± 0.23	2.37 ± 0.40	2.47 ± 0.30	1.86 ± 0.36	2.01 ± 0.40
C6:0	1.97 ± 0.21	2.08 ± 0.17	1.96 ± 0.16	1.97 ± 0.19	2.32 ± 0.38	2.39 ± 0.47
C8:0	1.15 ± 0.15	1.21 ± 0.14	1.82 ± 0.05	1.78 ± 0.23	2.69 ± 0.44	2.70 ± 0.47
C10:0	2.91 ± 0.47	3.05 ± 0.47	6.07 ± 0.14	6.00 ± 0.99	9.64 ± 1.60	9.71 ± 1.48
C10:1	0.79 ± 0.16	0.84 ± 0.16	0.58 ± 0.09	0.52 ± 0.09	0.68 ± 0.14	0.69 ± 0.14
C12:0	3.16 ± 0.51	3.28 ± 0.53	3.15 ± 0.15	3.15 ± 0.53	4.22 ± 0.52	4.26 ± 0.63
C14:0	11.88 ± 0.96	12.15 ± 0.99	11.76 ± 0.56	11.21 ± 1.00	9.56 ± 0.74	9.63 ± 0.72
C14:1	0.99 ± 0.15	1.02 ± 0.15	0.16 ± 0.01	0.17 ± 0.03	0.14 ± 0.03	0.14 ± 0.02
C15:0	1.05 ± 0.17	1.09 ± 0.17	0.81 ± 0.07	0.85 ± 0.11	0.66 ± 0.09	0.67 ± 0.10
C16:0	32.12 ± 1.68	32.19 ± 1.58	29.69 ± 3.34	29.32 ± 2.02	27.70 ± 2.45	27.69 ± 1.76
C16:1	1.37 ± 0.14	1.38 ± 0.15	1.09 ± 0.09	1.04 ± 0.07	0.70 ± 0.08	0.70 ± 0.08
C17:0	0.56 ± 0.07	0.57 ± 0.06	0.51 ± 0.07	0.55 ± 0.20	0.50 ± 0.04	0.51 ± 0.07
C17:1	0.30 ± 0.06	0.32 ± 0.05	0.24 ± 0.04	0.31 ± 0.07	0.26 ± 0.05	0.27 ± 0.05
C18:0	9.14 ± 1.74	8.93 ± 1.81	11.04 ± 0.68	11.75 ± 1.20	9.88 ± 1.67	9.66 ± 1.94
Total *trans* C18:1	2.65 ± 0.80	2.59 ± 0.81	2.36 ± 0.02	2.39 ± 0.55	2.62 ± 0.57	2.57 ± 0.43
Total *cis* C18:1	22.93 ± 2.05	22.21 ± 1.91	22.28 ± 1.52	22.45 ± 2.57	21.87 ± 2.45	21.70 ± 2.32
cis-9,*cis*-12 C18:2	3.25 ± 0.35	3.00 ± 0.91	2.85 ± 0.01	2.73 ± 0.39	3.71 ± 0.73	3.74 ± 0.70
cis-9,*trans*-11 C18:2 (CLA)	0.38 ± 0.16	0.38 ± 0.18	0.48 ± 0.10	0.52 ± 0.17	0.53 ± 0.15	0.51 ± 0.13
αC18:3	0.25 ± 0.04	0.24 ± 0.09	0.51 ± 0.38	0.53 ± 0.41	0.28 ± 0.05	0.28 ± 0.06
C20:0	0.11 ± 0.02	0.27 ± 0.49	0.29 ± 0.08	0.29 ± 0.09	0.18 ± 0.04	0.17 ± 0.04
Σ SFA	67.09 ± 2.79	68.02 ± 2.56	69.47 ± 2.76	69.35 ± 2.87	69.21 ± 3.04	69.40 ± 2.60
Σ MUFA	29.03 ± 2.56	28.36 ± 2.34	26.70 ± 2.38	26.87 ± 2.53	26.27 ± 2.63	26.07 ± 2.36
Σ PUFA	3.89 ± 0.36	3.61 ± 0.98	3.84 ± 0.38	3.78 ± 0.76	4.52 ± 0.85	4.53 ± 0.76
SFA/UFA[2]	2.06 ± 0.27	2.15 ± 0.26	2.27 ± 0.35	2.29 ± 0.32	2.28 ± 0.33	2.29 ± 0.28

[1]FM = Folch method modified by Iverson et al. (2001).

[2]UFA = unsaturated FA.

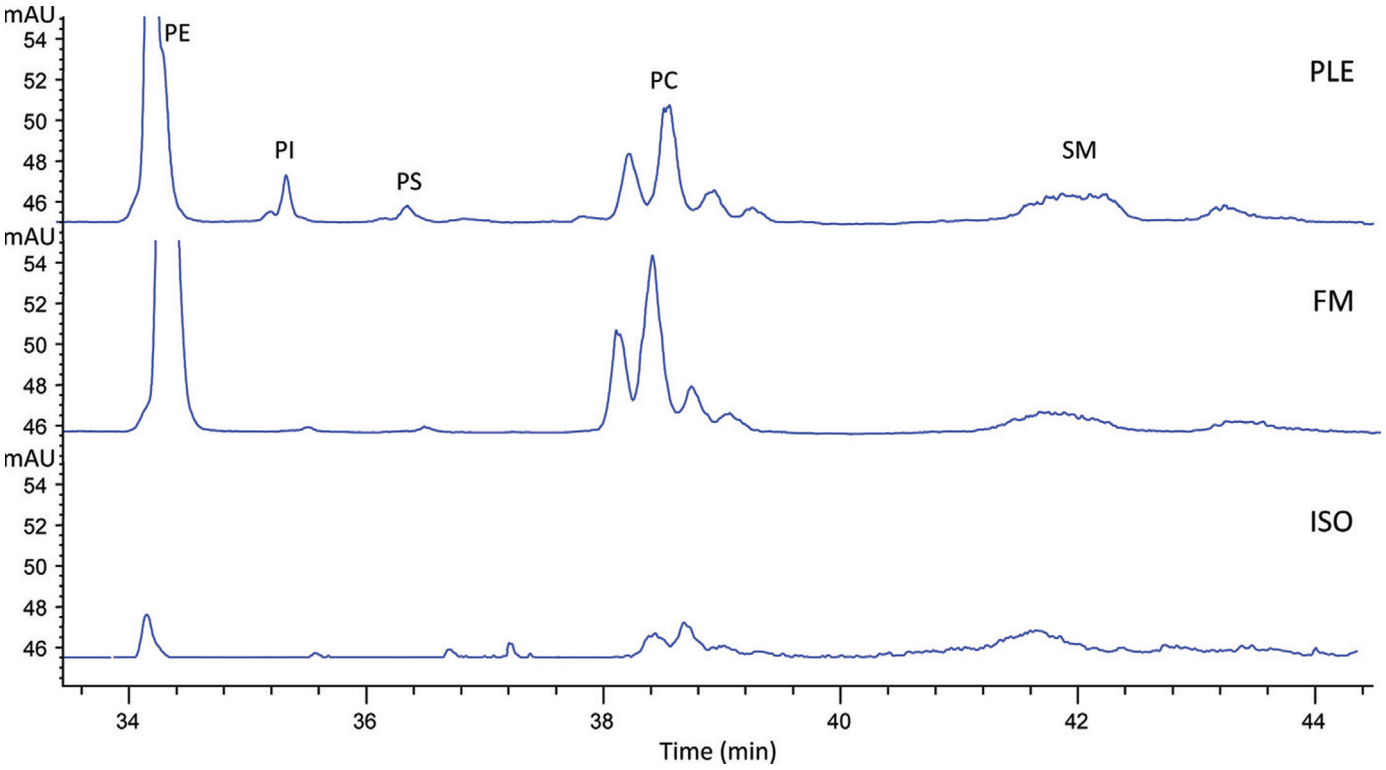


Figure 2. Chromatogram profile area of phospholipids and sphingomyelin from milk fat extracted by the pressurized liquid extraction (PLE), Folch (Folch et al., 1957), and International Organization for Standardization (ISO) methods. ISO = Röse-Gottlieb method, which is the official extraction procedure for milk fat (ISO, 2001); FM = Folch method, modified by Iverson et al. (2001); PLE = PLE with optimized conditions; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylserine; SM = sphingomyelin. Color version available in the online PDF.

traction of polar lipids (Figure 2). This is in agreement with a previous statement that this method does not extract all components and the same amount of polar lipids as some phospholipids (Avalli and Contarini, 2005).

In milk and dairy products, total lipids are dominated by TAG (~98%), whereas polar lipids are only found in trace amounts (0.1%), in agreement with Lopez et al. (2008), who observed less than 0.3% in cow milk fat. As expected, the neutral lipids were the major fraction (at more than 99%) in all samples. Triacylglycerides were the main components, followed by diacylglycerides and CHOL plus FFA. On the other hand, cholesterol esters, monoacylglycerides, glucosylceramides, and lactosylceramides were present in minor amounts. All these components did not show significant differences in their compositions when they were extracted either by the PLE or Folch method. This is in agreement with the results reported by Cescut et al. (2011) for yeast lipids isolated using both a chloroform:methanol extraction method (Bligh and Dyer, 1959) and a PLE method with the same solvents.

The phospholipid concentration was very low in milk (Table 3) and dairy products, except for some by-products of the butter process, such as dried butter serum and buttermilk and also in skim milk powder. Rombaut et al. (2006) obtained a polar lipids amount of 33.05 and 29.06% from fat in buttermilk and butter serum and 19.06% in skim milk, whereas less than 1% was obtained from milk.

As can be seen in Table 3, although the total amount of the polar lipid fraction did not present significant differences among extraction methods used, some of the individual species of phospholipids contained in this fraction, such as PI and PS, were significantly higher when using PLE extraction than with the Folch method. No significant differences were found in the other phospholipids PE, PC, and SM, which were not affected by the extraction method. Zhou et al. (2010) observed similar results after lipid extraction by the Folch method of soybeans, egg yolk, calf brain, and ox liver and reported a recovery of less than 78% of total PI, whereas PE and PC were recovered at more than 90%. Moreover, Cescut et al. (2011) observed higher

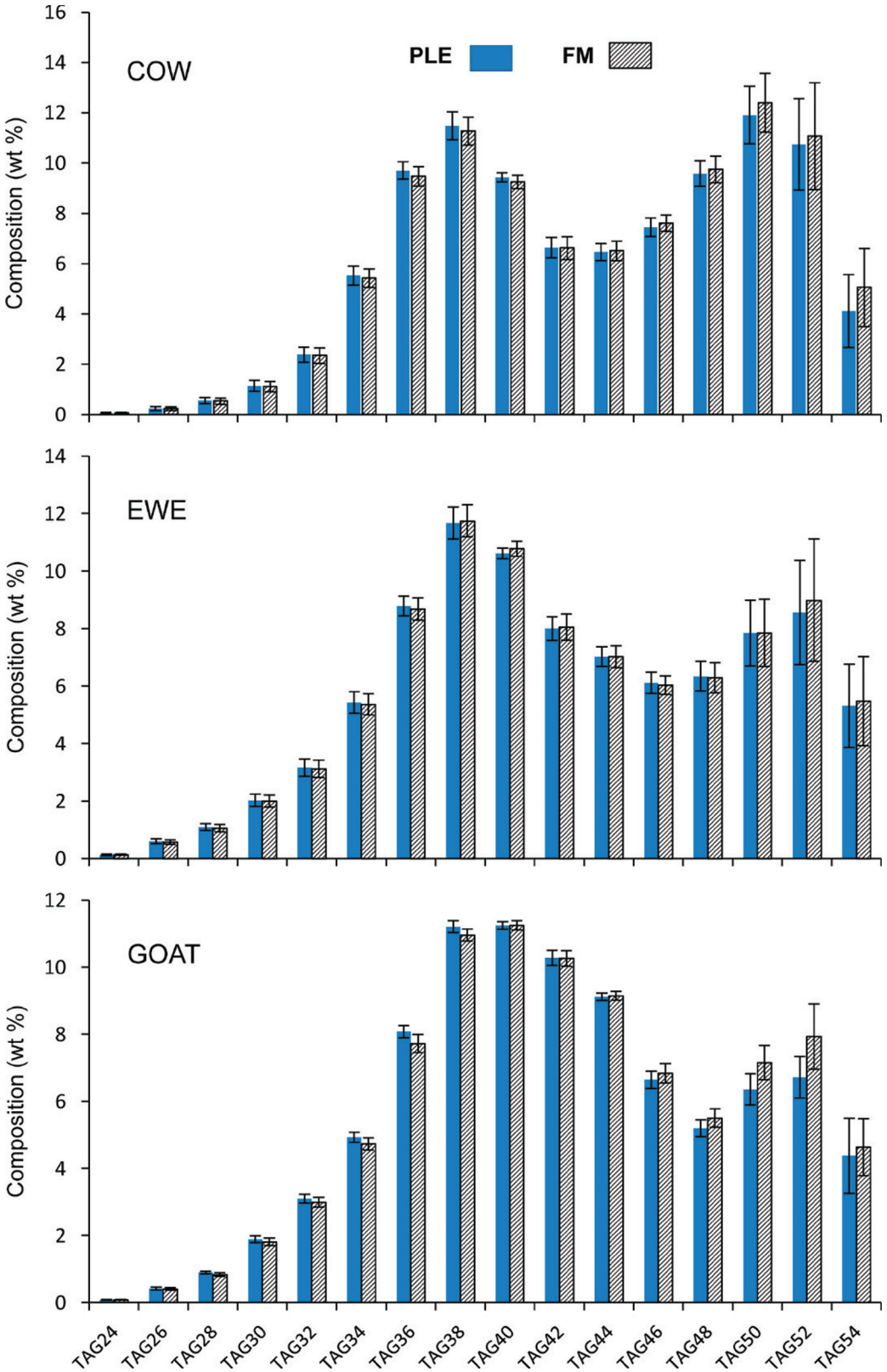


Figure 3. Effect of milk fat extraction by pressurized liquid extraction (PLE) and Folch (Folch et al., 1957) procedures on triacylglyceride (TAG) composition (wt %) from carbon number 24 to 54 (cow, ewe, and goat milk). FM = Folch method, modified by Iverson et al. (2001). Error bars represent SD values (n = 10). Color version available in the online PDF.

PI extraction from yeast when using the PLE method than the Bligh and Dyer (1959) method.

FA Analysis (FAME)

The FAME composition of the milk fat samples extracted with the PLE and Folch methods is shown in Table 4. With both methods, the major FAME in cow, ewe, and goat milk were C16:0, total *cis* C18:1, C14:0, and C18:0, showing values between 27.7 to 32.2%, 21.7 to 22.9%, 9.5 to 12.1%, and 8.9 to 11.7%, respectively. Although short-chain FA (C4–C10) had a slight trend to increase with milk extracted with the Folch method modified by Iverson et al. (2001), neither of the FAME displayed significant differences ($P < 0.05$) among the extraction methods. This is in agreement with Toschi et al. (2003), Mulbry et al. (2009), and Cescut et al. (2011), who did not find differences in FAME composition in poultry meat, algae, and yeast, respectively, when comparing a similar Folch method with PLE extraction with chloroform:methanol.

It might be reasonable to expect differences in the FAME profile, given that the PLE method extracted higher amounts of some phospholipids, such as PI and PS, than the Folch method. However, being minor compounds, this fact did not significantly affect to the total FAME profile.

TAG and CHOL Composition

A great deal of interest exists in the determination of TAG and CHOL composition of dairy fats because of their influence on technological properties (melting point and crystallization behavior, among others), but especially because of their role in nutrition and cardiovascular diseases. Figure 3 shows the profiles of TAG from cow, ewe, and goat milk fat, considering the quantification of 16 groups, corresponding to TAG of 24 to 54 carbon atoms. The TAG composition presented the same distribution as those in previous studies of TAG of milk fat from different species reported under similar experimental conditions (Fontecha et al., 2005). Low-molecular-weight TAG (C24–C40) are related to those containing short-chain FA, (e.g., C4, C6, or C8), and the medium-chain TAG (C42–C46), which include most C10 and C12, are TAG mainly present in dairy fats. These TAG (short chain and medium chain) are generally considered a good biologically inert source of energy because they diffuse from the gastrointestinal tract to the portal system without requirement for modification and, therefore, can help in the process of excess calorie burning and weight loss (Tsuji et al., 2001; Marten et al., 2006). The rest of TAG with high molecular weight (C48–C54) contain the 3 long-chain FA. This distribution is also related to the need for maintaining the fluid condition of the fat at physiological temperatures.

Although a tendency exists for slightly higher extraction of higher-molecular-weight TAG than the lower-molecular-weight TAG by the Folch method compared with the PLE method, none of the TAG groups described showed significant differences ($P < 0.05$) between the extraction methods tested. With regard to CHOL, also no significant differences in the amount of CHOL from different milk fat species were observed when using the PLE or Folch extraction methods. Cholesterol values (in g/100 g of total fat) were 0.43 ± 0.08 and 0.43 ± 0.05 in cows, 0.35 ± 0.04 and 0.37 ± 0.04 in ewes, and 0.37 ± 0.02 and 0.39 ± 0.02 in goats for the PLE and Folch methods, respectively.

CONCLUSIONS

The optimized PLE method proved to be capable of extracting milk lipids efficiently. Dichloromethane:methanol extracts gave the best results in terms of fat yield and 60°C did not alter the milk fat chemical composition. Furthermore, although TAG, FAME, and CHOL extraction did not present differences between methods, the PLE method extracted the phospholipids PI and PS much better and faster (only 10 min) than the official ISO method and most of the methods usually used to extract lipids from milk. In conclusion, the PLE method could be a valuable alternative to extract milk fat as a routine method. The PLE method used less than the half of the solvent mixture necessary to carry out the milk fat extraction compared with other methods, and the time applied per sample was significantly lower and offers the possibility of automation.

ACKNOWLEDGMENTS

This work was financially supported by the projects AGL2011-26713 and FUN-C-FOOD CSD2007-00063 provided by the Spanish Ministry of Science and Innovation and the ALIBIRD P2009/AGR-1469 project from the Community of Madrid.

REFERENCES

Avalli, A., and G. Contarini. 2005. Determination of phospholipids in dairy products by SPE/HPLC/ELSD. J. Chromatogr. A 1071:185–190.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.

Boselli, E., V. Velazco, M. F. Caboni, and G. Lercker. 2001. Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food. J. Chromatogr. A 917:239–244.

Castro-Gómez, P., J. Fontecha, and L. M. Rodríguez-Alcalá. 2014. A high-performance direct transmethylation method for total fatty

acids assessment in biological and foodstuff samples. Talanta 128:518–523.

Cescut, J., E. Severac, C. Molina-Jouve, and J. L. Uribelarrea. 2011. Optimizing pressurized liquid extraction of microbial lipids using the response surface method. J. Chromatogr. A 1218:373–379.

Conte, E., R. Milani, G. Morali, and F. Abballe. 1997. Comparison between accelerated solvent extraction and traditional extraction methods for the analysis of the herbicide diflufenican in soil. J. Chromatogr. A 765:121–125.

Feng, S., A. L. Lock, and P. C. Garnsworthy. 2004. A rapid method for determining fatty acid composition of milk. J. Dairy Sci. 87:3785–3788.

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.

Fontecha, J., H. Goudjil, J. J. Ríos, M. J. Fraga, and M. Juárez. 2005. Identity of the major triacylglycerols in ovine milk fat. Int. Dairy J. 15:1217–1224.

Gallier, S., D. Gragson, C. Cabral, R. Jiménez-Flores, and D. W. Everett. 2010. Composition and fatty acid distribution of bovine milk phospholipids from processed milk products. J. Agric. Food Chem. 58:10503–10511.

Hara, A., and N. S. Radin. 1978. Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90:420–426.

Heinze, V. M., and A. B. Actis. 2012. Dietary conjugated linoleic acid and long-chain n-3 fatty acids in mammary and prostate cancer protection: A review. Int. J. Food Sci. Nutr. 63:66–78.

Herrero, M., P. J. Martín-Álvarez, F. J. Señoráns, A. Cifuentes, and E. Ibáñez. 2005. Optimization of accelerated solvent extraction of antioxidants from Spirulina platensis microalga. Food Chem. 93:417–423.

Hilmarsson, H., L. V. Larusson, and H. Thormar. 2006. Virucidal effect of lipids on visna virus, a lentivirus related to HIV. Arch. Virol. 151:1217–1224.

ISO (International Organization for Standardization). 2001. Milk and milk products—Extraction methods for lipids and liposoluble compounds. ISO 14156:2001-IDF 172:2001. ISO, Geneva, Switzerland.

ISO (International Organization for Standardization). 2002. Milk fat—Preparation of fatty acid methyl esters. ISO 15884-IDF 182:2002. ISO, Geneva, Switzerland.

Iverson, S. J., S. L. C. Lang, and M. H. Cooper. 2001. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36:1283–1287.

Jansen, B., K. G. J. Nierop, M. C. Kotte, P. de Voogt, and J. M. Verstraten. 2006. The applicability of accelerated solvent extraction (ASE) to extract lipid biomarkers from soils. Appl. Geochem. 21:1006–1015.

Küllenberg, D., L. A. Taylor, M. Schneider, and U. Massing. 2012. Health effects of dietary phospholipids. Lipids Health Dis. 11:3.

Lopez, C., V. Briard-Bion, O. Menard, F. Rousseau, P. Pradel, and J.-M. Besle. 2008. Phospholipid, sphingolipid, and fatty acid compositions of the milk fat globule membrane are modified by diet. J. Agric. Food Chem. 56:5226–5236.

Macnaughton, S. J., T. L. Jenkins, M. H. Wimpee, M. R. Cormiér, and D. C. White. 1997. Rapid extraction of lipid biomarkers from pure culture and environmental samples using pressurized accelerated hot solvent extraction. J. Microbiol. Methods 31:19–27.

Marten, B., M. Pfeuffer, and J. Schrezenmeir. 2006. Medium-chain triglycerides. Int. Dairy J. 16:1374–1382.

Mulbry, W., S. Kondrad, J. Buyer, and D. L. Luthria. 2009. Optimization of an oil extraction process for algae from the treatment of manure effluent. J. Am. Oil Chem. Soc. 86:909–915.

Rodríguez-Alcalá, L. M., and J. Fontecha. 2010. Major lipid classes separation of buttermilk, and cows, goats and ewes milk by high performance liquid chromatography with an evaporative light scattering detector focused on the phospholipid fraction. J. Chromatogr. A 1217:3063–3066.

Rombaut, R., J. V. Camp, and K. Dewettinck. 2006. Phospho- and sphingolipid distribution during processing of milk, butter and whey. J. Food Technol. 41:435–443.

Schäfer, K. 1998. Accelerated solvent extraction of lipids for determining the fatty acid composition of biological material. Anal. Chim. Acta 358:69–77.

Señoráns, F. J., and P. Luna. 2012. Sample preparation techniques for the determination of fats in food. Pages 203–211 in Comprehensive Sampling and Sample Preparation. J. Pawliszyn, ed. Academic Press, Oxford, UK.

Singh, H. 2006. The milk fat globule membrane—A biophysical system for food applications. Curr. Opin. Colloid Interface Sci. 11:154–163.

Toschi, T. G., A. Bendini, A. Ricci, and G. Lercker. 2003. Pressurized solvent extraction of total lipids in poultry meat. Food Chem. 83:551–555.

Tsuji, H., M. Kasai, H. Takeuchi, M. Nakamura, M. Okazaki, and K. Kondo. 2001. Dietary medium-chain triacylglycerols suppress accumulation of body fat in a double-blind, controlled trial in healthy men and women. J. Nutr. 131:2853–2859.

Zhou, L., J. Le Grandois, E. Marchioni, M. Zhao, S. Ennahar, and F. Bindler. 2010. Improvement of total lipid and glycerophospholipid recoveries from various food matrices using pressurized liquid extraction. J. Agric. Food Chem. 58:9912–9917.

1.2. A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples.

Talanta 128 (2014) 518–523

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples

Pilar Castro-Gómez, Javier Fontecha, Luis M. Rodríguez-Alcalá *

Department of Bioactivity and Food Analysis, Lipid Group, Institute of Food Science Research (CIAL), CSIC-UAM, Autónoma University of Madrid, Nicolás Cabrera, 9, 28049, Madrid, Spain

ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form
16 May 2014
Accepted 23 May 2014
Available online 2 June 2014

Keywords:
Direct derivatization
Fatty acid analysis
Fatty acid methyl esters
Milk fat
CLA
Plasma

ABSTRACT

Isolation is the main bottleneck in the analysis of fatty acids in biological samples and foods. In the last few decades some methods described direct derivatization procedures bypassing these steps. They involve the utilization of methanolic HCL or BF3 as catalysts, but several evidences from previous works suggest these reagents are unstable, lead to the formation of artifacts and alter the distribution of specific compounds as hydroxy fatty acids or CLA. However, the main issue is that they are excellent esterification reagents but poor in transterification, being not suitable for the analysis of all lipid classes and leading to erroneous composition quantitations. The present research work is a comprehensive comparison of six general methylation protocols using base, acid or base/acid catalysts plus a proposed method in the analysis of total fatty acids in lipid standards mixtures, foodstuff and biological samples. The addition of aprotic solvents to the reaction mixture to avoid alterations was also tested. Results confirmed that procedures solely involving acid catalyst resulted in incomplete derivatizations and alteration of the fatty acid profile, partially corrected by addition of the aprotic solvent. The proposed method combining sodium methoxyde and sulfuric acid showed absence of alteration of the FAME profile and the best values for response factors (short chain fatty acids to PUFA), accuracy in the determination of total cholesterol and derivatization performance, thus showing a high reliability in the determination of the total fatty acid composition in biological samples and foods.

1. Introduction

In the recent years analysis of lipids has gained much attention as it was demonstrated that specific fatty acids (FA) may exert an important role in the prevention of human diseases [1]. Conjugated linoleic acid isomers (CLA), mainly found in dairy products, are described as a potent anticarcinogenic agent [2]. Polyunsaturated fatty acids (PUFAs) have been associated with prevention of sudden cardiac death and arrhythmia [3], participation in development of newborn's nervous system [4] and positive effects on depression or Alzheimer's disease [5,6]. FA compounds are routinely analyzed by gas liquid chromatography as methyl esters after isolation of lipids, which is time-consuming and cumbersome. One-step direct derivatization (DT) procedures bypass these extraction steps. Transesterification of glycerolipids is catalyzed by alkali in methanol (potassium hydroxide, sodium methoxide at room temperature, less than 5 min) [7]. On the other hand acids (BF_3, HCl or H_2SO_4, 50–100 °C, 5–60 min) are suitable for the esterification of free fatty acids (FFA) and transmethylation of sphingomyelins [8]. However, some issues arise from the use of acid reagents: first, they lead to protonation of alcohols, which react with CLA and hydroxy FA to form artifacts and *trans* isomers of FA, resulting in erroneous quantitative and qualitative compositions [9]. Aprotic solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO) added to the reaction mixture can avoid these alterations, preventing the addition of protonated methanol to the double bonds [10].

While methanolic hydrochloric acid can be prepared bubbling hydrogen chloride into dry methanol or from acetyl chloride, HCL reacts violently with methanol resulting in chloromethane, water and acidic gas, leading to the loss of 50% of the tritratable acid in six weeks at room temperature [11]. Moreover commercial BF_3 in methanol (14%) is one of the most common reagents; several authors have reported great instability and formation of artifacts [12,13]. Finally, although methanol in the presence of sulfuric acid can form dimethyl sulfate it can be prepared daily adding acid to cooled alcohol showing a good stability. Then the selection of the acid catalyst arises as an important question as to assess total FA composition in large studies of foods and biological samples a high-throughput procedure has to be rapid, accurate and complete. This latter requirement is very important for the calculation of the real concentration of each compound as well as to avoid degradation of the capillary column. In general, derivatization

* Corresponding author. Tel.: +34 91 0017900; fax: +34 91 0017905.

http://dx.doi.org/10.1016/j.talanta.2014.05.051
0039-9140/

methods involve one single reagent; then a basic/acid method seems to be the most suitable approach as that of Bondia-Pons et al. [14] using sodium methoxide/BF_3. Even more, it would be interesting to test if H_2SO_4/MeOH solutions, according to their ease of preparation, can be used after the transesterification reagent.

The aim of the present work is to compare the most commonly used DT procedures plus one proposed method involving both base catalyst and sulfuric acid to select the most appropriate procedure for the analysis of total FA in samples from clinical trials or food quality control.

2. Materials and methods

2.1. Chemicals and standards

Hexane, dichloromethane, N,N-dimethylformamide, 2-propanol (HPLC grade) and sulfuric acid (98%) were purchased from LABSCAN (Dublin, Ireland) while toluene (Analytic grade) was from Carlo Erba Reagents-SdS (Sabadell, Spain). Potassium hydroxide (85%), anhydrous hydrogen sulfate (97%), sodium chloride (99%) and sodium bicarbonate (99.9%) were from Panreac (Barcelona, Spain). Supelco 37 fame mix, sodium methoxide, acetyl chloride (puriss, 99%), boron trifluoride (14% in methanol), cholesterol (99%; Ch), cholesteryl palmitate (98%; CE-C16), dilinolein (98%; DG-C18:2), methyl nonadecanoate (99.5%; FAME-C19) and methyl tricosanoate (99%; FAME-C23) were obtained from Sigma (St. Louis, Missouri, USA). Glyceryl tritridecanoate (99%; TG-C13), monolinolenin (99%; MG-C18:3) and margaric acid (99%; FFA-C17) were purchased from Nu-Chek Prep, Inc. (Elysian, Minnesota, USA). Skim milk was purchased from Scharlau (Sentmenat, Barcelona, Spain) while reference butterfat CRM-164 (EU Commission; Brussels, Belgium) was from Fedelco Inc. (Madrid, Spain). Commercial CLA-rich oil (Tonalin® TG-80; 80% g CLA/100 oil) was kindly donated by Cognis (Düsseldorf, Germany). As plasma samples, an animal-based control serum was used (seronorm lipid, SERO AS, Stasjonsveien, Norway).

2.2. Lipid standard solutions and mixtures

Stock solutions of TG-C13, DG-C18:2, MG-C18:3, Ch, CE-C16, FFA-C17 and FAME-C19 were prepared in dichloromethane (25 mg/mL). Serial dilutions for each compound (1.5, 1, 0.75, 0.50, 0.25 and 0 mg/mL) were used to obtain the corresponding calibration curves. A solution of TG-C13, DG-C18:2, MG-C18:3, Ch, CE-C16 and FFA-C17 (5 mg compound/mL) was prepared in duplicate and labeled as LM1 and LM2. Tritridecanoin and methyl tricosanoate were accurately weighed to obtain a mixture of 1.40 mg/mL for each compound (EM).

2.3. Derivatization methods

In the present study six general methylation protocols using base, acid or base/acid catalysts were compared: potassium hydroxide (KOH) [15], sodium methylate/boron trifluoride in methanol (MBF) [14], boron trifluoride in methanol (BF3) [16], acetyl chloride in methanol (ACL) [17] and sulfuric acid (H_2SO_4) assayed according to Christie et al. [18] as follows: samples were incubated (details about temperature and time are given in the subsection*Experiment 1*) with 3 mL 1 M H_2SO_4 in methanol *then cooled in an ice bath* followed by pipetting 1 mL hexane, 1 min vortex (waiting 5 min for the separation of layers), addition of 7.5 mL 6% w/v Na_2CO_3 and finally 3500 rpm, 10 min. Upper layer was collected and transferred into vials.

Two proposed basic/acid methods were assayed. The first (MHS) involved a sodium methoxide (MetNa) transesterification (2.5 mL 0.5 M, 80 °C, 10 min) and the second (KHS) was with potassium hydroxide (0.250 mL 2 N plus 1 mL hexane at room temperature 5 min) and both followed by 1 M H_2SO_4.

All reactions were accomplished in 16 mL borosilicate glass tubes (16×125 mm^2) with acid/heat resistant cap sealed with a PFTE thread tape (5 cm × 12 mm × 0.10 mm) (Fisher scientific, Madrid, Spain). Tubes were placed into an incubator with shaker function (1250 rpm/min) (TR100-G; JP Selecta, Barcelona, Spain).

Methods involving an acid catalyst were studied with the addition of the aprotic solvents DMF and DMSO in the reaction mixture (one-third of the total volume incorporated prior to reagent) to test, qualitatively and quantitatively, the prevention of alterations in the FA composition with special attention to the CLA isomer profile.

2.4. Experiments

2.4.1. Experiment 1

As part of the development of the proposed method (MHS, KHS), an assay was carried out to select the most suitable temperature and time conditions for acid catalyst: 200 μL of lipid mixtures (LM1, LM2) was placed in separated tubes and dried with a N_2 stream. Mixtures were incubated at 30 °C/60 °C/100 °C during 5/30/60 min according to the H_2SO_4 method. The selected conditions were used in further experiments for MHS and KHS.

2.4.2. Experiment 2

This assay was intended to assess the efficiency of different DT methods MBF, BF3, ACL plus the proposed MHS and KHS in the derivatization of 200 μL of samples LM1 and LM2 (previously evaporated to addition of reagents). To know the suitability of the basic catalyst in MHS and KHS, KOH and MetNa were also tested with the lipid mixtures. The impact of utilization of aprotic solvents when using an acid catalyst was also studied.

2.4.3. Experiment 3

This assay was designed to know the possible alteration of the fatty acid profile focusing in CLA when using BF3, ACL, MBF and MHS and the protective properties of aprotic solvents in the derivatization of CLA-rich oil (Tonalin®; 5 mg). KOH was used as the control method as it is an international standardized protocol using a non-altering reagent.

2.4.4. Experiment 4

A response factor (RF) quantification assay to test the feasibility of the obtained FA composition was carried out using 500 μL of reconstituted skim milk powder (10% w/v) spiked with CRM-164 to a final concentration of 10 mg/mL. As a methylation control, the KOH method was used after lipid extraction [19].

2.4.5. Experiment 5

Finally, methods showing a complete derivatization, which did not alter the composition of Tonalin® oil and with good RF, were used in the FAME analysis of 100, 250 and 500 μL of an animal-based control serum. Samples were spiked with 200 μL of EM solution to test derivatization efficiency.

2.5. Analytical methods

Analysis of lipids and the corresponding FAME after derivatization in Experiments 1 and 2 were accomplished in a CLARUS 400 gas chromatograph (Perkin Elmer, Massachusetts, USA) equipped with a FID detector and a Rtx-65TG column (30 m × 0.25 mm × 0.10 μm; Resteck Corporation, Bellefonte, PA, USA). Temperature program conditions were 120 °C held for 30 s, 10 °C/min to 220 °C held for 30 s, and 6 °C/min to 350 °C held for 30 min. Injector and FID temperatures were 355 °C and 370 °C, respectively. Helium was used

as carrier gas (25 psig; split ratio 4:1) and the injection volume was 0.5 μL.

FAME formation (measure moles × 100)/theoretical moles), disappearance of TG, CE, FFA, DG and MG (100-(final moles × 100/initial moles) and recovery total cholesterol (measure moles (Free+CE) × 100/theoretical moles (Free+CE)) were calculated using concentration values from the corresponding calibration curve.

In experiments 1–3 FAME were analyzed (1 μL; 1:10 split ratio) in a 6890 Agilent GLC (Palo Alto, CA, USA) fitted with an MS detector (Agilent 5973N) operated in the scan mode (50–550 Da) and equipped with 100 m a CPSil-88 capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, Chrompack, Middelburg, the Netherlands). Chromatographic conditions were as in Rodriguez-Alcala and Fontecha [20].

2.6. Statistical analysis

All the experiments were carried out in triplicate. Data were analyzed according to the general linear model (GLM) using Bonferroni's test for multiple comparisons and Pearson correlation of SPSS Statistics software v22.0 for Mac (IBM, Armonk, NY, USA). Level of significance was $p < 0.05$.

3. Results and discussion

3.1. Experiment 1

Table 1 shows the obtained results for FAME formation, lipids disappearance and total cholesterol recovery after derivatization of LM solutions with H_2SO_4 assaying 30/60/100 °C and 5/30/60 min of reaction time. Triglyceride (TG-C13), diglyceride (DG-C18:2) and cholesterol esters (CE-C16) were not completely transesterified although temperature and time increased FAME formation and lipid disappearance ($p < 0.05$). Significant differences were not found in the results using conditions above 60 °C and 30 min. However it was observed that an increment in FAME C18:3 formation was accompanied by higher values for FAME C13 and FAME C18:2 ($p < 0.05$). This effect can be explained attending to the emulsifying properties of MG; in contact with methanol this compound forms micelles emulsifying TG and DG and protecting them from transesterification. Thus, some authors have reported a low derivatization rate for MG [21]. When hexane is added for extraction, micelles locate in the interface. In order to avoid collecting methanol, the organic layer was not completely gathered. As MG are converted into FAME, it increases the availability of TG and DG to react. On the other hand, the rapid derivatization of FFA acids agrees with the fact that they are polar compounds dissolving in methanol. Therefore, kinetics of reaction is influenced by solubility in methanol.

In further experiments, the selected acid derivatization conditions for H_2SO_4 were 60 °C and 30 min.

3.2. Experiment 2

In previous trials carried out by the authors assaying DMSO in DT, a peak coeluted with the same retention time of pentadecanoic acid (C15:0), whereas DMF did not interfere with any FA peak. Therefore only DMF was selected in the tested reaction mixtures of this work. In this experiment the selected acid conditions for H_2SO_4 were tested in basic/acid derivatization of a lipid mixture using sodium methoxyde (MHS) or potassium hydroxide (KHS) and compared versus other DT procedures.

According to the obtained results (Table 2), complete derivatization was only accomplished by the MBF and MHS methods. The obtained results for BF3 and ACL did not agree with the studies where those methods were developed as seen by the fact that diglycerides and free fatty acids were converted into FAME. In the present research work this was only observed for FFA C17 in accordance with the results of experiment 1 and others elsewhere reviewed stating that acid catalysts are suitable for esterification [22].

Although results from the transesterification of TG C13, DG C18:2 and MG C18:3 using KOH were promising, when combined with sulfuric (KHS), FAME formation for the corresponding compounds decreased ($p < 0.05$). Elsewhere it has been reported that hydroxide bases can lead to the hydrolysis of FAME [7]. The low conversion rates of glycerolipids observed for MetNa suggest that emulsifying compounds can interfere with derivatization. This agrees with the increments in reaction performance when DMF was added, for all the methods involving an acid catalyst. Data suggest that DMF may contribute to decrease the surface tension and thus the size of the emulsion droplets. It was observed that using MBF leads to the overestimation of FAME formation rates (C13, C16, C17, C18:2 and C18:3) but addition of this solvent improved the results ($p < 0.05$).

The KHS method was showed to be inaccurate in the analysis of FAME from CE and MG as well as it did not allow the determination of total cholesterol (sum of free and from CE). Therefore, it was discarded for further experiments.

Table 1
TG-C13, CE-C16, FFA-C17, DG-C18:2 and MG-C18:3 disappearance (%), FAME formation (%) and recovery of cholesterol after assaying the H_2SO_4 method at 30, 60 and 100 °C during 5, 30 and 60 min (Experiment 1).

	30 °C			**60** °C			**100** °C		
	5 min	**30 min**	**60 min**	**5 min**	**30 min**	**60 min**	**5 min**	**30 min**	**60 min**
FAME-C13	$n.d^{D}$	4 ± 1^{C}	10 ± 0.1^{B}	5 ± 0.1^{C}	31 ± 1^{A}	40 ± 3^{A}	33 ± 1^{A}	34 ± 1^{A}	33 ± 0.1^{A}
TG-C13	23 ± 7^{C}	31 ± 7^{B}	41 ± 1^{B}	41 ± 5^{B}	67 ± 1^{A}	73 ± 2^{A}	67 ± 0.1^{A}	67 ± 1^{A}	67 ± 5^{A}
FAME-C16	$n.d^{B}$	$n.d^{B}$	$n.d^{B}$	$n.d^{B}$	23 ± 0.1^{A}	30 ± 3^{A}	23 ± 6^{A}	21 ± 1^{A}	25 ± 4^{A}
CE-C16	28 ± 8^{D}	30 ± 9^{C}	30 ± 2^{C}	42 ± 2^{B}	54 ± 7^{AB}	65 ± 3^{A}	55 ± 0.1^{A}	50 ± 3^{AB}	56 ± 1^{A}
FAME-C17	69 ± 7^{C}	100 ± 3^{B}	104 ± 1^{B}	92 ± 8^{B}	104 ± 1^{AB}	100 ± 9^{AB}	107 ± 1^{A}	107 ± 2^{A}	105 ± 2^{A}
FFA-C17	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1
FAME-C18:2	2 ± 1^{D}	8 ± 1^{C}	16 ± 1^{B}	9 ± 0.1^{C}	39 ± 2^{A}	46 ± 2^{A}	42 ± 2^{A}	43 ± 2^{A}	41 ± 0.1^{A}
DG-C18:2	48 ± 4^{C}	61 ± 2^{B}	63 ± 6^{B}	58 ± 3^{B}	80 ± 2^{A}	89 ± 1^{A}	79 ± 3^{A}	79 ± 2^{A}	81 ± 1^{A}
FAME-C18:3	10 ± 1^{D}	20 ± 2^{C}	28 ± 1^{C}	49 ± 0.1^{B}	54 ± 8^{AB}	63 ± 2^{A}	61 ± 3^{A}	62 ± 2^{A}	60 ± 1^{A}
MG-C18:3	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 0.1	100 ± 1	100 ± 1
Total-Ch	45 ± 1^{C}	52 ± 5^{B}	52 ± 2^{B}	48 ± 1^{B}	63 ± 2^{A}	60 ± 1^{A}	59 ± 1^{A}	63 ± 1^{A}	62 ± 2^{A}

Results expressed as mean ± SD ($n=3$). **FAME formation**: $(moles_{EXP} \times 100)/moles_{TH}$. (EXP for measure amount, TH for calculated amount). **Disappearance of TG, CE, FFA, DG and MG**: 100 − (moles $c_F \times 100$/moles c_O) (CF for final concentration, CO for initial). **Recovery of Total Cholesterol:** moles $(Free+CE)_{EXP} \times 100$/ moles $(Free+CE)_{TH}$; **n.d:** not detected. Superscript letters in a row for significant differences by conditions ($p < 0.05$).

Table 2
TG-C13, CE-C16, FFA-C17, DG-C18:2 and MG-C18:3 dissapearance (%), FAME formation (%) and recovery of cholesterol by different methods and the effect of adding DMF when using acid catalyst (Experiment 2).

	FAME C13	TG C13	FAME C16	CE C16	FAME C17	C17 FFA	FAME C18:2	DG C18:2	FAME C18:3	MG C18:3	Total Ch
No DMF											
KOH	104 ± 3^{AB}	100 ± 0.1^{A}	2 ± 0^{h}	56 ± 2^{E}	n.d^{E}	20 ± 4^{B}	105 ± 3^{C}	100 ± 0.1^{A}	78 ± 3^{C}	100 ± 0.1^{A}	45 ± 1^{E}
MetNa	54 ± 10^{D}	100 ± 0.1^{A}	54 ± 10^{E}	100 ± 0.1^{A}	n.d^{E}	100 ± 0.1^{A}	57 ± 10^{F}	100 ± 0.1^{A}	64 ± 10^{DC}	100 ± 0.1^{A}	44 ± 10^{DE}
BF3	53 ± 2^{D}	77 ± 3^{B}	23 ± 2^{F}	40 ± 0.1^{F}	107 ± 7^{B}	100 ± 0.1^{A}	80 ± 1^{E}	100 ± 0.1^{A}	67 ± 20^{DC}	100 ± 0.1^{A}	63 ± 4^{C}
ACL	29 ± 3^{E}	77 ± 2^{B}	20 ± 2^{F}	59 ± 2^{DE}	84 ± 8^{D}	100 ± 0.1^{A}	56 ± 5^{F}	92 ± 1^{B}	52 ± 10	100 ± 0.1^{A}	54 ± 5^{D}
MBF	112 ± 3^{A}	100 ± 0.1^{A}	141 ± 0^{A}	100 ± 0.1^{A}	145 ± 4^{A}	100 ± 0.1^{A}	123 ± 2^{B}	100 ± 0.1^{A}	116 ± 1^{A}	100 ± 0.1^{A}	29 ± 0^{F}
MHS	102 ± 4^{AB}	100 ± 0.1^{A}	104 ± 1^{C}	100 ± 0.1^{A}	110 ± 1^{B}	100 ± 0.1^{A}	101 ± 1^{D}	100 ± 0.1^{A}	83 ± 10^{C}	100 ± 0.1^{A}	88 ± 4^{B}
KHS	88 ± 7^{C}	100 ± 0.1^{A}	16 ± 5^{FG}	86 ± 3^{C}	89 ± 10^{D}	100 ± 0.1^{A}	80 ± 10^{E}	100 ± 0.1^{A}	78 ± 10^{C}	100 ± 0.1^{A}	44 ± 7^{DE}
DMF											
BF3	110 ± 5^{A}	100 ± 0.1^{A}	87 ± 7^{D}	95 ± 0.1^{B}	107 ± 8^{B}	100 ± 0.1^{A}	113 ± 6^{C}	100 ± 0.1^{A}	98 ± 10^{B}	100 ± 0.1^{A}	45 ± 9^{DE}
ACL	79 ± 3^{C}	100 ± 0.1^{A}	77 ± 3^{D}	100 ± 0.1^{A}	79 ± 1^{D}	100 ± 0.1^{A}	83 ± 3^{E}	100 ± 0.1^{A}	60 ± 10^{D}	100 ± 0.1^{A}	51 ± 2^{D}
MBF	100 ± 1^{B}	100 ± 0.1^{A}	128 ± 2^{B}	100 ± 0.1^{A}	116 ± 1^{B}	100 ± 0.1^{A}	137 ± 3^{A}	100 ± 0.1^{A}	105 ± 1^{B}	100 ± 0.1^{A}	59 ± 1^{C}
MHS	108 ± 1^{A}	100 ± 0.1^{A}	102 ± 2^{C}	100 ± 0.1^{A}	100 ± 1^{C}	100 ± 0.1^{A}	105 ± 3^{C}	100 ± 0.1^{A}	101 ± 1^{B}	100 ± 0.1^{A}	99 ± 0.1^{A}
KHS	106 ± 5^{A}	100 ± 0.1^{A}	13 ± 1^{G}	64 ± 2^{D}	101 ± 7^{BC}	100 ± 0.1^{A}	107 ± 6^{C}	100 ± 0.1^{A}	78 ± 10^{C}	100 ± 0.1^{A}	60 ± 3^{CD}

Results expressed as mean ± SD ($n=3$). **FAME formation**: $(\text{moles}_{EXP} \times 100)/\text{moles}_{TH}$. (EXP for measure amount, TH for calculated amount). **Disappearance of TG, CE, FFA, DG and MG**: $100-(\text{moles } c_F \times 100/\text{moles } c_O)$ (CF for final concentration, CO for initial). **Total Cholesterol:** moles $(\text{Free}+\text{CE})_{EXP} \times 100/\text{moles } (\text{Free}+\text{CE})_{TH}$; **n.d:** not detected. Superscript letters in a column for significant differences by method ($p < 0.05$).

Table 3
Fatty acid composition (mg FA/g oil) of CLA-rich oil (Tonalin® TG80) obtained assaying five different procedures based on direct transesterification with or without dimethylformamide (DMF) added to the reaction mixture (Experiment 3).

	RM	No DMF				DMF			
	KOH	BF3	ACL	MBF	MHS	BF3	ACL	MBF	MHS
C16	3 ± 0.1^{C}	3 ± 0.1^{C}	5 ± 0.1^{A}	3 ± 0.1^{C}	2.6 ± 0.1^{D}	3 ± 0.1^{C}	3 ± 0.1^{B}	3 ± 0.1^{C}	3 ± 0.1^{C}
C18	23 ± 0.1^{E}	23 ± 0.1^{E}	89 ± 1^{A}	26 ± 0.1^{D}	28 ± 3^{CD}	30 ± 0.1^{C}	51 ± 0.7^{B}	30 ± 0.1^{C}	32 ± 1^{C}
C18:1 c9	126 ± 1^{C}	134 ± 1^{B}	36 ± 0.8^{E}	138 ± 0.5^{B}	135 ± 9^{B}	151 ± 0.6^{A}	284 ± 1^{A}	151 ± 0.6^{A}	122 ± 1^{C}
18:2 Non-conjugated isomers									
c9, c12	2 ± 0.1^{B}	2 ± 0.1^{B}	1 ± 0.1^{B}	2 ± 0.2^{B}	2 ± 0.1^{B}	2 ± 0.1^{B}	6 ± 0.2^{A}	2 ± 0.1^{B}	2 ± 0.1^{B}
Total t, t	2 ± 0.1^{C}	2 ± 0.1^{B}	0.5 ± 0.1^{E}	2 ± 0.1^{B}	1 ± 0.1^{D}	2 ± 0.1^{B}	5 ± 0.2^{A}	2 ± 0.1^{B}	1 ± 0.3^{C}
Total c, t	2 ± 0.1^{B}	2 ± 0.1^{B}	1 ± 0.1^{D}	2 ± 0.1^{B}	1 ± 0.1^{C}	2 ± 0.1^{B}	4 ± 0.2^{A}	2 ± 0.1^{B}	2 ± 0.4^{B}
18:2 Conjugated isomers									
c9, t11	402 ± 0.6^{A}	368 ± 0.4^{C}	315 ± 10^{D}	395 ± 0.1^{A}	400 ± 3^{A}	390 ± 0.1^{A}	335 ± 10^{D}	390 ± 0.1^{A}	412 ± 8^{A}
t10, c12	422 ± 2^{A}	386 ± 0.1^{D}	263 ± 7^{E}	407 ± 0.6^{B}	407 ± 1^{B}	400 ± 0.7^{CD}	242 ± 5^{E}	400 ± 0.7^{CD}	409 ± 8^{AB}
Total c, c	10 ± 0.1^{C}	11 ± 0.1^{C}	14 ± 0.2^{B}	10 ± 0.1^{C}	13 ± 2^{B}	10 ± 0.1^{C}	23 ± 0.1^{A}	10 ± 0.1^{C}	10 ± 0.9^{C}
Total t, t	10 ± 0.1^{D}	17 ± 0.2^{B}	17 ± 0.6^{B}	11 ± 0.1^{C}	9 ± 0.5^{D}	10 ± 0.1^{D}	39 ± 0.5^{A}	10 ± 0.1^{D}	10 ± 0.7^{D}
Total CLA	844 ± 1^{A}	782 ± 0.1^{D}	698 ± 5^{E}	824 ± 0.4^{A}	829 ± 10^{A}	809 ± 0.8^{C}	640 ± 9^{E}	809 ± 0.8^{C}	840 ± 2^{A}
Artifact	n.d^{D}	52 ± 1^{B}	168 ± 7^{A}	3 ± 0.1^{C}	1 ± 0.1^{D}	n.d^{D}	n.d^{D}	n.d^{D}	n.d^{D}

RM: Reference method; **DMF**: N,N dimethylformamide; **FA**: fatty acid; ***c***: cis double bond; ***t***: trans double bond. Superscript letters in a row for significant differences by methods ($p < 0.0.5$). $n=3$ in all tests.

In general, results obtained with MHS were better than with the rest of the methods. Total cholesterol measure was only possible with this method.

3.3. Experiment 3

In this next experiment (Table 3) the possible alteration of the FA profile of a CLA-rich oil (Tonalin® TG80) was studied when assaying BF3, ACL, MBF and the proposed MHS. KOH was used as the reference method since reaction is carried out by potassium hydroxide and therefore does not alter the FAME profile. The protective effect of DMF was also tested.

The results from ACL showed higher concentrations of C16 and C18 than in KOH ($p < 0.05$). On the other hand contents for C18:1 c9 and the non-conjugated C18:2 isomers were significantly lower than in the reference method. The utilization of DMF led to overestimation of those compounds. DT with ACL is an exothermic reaction that may alter the sample, yielding higher levels of palmitic (C16) and oleic acid (C18:1 *c*9), except when the reaction is carried out overnight at room temperature [23]. In addition, a recent study confronted the utilization of NaOH followed by $NaHSO_4$ or BF_3 in the FA analysis of phospholipids from reindeer and fish muscle and found that the latter reagent gave higher concentrations of C18, C18:1 *t*, C18:2 *c*9, *c*12, C18:3 *c*9, *c*12, *c*15, C20:2 n6 and C20:3 n3 [24]. Similar variations were found for C18 and C18:1 c9 using MBF with and without DMF. When using the proposed method MHS, the aprotic solvent resulted in values similar to that in KOH except for C18, which was slightly higher ($p < 0.05$).

Formation of artifacts was associated with increments in the concentration of *trans* isomers of CLA (CLA *t*, *t*) and therefore decreases of the major isomers of CLA present in Tonalin® oil such as C18:2 *c*9, *t*11 and C18:2 *t*10, *c*12. It is stated that at low pH, pronated alcohols interact with hydroxy fatty acids and double bonds of CLA, thus forming methoxy compounds and CLA *t*, *t* isomers [10].

Although these side reactions are well known, many investigations are carried out without considering these points. Massod et al. [25] and recently Glaser et al. [26] reported the FA composition in human plasma using methanolic hydrochloric acid, respectively, without the addition of DMF or DMSO. Their analysis did not show the presence of CLA isomers but the results from the present study suggest that concentrations of palmitic, stearic and oleic acid could be overestimated if the aprotic solvent is not

Table 4
Fatty acid composition of commercial animal-based control serum (SERNORM lipid) using MHS and MBS methods at three levels of the sample amount (Experiment 5).

Fatty acid	MHS						MBF					
	100 μL		250 μL		500 μL		100 μL		250 μL		500 μL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C12	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.03	0.1	0.01
C14 DMA	0.1^{D}	0.01	0.1^{CD}	0.02	0.1^{CD}	0.01	0.2^{B}	0.01	0.3^{B}	0.03	0.4^{A}	0.01
C15i DMA	0.2^{C}	0.01	0.2^{C}	0.01	0.2^{C}	0.01	0.3^{B}	0.01	0.3^{B}	0.03	0.5^{A}	0.02
C15ai DMA	0.4^{C}	0.01	0.4^{C}	0.02	0.4^{C}	0.01	0.6^{B}	0.02	0.7^{B}	0.04	1^{A}	0.02
C14	2	0.01	2	0.03	2	0.06	2	0.05	2	0.10	2	0.03
C15ai	0.2	0.01	0.3	0.02	0.3	0.01	0.2	0.04	0.3	0.01	0.3	0.02
C15i	0.4^{B}	0.01	0.6^{AB}	0.02	0.5^{AB}	0.01	0.5^{B}	0.05	0.5^{AB}	0.04	0.6^{A}	0.02
C14:1 c9	0.1^{B}	0.01	0.1^{AB}	0.01	0.1^{A}	0.01	$n.d^{C}$	n.a	0.1^{AB}	0.01	0.1^{AB}	0.01
C15	0.4^{AB}	0.01	0.4^{A}	0.01	0.4^{A}	0.01	0.3^{B}	0.03	0.4^{AB}	0.01	0.4^{A}	0.01
C16 DMA	0.4^{D}	0.01	0.5^{D}	0.02	0.5^{D}	0.01	0.7^{C}	0.01	0.9^{B}	0.01	1^{A}	0.10
C16i	0.2	0.01	0.2	0.01	0.2	0.01	0.2	0.03	0.2	0.01	0.2	0.02
C16	18	0.04	19	1	18	1	19	0.20	19	1	19	0.20
C17ai	0.1	0.01	0.1	0.01	0.1	0.01	0.1	0.02	0.1	0.02	0.1	0.01
C16:1 c7	0.3^{BC}	0.01	0.4^{AB}	0.01	0.4^{A}	0.03	0.3^{C}	0.03	0.4^{AC}	0.01	0.4^{AB}	0.01
C16:1 c9	1^{BC}	0.03	2^{A}	0.04	2^{A}	0.10	1^{C}	0.02	2^{B}	0.04	2^{AB}	0.01
C16 PhyAc	3^{B}	0.03	2^{C}	0.02	2^{C}	0.01	3^{A}	0.20	2^{C}	0.04	1.3^{D}	0.10
C17ai	0.3	0.01	0.4	0.01	0.4	0.01	0.4	0.20	0.3	0.01	0.3	0.01
C18 DMA	0.1^{C}	0.01	0.1^{C}	0.01	0.1^{C}	0.01	0.1^{BC}	0.02	0.1^{AB}	0.01	0.2^{A}	0.01
C17:1 unk	0.2^{BC}	0.01	0.2^{A}	0.01	0.2^{A}	0.01	0.1^{C}	0.01	0.2^{AB}	0.01	0.2^{AB}	0.01
C17:1 c8	0.1^{B}	0.01	0.1^{B}	0.01	0.2^{A}	0.01	0.1^{B}	0.02	0.1^{B}	0.01	0.1^{A}	0.01
C17:1 c9	0.1	0.02	0.1	0.02	0.2	0.01	0.1	0.02	0.1	0.01	0.2	0.04
C18	14^{A}	0.01	13^{B}	0.10	13^{B}	0.20	14^{A}	0.02	13^{AB}	0.40	13^{B}	0.01
C18:1 t11	0.2	0.01	0.2	0.01	0.3	0.01	0.2	0.03	0.2	0.01	0.2	0.01
C18:1 c9	22	0.04	21	0.01	21	0.04	22	0.20	22	0.40	21	0.10
C18:1 c11	0.8	0.02	1	0.03	0.9	0.01	0.8	0.10	0.8	0.01	0.8	0.01
C18:1 c12	0.1^{A}	0.01	0.1^{A}	0.01	0.2^{A}	0.03	$n.d^{B}$	n.a	0.1^{A}	0.01	0.1^{A}	0.01
C18:2 t, t	0.1	0.01	0.1	0.01	0.2	0.03	0.1	0.02	0.2	0.01	0.1	0.01
C18:2 c9, c12	25	0.10	26	0.30	26	0.40	24	0.30	25	0.30	25	0.20
C18:3 c6, c9, c12	0.3^{C}	0.03	0.4^{AB}	0.01	0.4^{A}	0.01	0.2^{D}	0.01	0.3^{C}	0.01	0.3^{BC}	0.02
C18:3 c9, c12, c15	5^{CD}	0.01	6^{B}	0.10	6^{A}	0.04	4^{E}	0.10	5^{D}	0.03	5^{C}	0.01
C18:4 n3	0.1	0.01	0.2	0.02	0.2	0.02	0.1	0.10	0.1	0.01	0.1	0.01
C20:2 n6	0.1^{A}	0.01	0.05^{A}	0.01	0.05^{A}	0.01	$n.d^{B}$	n.a	0.05^{A}	0.01	0.1^{A}	0.02
C20:3 n6	0.9	0.01	0.8	0.04	0.9	0.04	0.8	0.04	0.8	0.06	0.8	0.01
C20:4 AA	1^{A}	0.01	1.3^{A}	0.10	1^{A}	0.04	1^{B}	0.01	1^{B}	0.03	1^{B}	0.05
C20:4 n3	0.9^{A}	0.10	0.7^{B}	0.04	0.7^{B}	0.02	0.8^{A}	0.10	0.6^{B}	0.05	0.6^{B}	0.01
C20:5 EPA n3	0.9^{A}	0.02	0.8^{AB}	0.05	0.9^{A}	0.01	0.6^{C}	0.03	0.7^{C}	0.01	0.7^{BC}	0.04
C22:5 DPA n3	0.4	0.02	0.4	0.05	0.4	0.02	0.5	0.01	0.5	0.10	0.4	0.10
C22:6 DHA	0.2	0.02	0.2	0.01	0.2	0.01	0.3	0.04	0.3	0.10	0.4	0.10
μg FA/ mL serum	658	60	683	90	707	60	666	77	684	40	705	40

DMA: dimethylactetal; ***ai***: anteiso; **i**: iso; ***PhyAc***: Phytanic acid; ***c***: cis double bond; ***t***: trans double bond; ***n3***: omega 3 fatty acid; ***n6***: omega 6 fatty acid; ***AA***: Arachidonic acid; ***EPA***: eicosapentanoic acid; ***DPA***: docosapentanoic acid; ***DHA***: docosahexanoic. Superscript letter for significant differences in the fatty acid composition among samples ($p < 0.0.5$). $n=3$ in all tests.

added. MBF has been assayed in the analysis of the FA composition in human milk [27] and human plasma with emphasis on CLA contents [28]. According to the present research work that methodology can yield a slight production of *trans* isomers and erroneous oleic acid concentration.

Among the assayed methods, the proposed MHS showed the lowest capacity of alteration of the CLA profile in the assayed methods. Such modifications were corrected with the utilization of DMF.

3.4. Experiment 4

The feasibility of the obtained FA composition was tested by means of calculations of response factors (RF) of a skimmed milk added with a reference butterfat from the analysis using the DT methods BF3, ACL, MBF and MHS. KOH was used as the reference method.

According to the obtained results (data not shown), MHS presented the best RF values, close to 1 for most of the FA (except for C4 and C6, 2.72 and 1.80, respectively) including CLA isomers (0.97). Nevertheless when MBF and BF3 methods were applied the RF values for short-chain FA (C4–C10) ranged from 5.99 (C6 with BF3) to 1.62 (C10 with MBF) while C4 was not detected. The results are the consequence of loses of these compounds. The opposite occurred for ACL (RF < 0.5), pointing out the presence of artifacts or co-elution in short FA moieties.

3.5. Experiment 5

According to the results obtained in the previous experiments of the current research work, MHS and MBF methods showed the best results in the derivatization of lipids into FA. Thus, they were tested in the analysis of an animal-based control serum at three different sample volumes (Table 4).

Finally the MHS and MBF methods were selected according to completeness of reaction and lack of alteration of the fatty acid profile to be tested with animal plasma samples at three different volumes (100, 250 and 500 μL).

The derivatization performance (Dp) was calculated as EM was added to plasma prior to derivatization. Dp values were not significantly different in any of the assayed sample amounts with MBF (mean value 74% ± 5). However for MHS, Dp was 77% ± 0.8 for 100 μL, 87% ± 2 in 500 μL ($p > 0.05$) while 93% ± 2 using 250 μL ($p < 0.05$). Except using 100 μL, Dp values were significantly better

with MHS than with MBF. These results confer a superior reliability to the composition obtained using MHS: without application of Dp, total concentration was lower in MBF ($p < 0.05$). After correction, a slight increase was found with the sample volume but differences among methods and amounts were not significant. At the lowest sample level C14:1 *c*9, C18:1 *c*12 and C20:2 n6 were not detected using MBF. Using higher volumes did not result in any difference in their concentrations when comparing methods.

On the other hand C16 PhyAc and C18 showed higher concentrations when using 100 μL than with 250 μL and 500 μL in both methods ($p < 0.05$). It could be attributed to low Pt in these samples but the same effect was not found in MBF with 250 μL and 500 μL, which also registered Dp < 75%. Some minor compounds (C14 DMA, C15i DMA, C15ai DMA, C16 DMA, C18 DMA) showed lower amounts in MHS while some PUFA (C18:3 *c*6, *c*9, *c*12, C18:3 *c*9, *c*12, *c*15, C20:4 AA and C20:5 EPA n3) had higher contents ($p < 0.05$). Such differences could be attributable to a difficulty of MBF in the derivatization of lipids containing PUFA.

Results showed that in general there are no major differences when using 250 μL or 500 μL as sample size in both compared methods. However, utilization of the lowest sample amount possible is a desirable matter in order to accomplish further or complementary determinations.

4. Conclusion

The results of the present research work showed that among the assayed DT methods, the proposed MHS was the only one suitable for the analysis of total FA in foodstuff and biological samples as it showed a high derivatization performance, absence of alteration of the FAME profile mainly CLA isomers, good RF values for short, medium and long chain FA and allowed the measurement of total cholesterol.

Acknowledgments

This work was supported by AGL2011-26713, FUN-C-FOOD CSD2007-00063 and ALIBIRD P2009/AGR-1469. The authors wish to thank Dr. Charlie Scrimgeour (Mylnefield Lipid Analysis, The James Hutton Institute, Dundee, Scotland) for helpful discussions concerning this work.

References

[1] J. Fontecha, L.M. Rodriguez-Alcala, M.V. Calvo, M. Juarez, Curr. Nutr. Food Sci. 7 (2011) 155–159.
[2] R. Shiraishi, R. Iwakiri, T. Fujise, T. Kuroki, T. Kakimoto, T. Takashima, Y. Sakata, S. Tsunada, Y. Nakashima, T. Yanagita, K. Fujimoto, J. Gastroenterol. 45 (2010) 625–635.
[3] W.S. Harris, Pharmacol. Res. 55 (2007) 217–223.
[4] G. Hornstra, Am. J. Clin. Nutr. 71 (2000) 1262S–1269S.
[5] L.A. Horrocks, Y.K. Yeo, Pharmacol. Res. 40 (1999) 211–225.
[6] K.M. Appleton, R.C. Hayward, D. Gunnell, T.J. Peters, P.J. Rogers, D. Kessler, A. R. Ness, Am. J. Clin. Nutr. 84 (2006) 1308–1316.
[7] W.W. Christie, J. Lipid Res. 23 (1982) 1072–1075.
[8] A.I. Carrapiso, C. García, Lipids 35 (2000) 1167–1177.
[9] K. Nuernberg, D. Dannenberger, K. Ender, G. Nuernberg, J. Agric. Food Chem. 55 (2007) 598–602.
[10] M. Yamasaki, K. Kishihara, I. Ikeda, M. Sugano, K. Yamada, J. Am. Oil Chem. Soc. 76 (1999) 933–938.
[11] Y. Kishimoto, N.S. Radin, J. Lipid Res. 6 (1965) 435–436.
[12] Y. Park, K.J. Albright, Z.Y. Cai, M.W. Pariza, J. Agric. Food Chem. 49 (2001) 1158–1164.
[13] J. Kramer, V. Fellner, M. Dugan, F. Sauer, M. Mossoba, M. Yurawecz, Lipids 32 (1997) 1219–1228.
[14] I. Bondia-Pons, A.I. Castellote, M.C. López-Sabater, J. Chromatogr. B 809 (2004) 339–344.
[15] I.S. ISO-IDF, ISO 15884-IDF (2002) 182:2002.
[16] J.V. O'Fallon, J.R. Busboom, M.L. Nelson, C.T. Gaskins, J. Anim. Sci. 85 (2007) 1511–1521.
[17] G. Lepage, C. Roy, J. Lipid Res. 25 (1984) 1391–1396.
[18] W.W. Christie, Gas Chromatography and Lipids, A Practical Guide, The Oily Press, Ayr, Scotland, 1990.
[19] J. Folch, M. Lees, G.H.S. Stanley, J. Biol. Chem. 226 (1957) 497–509.
[20] L.M. Rodriguez-Alcala, J. Fontecha, J. Dairy Sci. 90 (2007) 2083–2090.
[21] C. Stavarache, M. Vinatoru, Y. Maeda, Ultrason. Sonochem. 14 (2007) 380–386.
[22] K.S. Liu, J. Am. Oil Chem. Soc. 71 (1994) 1179–1187.
[23] Z. Xu, K. Harvey, T. Pavlina, G. Dutot, G. Zaloga, R. Siddiqui, Lipids 45 (2010) 199–208.
[24] S. Sampels, J. Pickova, Food Chem. 128 (2011) 811–819.
[25] M. Masood, N. Salem, Lipids 43 (2008) 171–180.
[26] C. Glaser, H. Demmelmair, B. Koletzko, J. Lipid Res. 51 (2010) 216–221.
[27] C. Moltó-Puigmartí, M. Permanyer, A.I. Castellote, M.C. López-Sabater, Food Chem. 124 (2011) 697–702.
[28] J. Mayneris-Perxachs, I. Bondia-Pons, C. Moltó-Puigmartí, M. Pairó, A. I. Castellote, M.C. López-Sabater, Food Chem. 123 (2010) 296–305.

1.3. Comprehensive study of the lipid classes of krill oil by fractionation and identification of triacylglycerols, diacylglycerols and phospholipid molecular species by using UPLC-QTOF-MS.

Food Anal. Methods
DOI 10.1007/s12161-015-0150-6

Comprehensive Study of the Lipid Classes of Krill Oil by Fractionation and Identification of Triacylglycerols, Diacylglycerols, and Phospholipid Molecular Species by Using UPLC/QToF-MS

María Pilar Castro-Gómez[1] · Francisca Holgado[1] · Luis Miguel Rodríguez-Alcalá[1] · Olimpio Montero[2] · Javier Fontecha[1]

Received: 9 January 2015 / Accepted: 5 March 2015

Abstract Krill oil represents an interesting source of bioactive lipid components, being suitable as a functional ingredient. This oil is characterized by its high concentration of long-chain omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The contents of EPA and DHA were similar to those in fish oils, but with the difference that almost the half are located in phospholipids (mainly phosphatidylcholine). This might explain its higher absorption and bioavailability. This highly unsaturated oil maintains stable due to the presence of astaxanthin, a potent antioxidant, which assures the stability of the omega-3 fatty acids. However, there is lack of investigations reporting a deep comprehensive description of the krill oil (KO) lipid composition. The characterization includes new data of its neutral and polar components and the identification of triacylglycerols, diacylglycerols, and molecular species that has been done by different chromatographic techniques as gas chromatography–mass spectrometry/flame ionization detector (GC-MS/FID), flash chromatography–evaporative light scattering detector (FC-ELSD), and HPLC-ELSD. Also phospholipid molecular species by using ultraperformance liquid chromatography/quadruple-time-of-flight mass spectrometry (UPLC/QToF-MS) have been determined.

✉ Javier Fontecha
j.fontecha@csic.es

[1] Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Universidad Autónoma de Madrid, Calle Nicolás Cabrera 9, 28049 Madrid, Spain

[2] Centre for Biotechnology Development (CDB, CSIC), Boecillo Technological Park, 47151 Valladolid, Spain

Published online: 26 March 2015

Keywords Fatty acid · Krill oil · Lipid classes · Phospholipid · Chromatography techniques

Introduction

Krill is a small marine crustacean (2.5–6-cm size and 2-g weight) whose 65 % of total dry weight are proteins and, depending on the species, age, and time from capture to freezing, the lipid content varies from 12 to 50 % (Kolakowska et al. 1994; Saether et al. 1986; Svetlova et al. 1985). Most of the commercially available krill are harvested in the Antarctic Ocean (*Euphausia superba*). Krill oil (KO) is characterized by the concentration and profile of long-chain omega-3 polyunsaturated fatty acids (n-3 LCPUFAs) and phospholipids (PLs). It also naturally contains astaxanthin, which is an antioxidant. Omega-3 fatty acids are known to exert positive effects on cardiovascular diseases (Eslick et al. 2009; Harris et al. 2008), because of their capacity to reduce plasma triglycerides (TAGs), cardiac arrhythmia, blood pressure, platelet aggregation, and inflammation markers as well as to enhance HDL cholesterol (Balk et al. 2006). Furthermore, positive effects have been reported against insulin resistance and some neurological diseases (Breslow 2006; Davidson et al. 2007). In fish oils, n-3 LCPUFAs are esterified to TAGs while in KO, a large portion of these fatty acids are located in PLs (Winther et al. 2010). Recent studies in rats have reported that KO has positive effects on different parameters of metabolic syndrome (Batetta et al. 2009; Tandy et al. 2009). There are also studies demonstrating that omega-3 fatty acids in PL form are more efficiently taken up into body tissues, and especially the brain (Di Marzo et al. 2010; Wijendran et al. 2002). Numerous clinical studies on KO have demonstrated its beneficial effects on blood lipids (Berge et al.

2014; Bunea et al. 2004), inflammation (Deutsch 2007), and premenstrual syndrome (Sampalis et al. 2003). Moreover, the KO seems to be more effective in increasing plasma levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), when compared to fish oil (Maki et al. 2009; Schuchardt et al. 2011; Ulven et al. 2011). Astaxanthin, which is taken up by krill from the algae and plankton consumed by the animal, is an antioxidant stronger than vitamins A and E and plays a role in assuring the stability of the KO (Suzuki and Shibata 1990). This antioxidant has also been proven to exert anti-inflammatory, analgesics, and hypolipidemic effects in human and animals (Deutsch 2007; Hussein et al. 2006; Ikeuchi et al. 2007).

As it is well known, KO is a suitable source of omega-3 fatty acids, and previous studies describe the effect of the different isolation processes on the KO (Ali-Nehari and Chun 2011; Gigliotti et al. 2011), but there is a lack of investigations reporting a deep comprehensive description of the KO lipid composition. Therefore the objective of this research work was to describe the composition of commercial krill oil and its isolated lipid fractions. The characterization includes new data of its neutral and polar components and the identification of TAGs, diacylglycerols (DAGs), monoacylglycerols (MAGs), and molecular species that has been done by several chromatographic techniques as gas chromatography–mass spectrometry/flame ionization detector (GC-MS/FID), flash chromatography–evaporative light scattering detector (FC-ELSD), and HPLC-ELSD. Also, ultraperformance liquid chromatography/quadruple-time-of-flight mass spectrometry (UPLC/QToF-MS) that has the potential to substantially improve the accuracy, sensitivity, and speed has been used for the determination and identification of phospholipid molecular species.

Materials and Methods

Sample

Antarctic KO obtained from *E. superba* was kindly donated by AKO3TM (Aker BioMarine Antarctic AS, Oslo, Norway). The sample was stored in amber vials flushed with nitrogen and kept at −40 °C until the analysis.

Chemicals and Reagents

All solvents were at least HPLC grade and MS grade when available. Chloroform, hexane, methanol, isooctane, isopropanol, ammonium hydroxide, arsenic formate, and acetonitrile were purchased from LABSCAN (Dublín, Ireland). Potassium hydroxide and sodium carbonate were obtained from PANREAC (Barcelona, Spain). Formic acid (98 %) and triethylamine (99.5 %); the TAG standards trinanoin and tritridecanoin; the free fatty acid (FFA) standards pelargonic (C9), tridecanoic (C13), myristic (C14), palmitic (C16), estearic (C18), araquidonic (AA, 20:4), eicosapentaenoic acid (EPA, 20:5), and docosahexaepentaenoic acid (DHA, 22:6); the sterols 5α-cholestane, cholesterol (CHOL), cholesterol ester (CE), desmosterol, campesterol, β-sitosterol and lanosterol, MAGs and DAGs, monostearin, and diolein; and PLs phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), sphingomyelin (SM) phosphatidylcholine (PC), and N-oleoylethanolamine were from Sigma (St. Louis, MO, USA). Reference samples as butter fat BCR 164 & BCR-519 (EU Commissions; Brussels, Belgium) were from Fedelco Inc. (Madrid, Spain); the omega-3 Ropufa from DSM (Derbyshire, UK) and microalgae oil from Martek (Martek Bioscience Corporation, Columbia, MD, USA) were used.

Chromatographic Techniques

Flash Chromatography

KO fractionation was carried out with a preparative Reveleris® Flash System Chromatography (Grace, Deerfield, IL, USA) equipped with an evaporative light scattering detector (ELSD). The KO was dissolved in hexane (50 mg/mL) and loaded onto a preconditioned 4-g silica cartridge (Grace Reveleris, Deerfield, USA). The elution solvent program consisted of hexane/diethyl ether (98:2), hexane/diethyl ether (95:5), and finally methanol. Each step was maintained during 5 min at a flow rate of 7 mL/min. Pressure during fractionation was below 100 psi. The three lipid fractions collected (F1, F2, and F3) were evaporated under nitrogen stream, weighed, and kept at −40 °C until further analysis. Assays were carried out in triplicate.

HPLC-ELSD Analysis

Separation of lipid classes was accomplished with a HPLC Agilent Technologies, model 1200 (Palo Alto, CA, USA) coupled to an ELSD detector (SEDERE, SEDEX 85 model, Alfortville Cedex, France) using prefiltered compressed air as the nebulizing gas at pressure of 3.5 bar, temperature of 60 °C, and the gain set at 3. Two columns of Zorbax Rx-SIL column (Agilent Technologies, Palo Alto, CA, USA) of 250 mm× 4.5 mm and 5-μm particle size were used in series along with a precolumn of the same packing. Samples of KO as well as the isolated KO fractions were prepared at 5 mg/mL, and the injection volume was 50 μL. Solvent gradient was as detailed in Castro-Gomez et al. (2014). Analyses were carried out with solvents freshly prepared. Lipid standards were analyzed under the same conditions and used for further identification. Analyses of KO and isolated fractions were carried out in triplicate.

GC-FID Analysis

Triacyglycerols The samples were injected (30 mg/mL) in a CLARUS 400 gas chromatograph (Perkin Elmer, Beaconsfield, UK) fitted with a RTX-65 TAG fused-silica capillary column Crossbond® with 65 % diphenyl/35 % dimethyl polysiloxane as stationary phase (30 m×0.25 mm ID×0.1 μm) (Restek Corporation, Bellefonte, PA, USA). Analysis of TAG by different carbon numbers (CNs) (Fontecha et al. 2006) and their different molecular species was carried out with the following temperature program: 120 °C held for 30 s, 10 °C/min to 220 °C held for 30s, and 6 °C/min to 350 °C held for 30 min. Injector and FID temperatures were 355 and 370 °C, respectively. Helium was used as carrier gas at 25 psi, and the injection volume was 0.5 μL. For TAG identification and quantification, the reference butter fat BCR-519 was used as well as the TAG trinanoin as internal standard (100 μL; 1 mg/mL). Assays with KO and each isolated fraction were analyzed in triplicate.

Cholesterol Determination Cholesterol determination was as described by Fraga et al. (Fraga et al. 2000). Briefly, KO and isolated fraction samples were derivatized following the ISO-IDF procedure (ISO 2002) using 2 N potassium hydroxide (KOH) in methanol after adding 5 μL of 5α-cholestane (13.75 mg/mL in hexane) as internal standard. A volume of 0.5 μL of the resulting solution was injected for GC analysis in a CLARUS 400 GC-FID (Perkin-Elmer, Beaconsfield, UK) fitted with a RTX-65 TAG fused-silica capillary column Crossbond® with 65 % diphenyl/35 % dimethyl polysiloxane as stationary phase (30 m×0.22 mm×0.10 μm) (Restek Corporation, Bellefonte, AP).

Spectrometric Techniques

MS Analysis

KO and isolated fraction samples were derivatized following the ISO-IDF procedure (ISO 2002). Briefly, an amount of 25 mg of fat sample was mixed with 200 μL of hexane with tritridecanoin, as internal standard (2 mg/mL). A volume of 50 μL of KOH 2 N in methanol was added and stirred, and after 5 min, the reaction was stopped with 125 mg of $NaHSO_4$. Then, the sample is centrifuged (12,000 rpm, 5 min, 4 °C), and 50 μL of supernatant was dissolved in 450 μL of hexane previous injection. Fatty acid methyl esters (FAMEs) were separated using a CP-Sil 88 fused-silica capillary column (highly substituted cyanopropyl phase, 100 m×0.25 mm×0.2 μm, Chrompack, Middelburg, the Netherlands) in an Agilent chromatograph (model 6890 N, Palo Alto, CA, USA) fitted with an MS detector (Agilent 5973 N) operated in the scan mode of 50 to 550 Da. Chromatographic conditions were as in Rodríguez-Alcalá and Fontecha (2007). Briefly, the column was held at 100 °C for 1 min after injection and temperature-programmed at 7 °C/min to 170 °C, held there for 55 min, and then at 10 °C/min to 230 °C and held there for 33 min. The injector temperature was set at 250 °C. Helium was used as carrier gas with a column inlet pressure of 30 psi. MS detector conditions were transfer line temperature of 250 °C, source temperature of 230 °C, quad temperature of 150 °C, and electron impact ionization at 70 eV. For peak identification, mass spectra obtained in our analysis were compared with those in the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA) library. The injection volume was 1 μL and split mode of 1:25 was used. For qualitative and quantitative analysis, response factors were calculated using anhydrous milk fat (reference material BCR-164) and Supelco 37 FAME mix (Sigma, St. Louis, MO). Tritridecanoine as internal standard (200 μL; 1.3 mg/mL) was also used. Assays were carried out in triplicate.

UPLC/QToF-MS Analysis

Phospholipid Molecular Species Determination Molecular analysis of KO samples was carried out by ultraperformance liquid chromatography (UPLC) using an ACQUITY UPLC® (Waters, Manchester, UK), which was equipped with a Sample Manager model and a Binary Solvent Manager model, whose outlet was connected to an Acquity HSS T3 1.8 μm, 2.1×100 mm column with a precolumn of the same packing material, VanGuard 1.8 μm, 2.1×10 mm (Waters, Barcelona, Spain). Separation was carried out with two different solvent gradient: initial, 100 % A; 1.0 min, 100 % A; 2.5 min, 20 % A; 4.0 min, 20 % A; 5.5 min, 0 % A; 8.0 min, 0 % A; 10.0 min, 100 % A; and 12.0 min, 100 % A; where solvents were (A) MeOH/H_2O (1:1) with 0.5 % formic acid and 5 mM ammonium formate pH 7.5, and (B) MeOH/acetonitrile (6:4) with 0.5 % formic acid and 5 mM ammonium formate. For quantification, an external standard of PC (10:0/10:0) was used to draw a correlation curve of chromatographic peak area to standard concentration (μg/mL). Mass spectrometry detection of PL was carried out with a quadruple-time-of-flight mass spectrometer (QToF-MS) SYNAPT HDMS G2 with electrospray ionization (ESI) source (WATERS, Manchester, UK). The chromatographic column outlet was directly connected to the ionization source. Data were acquired and analyzed with the software MassLynx®.

The isolated F3 fraction from KO enriched in PL was dissolved in MeOH/H_2O (9:1), at 0.023 mg/mL, and 7.5 μL was injected. PC and SM species were detected in positive mode as the [M+H]+ions. The conditions of MS analysis were 400 to 1000 scanning range, capillary voltage of 0.7 V, source temperature of 90 °C, desolvation temperature of 300 °C, gas flow of 30 L h^{-1}, and desolvation gas flow of 800 L h^{-1}. A MS^E method was operated for sample analysis, which includes a low-energy function (full-scan equivalent) and a high-energy function that renders fragments of the base peak

m/z through a collision-induced dissociation (CID) continuously; using this high energy function, the fragment at m/z 184.074 could be monitored for PC.

TAG and DAG Species Determination The isolated F1 fraction enriched in TAG and F2 fraction enriched in DAG were dissolved in a mixture of ethanol/acetone/2-propanol (1:1:1, *v*/*v*/*v*), and a volume of 7.5 μL (0.38 mg/mL) was injected. The same equipment as that above was used. Separation was carried out with the solvents A (acetonitrile/2-propanol/methanol (3:4:3, *v*/*v*/*v*)) and B (acetonitrile/2-propanol (3/7*v*/*v*), both with 0.1 % NH_4OH. The following elution gradient was used: initial, 100 % A; 3 min, 100 % A; 6 min, 98 % A; 8 min, 98 % A; 9.5 min, 95 % A; 11 min, 95 % A; 16 min, 100 % A; and 18 min, 100 % A. The flow rate was 0.4 mL/min. Quantification of TAG and DAG was done by drawing a correlation curve of the chromatographic peak area versus TAG (16:0/16:0/16:0) concentration (μg/mL) using an external standard (SIGMA-ALDRICH, CAS 555-44-2, reference T5888-100MG, >99 %). TAG and DAG species were detected with the ToF detector in positive mode as the $[M+NH_4]^+$ and $[M+ACN+NH_4]^+$ ions. For quantification, the $[M+ACN+NH_4]^+$ ion peak area was used. Mass spectrometer conditions were scan from 400 to 1000 Da, capillary voltage of 0.8 V, source temperature of 90 °C, sampling cone of 15 V, desolvation temperature of 280 °C, gas flow of 40 L/h, and desolvation gas flow of 700 L/h. A MS^E method was used with low (full-scan-like) and high (MS/MS-like) energy functions. Acyl groups esterifying the glycerol backbone could be identified by fragments detected in the high-energy function. Independent samples were measured in triplicate.

Statistical Analysis

The detection of possible significant differences in FAMEs among fractions was carried out with a non-parametric Mann-Whitney post-hoc test. A Student's *t* test between KO and the corresponding fraction was used for TAG (CN groups and theirs species) and cholesterol content. These were conducted with the aid of the SPSS package (SPSS 17.0 for Windows, SPSS Inc.).

Results and Discussion

Krill Oil Analysis by HPLC-ELSD, Fractionation by FC-ELSD, and Characterization of Lipid Classes

Figure 1 shows the profile of lipid classes of KO analyzed by HPLC-ELSD for the separation of neutral and/or polar lipids in qualitative and quantitative conditions following the procedure described by Castro-Gomez et al. (2014). The results revealed that KO was mainly composed of TAGs and PLs (43.7 and 48.9 %, respectively) and other minor compounds (Table 1). These contents were in agreement with the composition given by the KO producer for PLs (>46 %) and TAGs (<50 %), while information of the minor compound content was not indicated. These results are in good agreement with previous report by Tandy et al. (2009), Phleger et al. (1998)), and Ju et al. (2009) for different KO samples. These authors found values of 23 and 58 %, 37 and 51 %, and 38 and 55.4 % for TAGs and PLs, respectively. Moreover, percentage values reported for CHOL+FFA of 0.9 % (Phleger et al. 1998) and 0.6 % (Ju et al. 2009) were also comparable to the values observed in this study. Regarding the relative levels of the individual PL, amounts of PE, PC, and SM were found showing values of <0.1, 99.7, and 0.2 %, respectively, related to the total PL (Table 1). Ali-Nehari and Chun (2011) reported amounts of 80.4 % for PC and 14.9 % for PE, after extracting KO with supercritical carbon dioxide while SM was in trace level or not detected in KO (2011). Gigliotti et al. (2011) found a total PL content of 30 and 1–3 % of TAGs while almost 70 % of the total lipid content was accounted by non-PLs and lipids like DAGs, MAGs, CHOL, and FFAs. The differences among results might be due to TAG hydrolysis during sample processing, extraction procedure, and TLC separation.

In order to ensure better characterization of the KO sample, a solution in hexane (50 mg/mL) was loaded onto a preconditioned 4-g silica cartridge using flash chromatography (FC). Following this procedure that was proved to be very robust, reliable, and repeatable, we were able to fractionate KO into three fractions (F1, F2, and F3) of increasing polarity with a recovery of total lipid extracts close to 100 % (40.8, 14.5, and 44.7 % for F1, F2, and F3, respectively; Table 1). The isolated fractions were evaporated under flushing nitrogen, weighed and dissolved in chloroform/methanol 2:1 (5 mg/mL), and re-analyzed by HPLC-ELSD. The lipid class distribution of isolated fractions are shown in Fig. 1. All neutral lipids were thereby collectively isolated from the KO in two chromatographic fractions: F1 where TAGs were the major components (92.9 %) and F2 consisting of DAGs and MAGs and also some polar compounds that include sterols and free fatty acids (CHOL+FFA) that accounted of 67.9, 6.3, and 22.2 %, respectively).

Finally, F3 contained the total polar lipids consisted mainly in the phospholipid PC which was the major component accounted for 99.7 %. These results on lipid class profile of KO have not, to our knowledge, been reported before.

FAME Analysis by GC-MS of Krill Oil and Fractions

The FAME composition of KO and isolated fractions F1, F2, and F3 by GC-MS is shown in Table 2. It contained almost 43 % of saturated fatty acids (SFAs), mainly palmitic (16:0) and myristic (14:0) acid. Oleic (18:1c9) and vaccenic acid (18:1c11) were the major monounsaturated fatty acids (MUFAs) which represented 27.6 % of the FAs.

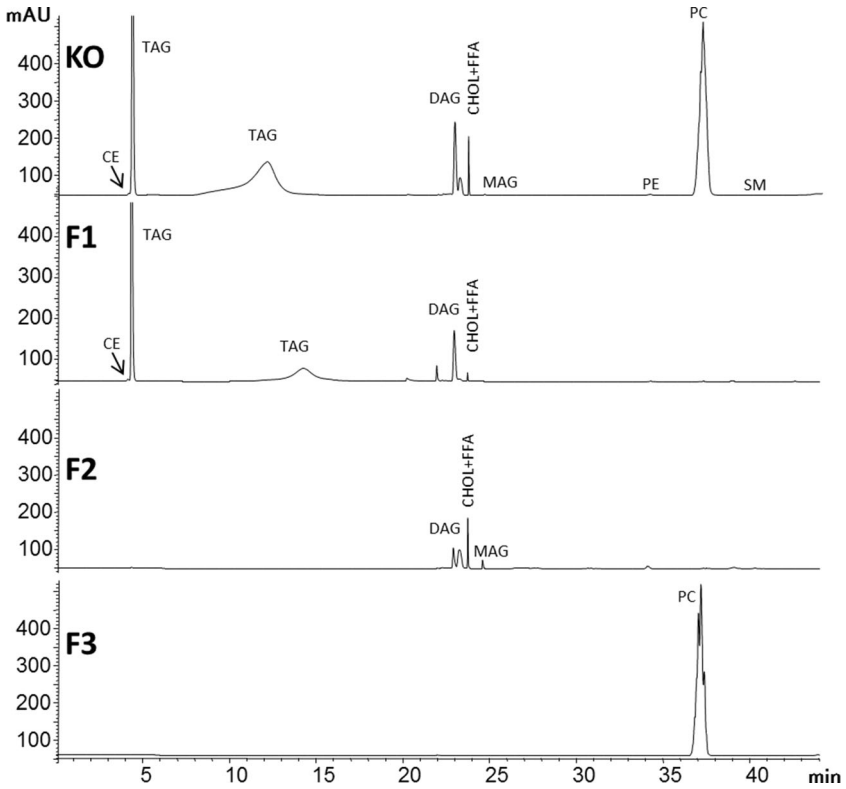


Fig. 1 Chromatographic profile of lipid classes of krill oil (KO) and the isolated fractions F1, F2, and F3 determined by HPLC-ELSD. *TAG* triacylglycerols, *DAG* diacylglycerols, *CHOL* cholesterol, *CE* cholesterol ester, *FFA* free fatty acids, *MAG* monoacylglycerols, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *SM* sphingomyelin

Polyunsaturated fatty acids (PUFAs) reached a level of 29.4 %, and the most abundant, as expected, were EPA and DHA, whose contents were to 15.9 and 5.5 %, respectively. Ju and Harvey (2004), Ulven et al. (2011), and Araujo et al. (2014) reported contents of 33, 34, and 38.1 % for SFA; 25, 28, and 24.0 % for MUFA; and 38, 42, and 37.9 % for PUFA, respectively. Hence, our results for these variables are consistent with these existing studies. Likewise, the value of 0.14 obtained for the omega-6/omega-3 ratio in this study was in the same range as those reported previously in other studies by Ju et al. (2009) and Kassis et al. (2012). The same happened for the EPA/DHA ratio of 2.9:1, which was in agreement with the value reported for the KO manufacturer of 2.5:1 and Ju et al. (2009) with 2.8:1 and somewhat higher than the value reported by Kolakowska et al. (1994) and Araujo et al. (2014) of 2:1 in comparable samples.

Table 1 Lipid class composition (%, *w*/*w*) of krill oil and isolated fractions F1, F2, and F3 analyzed by HPLC-ELSD

	KO Mean±SD	F1 Mean±SD	F2 Mean±SD	F3 Mean±SD
Yield	100	40.8±3.9	14.5±3.4	44.7±6.3
Lipid classes				
CE	<0.1	0.2±0.01		
TAG	43.7±2.7	92.9±3.3		
DAG	6.2±0.6	6.6±0.4	67.9±3.2	
CHOL+FFA	1.0±0.2	0.3±0.01	22.2±1.6	
MAG	<0.1		6.3±0.3	
Phospholipids (PL)				
PE	<0.1			
PC	48.9±1.9			99.7±0.1
SM	0.1±0.01			0.3±0.01

KO krill oil, *SD* standard deviation, *CE* cholesterol esters, *TAG* triacylglycerols, *DAG* diacylglycerols, *CHOL+FFA* cholesterol+free fatty acids, *MAG* monoacylglycerols, *PE* phosphatidylethanolamine, *PC* phosphatidylcholine, *SM* sphingomyelin

The FAME composition of isolated fractions from KO (F1, F2, and F3) showed, as expected, important differences between them. F1 was composed by significantly higher content of SFA (53 %) than F2 and F3 (36 and 38 %, respectively). These data suggest that nearly half of the SFAs present in KO are bound to TAGs. Among the three most important SFAs, 24 % of 14:0 was present in F1, whereas 16:0 and lauric acid (12:0) were especially present in the phospholipid fraction F3 (34 and 1 %, respectively). Nevertheless, stearic acid (18:0) was at similar values in the three fractions. These results are in agreement with the results showed by Araujo et al. (2014) in which the 14:0 represented 17.5 % in TAG fraction and only 4.2 % in PL while 16:0 was contained in 25.0 % in this latter fraction against 18.9 % in TAG. The MUFA content decreased as the polarity of the fraction increased, and hence, F1, F2, and F3 had contents of 35, 28, and 17 %, respectively. This trend was also seen with

Table 2 Mean values and standard deviations of the total fatty acid composition of krill oil and the isolated fractions F1, F2, and F3 by GC-MS

	Krill Oil	F1	F2	F3
C12:0	2.67 (0.42)±0.46	0.36 (0.15)±0.06 a	0.31 (0.15)±0.05 a	2.00 (1.02)±0.34 b
C14:0	86.95 (13.61)±11.07	58.01 (24.22)±7.38 a	28.95 (14.18)±3.68 b	n.d.
C15:0i	2.15 (0.34)±0.18	2.15 (0.90)±0.18	n.d.	n.d.
C15:0ai	1.00 (0.16)±0.14	1.00 (0.42)±0.14	n.d.	n.d.
C14:1	1.69 (0.26)±0.15	1.69 (0.71)±0.15	n.d.	n.d.
C15:0	3.48 (0.54)±0.28	0.98 (0.41)±0.08 a	1.32 (0.65)±0.11 b	1.18 (0.60)±0.09 ab
C16:0	147.99 (23.16)±17.75	47.15 (19.69)±5.66 a	34.44 (16.87)±4.13 a	66.40 (34.00)±7.97 b
C17:0i	4.51 (0.71)±0.76	1.63 (0.68)±0.27 a	1.46 (0.72)±0.25 a	1.43 (0.73)±0.24 a
C16:1 t	1.08 (0.17)±0.07	1.08 (0.45)±0.07	n.d.	n.d.
C16:1	39.58 (6.19)±4.6	20.78 (8.68)±2.42 a	14.20 (6.96)±1.65 b	4.60 (2.36)±0.53 c
C17:0	14.53 (2.27)±1.84	9.95 (4.15)±1.26 a	4.58 (2.24)±0.58 b	n.d.
C18:0ai	1.66 (0.26)±0.53	1.66 (0.69)±0.53	n.d.	n.d.
C17:1	4.76 (0.75)±0.56	2.30 (0.96)±0.27 a	2.46 (1.21)±0.29 a	n.d.
C18:0	8.39 (1.31)±0.81	2.83 (1.18)±0.27 ab	2.28 (1.12)±0.22 a	3.27 (1.67)±0.32 b
C18:1c9	74.30 (11.63)±9.74	37.40 (15.62)±4.90 a	24.19 (11.85)±3.17 b	12.71 (6.51)±1.67 c
C18:1c11	46.08 (7.21)±5.29	15.92 (6.65)±1.83 a	15.28 (7.49)±1.76 a	14.89 (7.62)±1.71 a
C18:1c12	3.10 (0.49)±0.7	3.10 (1.29)±0.7	n.d.	n.d.
C18:2 c11,t15	8.18 (1.28)±1.64	3.65 (1.52)±0.73 a	4.52 (2.21)±0.91 a	n.d.
C18:2	12.23 (1.91)±1.88	3.46 (1.44)±0.53 a	4.24 (2.08)±0.65 a	4.53 (2.32)±0.70 a
C20:0	1.19 (0.19)±0.09	1.19 (0.50)±0.09	n.d.	n.d.
C18:3c9,c12,c15	4.97 (0.78)±0.65	1.30 (0.54)±0.17 a	1.81 (0.89)±0.24 a	1.86 (0.95)±0.24 a
C20:1c9	3.92 (0.61)±0.54	1.28 (0.53)±0.18 a	1.16 (0.57)±0.16 a	1.48 (0.76)±0.20 a
C20:1c11	1.68 (0.26)±0.22	1.68 (0.70)±0.22	n.d.	n.d.
C18:4c6,c9,c12,c15	20.10 (3.15)±2.98	7.30 (3.05)±1.08 a	7.97 (3.90)±1.18 a	4.82 (2.47)±0.72 b
C20:4 AA	3.43 (0.54)±0.62	0.48 (0.20)±0.09 a	0.71 (0.35)±0.13 a	2.23 (1.14)±0.41 b
C20:5 EPA	101.77 (15.93)±15.54	8.18 (3.42)±1.25 a	40.39 (19.79)±6.17 b	53.20 (27.24)±8.12 c
C22:5 DPA	2.30 (0.36)±0.28	0.23 (0.10)±0.03 a	1.50 (0.73) ±	0.58 (0.30)±0.07 c
C22:6 DHA	35.24 (5.52)±6.57	2.77 (1.16)±0.52 a	12.35 (6.05)±2.30 b	20.11 (10.30)±3.75 c
Total	638.92 (100.00)±85.34	239.51 (100.00)±30.70 a	204.13 (100.00)±27.64 a	195.29 (100.00)±27.00 a
∑ SFA	274.52 (42.97)±33.75	126.90 (52.98)±15.80 a	73.34 (35.93)±9.01 b	74.28 (38.04)±8.95 b
∑ MUFA	176.19 (27.58)±21.69	85.23 (35.59)±10.58 a	57.29 (28.07)±7.00 b	33.67 (17.24)±4.11 c
∑ PUFA	188.22 (29.46)±29.96	27.38 (11.43)±4.35 a	73.50 (36.02)±11.66 b	87.33 (44.72)±13.96 b
∑ PUFA ω3	164.38 (25.73)±25.92	19.78 (8.26)±3.03 a	64.03 (31.37)±10.02 b	80.57 (41.26)±12.87 c
∑ PUFA ω6	23.83 (3.73)±4.04	7.60 (3.17)±1.32 a	9.48 (4.64)±1.65 a	6.76 (3.46)±1.08 a
SFA/MUFA	1.56±0.01	1.49±0.01 a	1.28±0.01 b	2.21±0.01 c
SFA/PUFA	1.46±0.05	4.65±0.15 a	1.00±0.03 b	0.85±0.03 b
ω6/ω3	0.14±0	0.38±0.01 a	0.15±0.00 b	0.08±0.00 c

Data are expressed in mg/g of oil. Values in parentheses are in %, *w*/*w*. Different letters mean significant differences for a given feature among fractions F1, F2, and F3 ($p<0.05$)

n.d. not detected, *i* iso, *ai* ante iso, *t* trans double bond

palmitoleic (16:1) and oleic acid, while myristoleic acid (14:1), 16:1 t, 18:1c12, and 20:1c11 were all bound to TAG (F1). PUFAs were present in F2 and F3 significantly higher than in F1 (31 and 41 % vs 11 %, respectively) mainly due to their high content of EPA and DHA. The highest levels of EPA and DHA were observed in F3 (27 and 20 %, respectively), which suggests that these FAs are mainly bound to PL. Comparable results were obtained when PLs were extracted from KO using carbon dioxide (Ali-Nehari and Chun 2011) or from freeze-dried krill (2011).

The omega-6/omega-3 value obtained for the F3 was almost half that for KO and five times lower than that for F1.

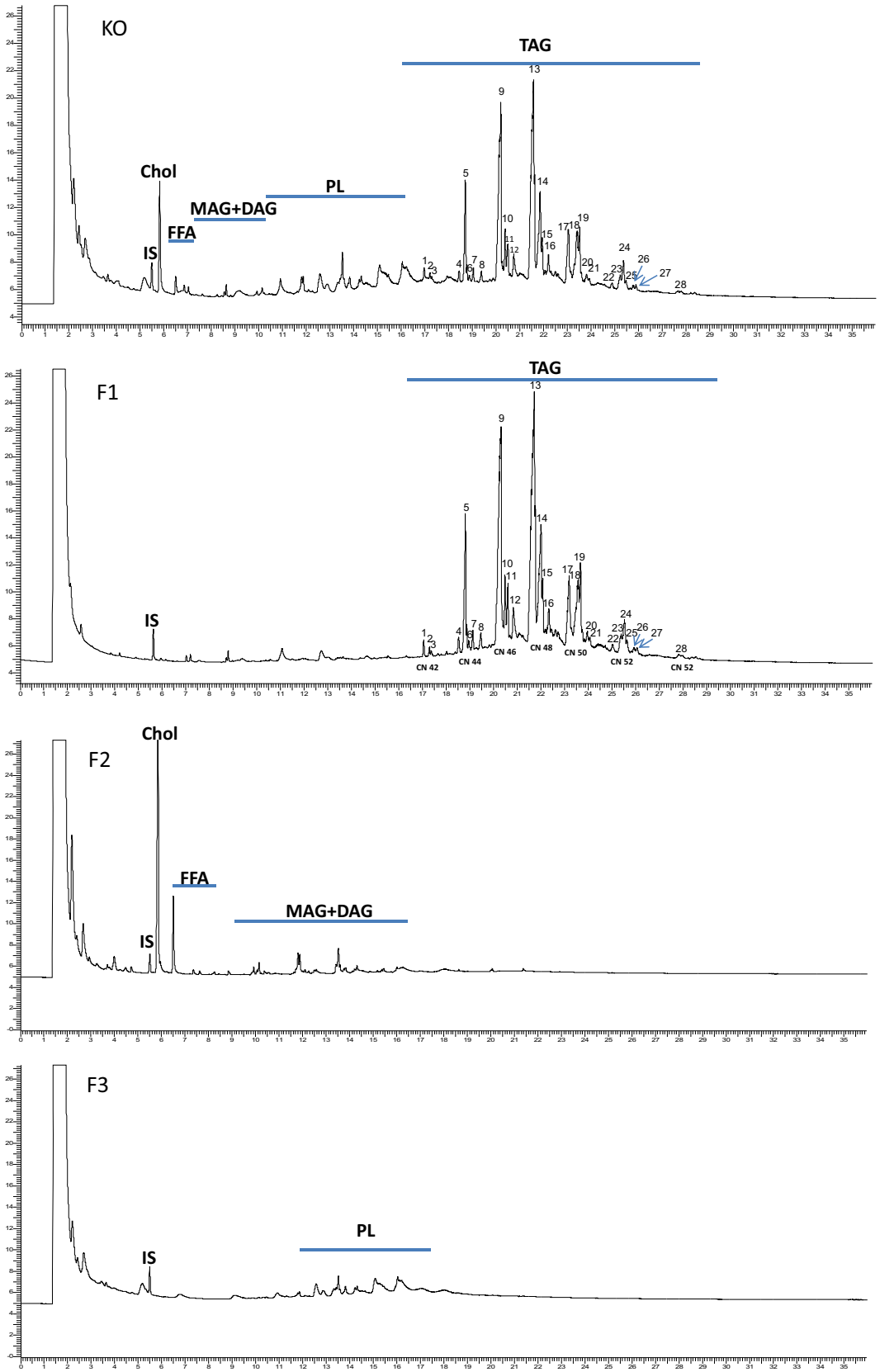


Fig. 2 Chromatographic profile obtained by GC-FID of krill oil (KO) and isolated fractions F1, F2, and F3, previously separated by flash chromatography–ELSD. *DAG+MAG* diacylglycerols+monoacylglycerols, *CHOL* cholesterol, *FFA* free fatty acids, *PLs* phospholipids, *CN* carbon number; *IS* internal standard

TAG and CHOL Analysis of Krill Oil and Isolated Fractions by GC-FID

Figure 2 shows the chromatographic profile of KO and the isolated fraction profiles by GC-FID. The mean values of the TAG content in KO and in fraction F1 are shown in Table 3. TAGs were classified by their carbon number (CN) following previous studies (Fontecha et al. 2006; Fraga et al. 1998). Seven CN groups (from CN42 to CN54) were found and quantified by GC analysis (Fig. 2) containing of a total of 28 different TAG molecular species. Within each CN group, the molecular species eluted from the most saturated to the most unsaturated TAG. Both KO and fraction F1 showed the same CN groups and TAG species, and there were no significant differences between them. The most abundant TAG was CN48 with about 38 % of total TAG which contained the major molecular species (peak 13) with almost its 70 %. In decreasing order of abundances were CN46 (26 %) and CN50 (17 %). As it have been described before, the F1 fraction contains a high content of SFA (14:0, 16:0) and MUFA (18:1) which are the major components in the TAG.

Table 3 Mean values of species (%, *w*/*w*) and standard deviations of the triacylglycerol (TAG) content by carbon number (CN) and their distribution molecular species (peak number in % of TAG) in krill oil and in the isolated fraction F1 determined by GC-FID

TAG	Krill Oil Mean (%)	Fraction 1 Mean (%)	*p*
TAG CN42 TOTAL	1.9±0.06	1.0±0.19	0.073
1	51.8±2.20	51.5±0.57	0.205
2	26.9±2.49	26.6±1.40	0.210
3	21.9±1.26	21.9±1.90	0.621
TAG CN44 TOTAL	9.1±1.02	8.3±0.48	0.131
4	6.7±0.20	6.8±0.10	0.397
5	72.7±0.99	71.9±0.38	0.267
6	4.9±0.71	5.3±0.53	0.335
7	8.5±0.67	8.4±0.15	0.078
8	7.3±0.39	7.6±0.22	0.271
TAG CN46 TOTAL	26.1±1.17	26.8±0.29	0.141
9	76.5±1.23	74.8±1.78	0.375
10	8.6±1.10	9.7±0.82	0.476
11	8.6±0.16	9.0±1.09	0.072
12	6.2±0.30	6.5±0.43	0.514
TAG CN48 TOTAL	37.6±1.05	39.4±0.49	0.175
13	69.9±0.26	70.0±0.29	0.769
14	22.8±0.76	23.4±0.33	0.115
15	4.6±0.38	4.0±0.48	0.588
16	2.7±0.22	2.6±0.14	0.510
TAG CN50 TOTAL	16.9±1.25	17.0±0.80	0.433
17	35.6±0.62	35.6±0.28	0.279
18	36.0±2.11	38.7±1.48	0.458
19	21.3±1.12	19.7±1.50	0.578
20	4.6±1.09	3.6±0.44	0.299
21	2.5±0.65	2.4±0.49	0.664
TAG CN52 TOTAL	6.8±1.52	6.1±0.96	0.618
22	8.3±0.12	7.8±0.69	0.050
23	25.3±0.43	25.8±1.41	0.139
24	44.2±2.12	42.6±1.30	0.308
25	11.1±2.46	11.7±1.29	0.344
26	5.6±0.89	6.4±0.43	0.395
27	5.4±0.46	5.6±0.90	0.181
TAG CN54 TOTAL	1.6±0.68	1.3±0.21	0.153
28	100	100	-

TAG triacylglycerols, *CN* carbon number; *p* level of significance ($p<0.05$)

With respect to the cholesterol (CHOL) content in KO, a total of 10 mg/g oil (1 % of KO) was found, which is in agreement with the previously reported range value from 1 to 50 mg/g oil (Gigliotti et al. 2011; Ju and Harvey 2004; Phleger et al. 1998). This value is also in agreement with the results obtained by HPLC-ELSD for CHOL+FFA (1.03 %), suggesting that the FFA content was less than 0.1 %. Figure 2 shows that CHOL was only present in fraction F2 in agreement with the results obtained by HPLC-ELSD described before. In comparison with the CHOL content reported for other seafood, the CHOL content present in the KO sample studied in this work is lower than in langoustine (about 75 mg/100 g product), lobster (131–144 mg/100 g product), crabs (131–144 mg/100 g product) and shrimp (152 mg/100 g product) (Barrento et al. 2010; Tou et al. 2007; Tsape et al. 2010).

Analysis of Krill Oil Isolated Fractions by UPLC/QToF MS

TAG Molecular Species

The isolated fractions F1 (containing mainly TAG), F2 (mainly DAG), and F3 (mainly PC) were individually submitted to UPLC/QToF-MS analysis for identification of the most probable molecular species and their FA composition. The results are given in Tables 4, 5, and 6 respectively.

Table 4 reveals the complexity of the TAG fraction (F1) examined, which included a mixture of species from CN42 to CN60 within 1 to 11 double bond (DB) ordered by elution retention time. The FAME information detailed before in Table 2 was used to ascertain the identities of the TAG, in particular when more than one TAG were identified from the diacyl fragment ion, and the final results were very consistent with the molecular weight of the TAG.

Araujo et al. (2014) described the TAG characterization of two KO supplements with very different compositions analyzed by LC-MS/MS. They reported a content of n-3 PUFA similar to fish oil with TAG structures in which the 21 % of n-

Table 4 Composition of triacylglycerol (TAG) molecular species (%, *w*/*w*) of the isolated fraction F1 from krill oil by UPLC/QToF-MS

Time (min)	Exact mass	Content (%)	CN/DB	Molecular species
1.63	816.63	0.89	50:9	TAG(12:0/18:4/20:5)
1.66	842.64	0.39	52:10	TAG(20:5/20:5/12:0)
1.82	818.64	0.75	50:8	TAG(18:4/18:4/14:0)
1.90	844.66	1.41	52:9	TAG(20:5/18:4/14:0)
1.94	870.67	1.05	54:10	TAG(20:5/20:5/14:0)
1.98	768.63	1.04	46:5	TAG(18:4/16:1/12:0)
2.03	794.64	0.74	48:6	TAG(20:5/16:1/12:0)
2.06	896.69	0.28	56:11	TAG(16:1/20:5/20:5)
2.07	820.66	0.70	48:7	TAG(14:1/14:1/20:5)
			42:1	TAG(14:0/12:0/16:1)
2.13	846.67	1.00	52:8	TAG(18:4/18:4/16:0)
2.21	898.71	0.24	56:10	TAG(20:5/18:1/18:4)
2.23	872.69	1.12	54:9	TAG(20:5/18:4/16:0)
2.35	796.66	3.38	52:6	TAG(18:1/20:5/14:0)
2.42	822.67	2.19	50:6	TAG(14:0/14:0/22:6)
2.53	874.71	0.30	54:8	TAG(20:5/18:2/16:1)
2.54	720.63	0.36	42:1	TAG(14:0/14:0/14:1),
			50:5	TAG(20:4/16:0/14:1)
2.58	746.64	0.88	44:1	TAG(12:0/16:0/16:1)
2.64	772.66	1.44	46:3	TAG(14:0/14:0/18:3)
2.78	824.69	1.80	50:5	TAG(20:5/16:0/14:0)
2.78	850.71	2.69	52:6	TAG(22:6/16:0/14:0),
2.81	798.67	3.22	48:4	TAG(14:0/16:0/18:4)
2.91	850.71	2.69	46:2	TAG(14:0/14:0/18:2), (14:1/14:0/18:1), (14:1/16:1/16:0)
2.95	824.69	2.69	50:5	TAG(18:4/18:1/14:0)
3.06	748.66	2.96	44:1	TAG(14:0/14:0/16:1)
3.14	774.67	3.03	46:2	TAG(16:1/16:1/14:0)
3.19	800.69	2.52	48:3	TAG(14:0/16:0/18:3)
3.31	878.74	1.79	54:6	TAG(16:1/18:4/20:1)
3.36	852.72	0.48	52:5	TAG(16:0/18:4/18:1),
3.54	878.74	0.54	54:6	TAG(20:5/18:1/16:0)
3.60	852.72	0.75	52:5	TAG(16:0/20:5/16:0)
3.66	802.71	2.70	48:2	TAG(18:1/16:1/14:0)
3.74	776.69	0.36	46:1	TAG(14:0/14:0/18:1)
3.83	802.71	5.92	48:2	TAG(16:0/16:1/16:1)
3.90	828.72	0.37	50:3	TAG(16:0/14:2/20:1), (16:0/14:0/20:3)
3.90	854.74	2.71	52:4	TAG(16:0/18:1/18:3)
4.01	790.71	1.49	47:1	TAG(15:0/15:0/17:1)
4.08	816.72	1.75	49:2	TAG(16:0/16:1/17:1), (14:0/18:1/17:1)
4.12	880.75	0.89	54:5	TAG(16:1/18:4/20:0)
4.15	790.71	0.49	47:1	TAG(14:0/15:0/18:1)
4.50	830.74	3.61	50:2	TAG(14:0/18:1/18:1)
4.63	804.72	7.97	48:1	TAG(14:0/16:0/18:1)
4.75	830.74	0.89	50:2	TAG(16:0/16:1/18:1)
4.88	858.77	0.49	52:2	TAG(16:0/18:0/18:2)

Table 4 (continued)

Time (min)	Exact mass	Content (%)	CN/DB	Molecular species
4.94	818.74	1.50	49:1	TAG(16:0/16:0/17:1)
5.04	844.75	1.30	51:2	TAG(16:0/17:1/18:1)
5.14	818.74	1.23	49:1	TAG(16:0/15:0/18:1)
5.50	884.78	1.22	54:3	TAG(18:1/18:1/18:1)
5.60	858.77	5.45	52:2	TAG(16:0/18:1/18:1)
5.73	832.75	4.83	50:1	TAG(16:0/16:0/18:1)
5.95	860.78	2.57	52:1	TAG(16:0/16:1/20:0)
6.61	874.80	0.39	53:1	TAG(16:0/17:1/20:0)
6.85	912.81	0.67	56:3	TAG(18:1/18:1/20:1)
6.96	886.80	3.39	54:2	TAG(16:0/18:1/20:1)
7.16	860.78	1.54	52:1	TAG(16:0/18:0/18:1)
7.26	914.83	0.66	56:2	TAG(16:1/20:1/20:0)
7.39	888.81	4.28	54:1	TAG(16:0/18:1/20:0)
7.39	970.89	0.48	60:2	TAG(20:1/20:1/20:0)
8.58	940.85	0.24	58:3	TAG(18:1/18:1/22:1)

All species were detected as $[M+ACN+NH_4]^+$

CN carbon number, *DB* double bonds

3 PUFAs were at the sn-2 position, although they only found TAG groups from CN28 to CN52.

In the present study, a total of 65 different molecular species of TAG were identified in F1 fraction, and against expected (due to high SFA content of this fraction; Table 2), none of them were fully saturated. That was explained because nearly 44 % of TAG of F1 fraction contained n-3 FA, again higher than expected taking into account the 8 % of n-3 as FAMEs, which could have been due to large distribution of n-3 FA within TAG. The most unsaturated TAG were located, as predictable, in those TAGs with high CN as 56:11 and 56:10.

In terms of relative amount, the most abundant TAG species were CN48 and CN50 with about 31 and 20 %, respectively (in agreement with the results provided previously by GC-FID (with about 38 and 17 %, respectively; see Table 3),

Table 5 Diacylglycerol (DAG) molecular species composition (%, *w*/*w*) in fraction F2 of krill oil by UPLC/QToF-MS

Time (min)	Exact mass	Content (%)	CN/DB	Molecular specie
0.92	660.48	22.62	40:10	DAG(20:5/20:5)
0.95	686.49	16.08	42:11	DAG(20:5/22:6)
0.99	712.51	4.25	44:12	DAG(22:6/22:6)
0.99	662.49	5.55	40:9	DAG(20:4/20:5)
1.04	612.48	4.03	36:6	DAG(18:4/18:2),(16:1/20:5),(14:0/22:6)
1.04	638.49	2.52	38:7	DAG(16:1/22:6)
1.04	586.46	2.62	34:5	DAG(18:4/16:1),(14:0/20:5)
1.09				
1.16	640.51	17.92	38:6	DAG(16:0/22:6),(18:1/20:5)
1.16	614.49	13.02	36:5	DAG(16:0/20:5),(18:4/18:1)
1.19	666.52	5.44	40:7	DAG(18:1/22:6)
1.34	566.49	2.05	32:1	DAG(14:0/18:1),(16:0/16:1)
1.34	592.91	1.50	34:2	DAG(16:0/18:2),(16:1/18:1)
1.54	594.52	2.40	34:1	DAG(18:1/16:0) ,(18:0/16:1),(14:0/20:1)

All species were detected as $[M+ACN+NH_4]^+$

DAG diacylglycerols, *CN* carbon number, *DB* double bonds

Table 6 Phosphatidylcholine (PC) species in F3 of krill oil as determined by UPLC/QToF-MS

Time (min)	Exact mass	Content (%)	CN/DB	Molecular specie
3.29	496.34	0.84	16:0	Lyso-PC(16:0)
3.37	520.34	NQ	18:2	Lyso-PC(18:2)
3.39	522.36	3.33	18:1	Lyso-PC(18:1)
6.68	760.58	6.67	34:1	PC(16:0/18:1)
6.77	826.53	2.97	40:10	PC(18:4/22:6, 20:5/20:5)
6.79	786.59	NQ	36:2	PC(18:0/18:2)
6.95	852.55	3.49	42:11	PC(20:5/22:6)
7.13	752.52	5.11	34:5	PC(14:0/20:5)
7.15	740.55	NQ	34:3	PC(P-16:0/18:3)
7.16	878.57	NQ	44:12	PC(22:6/22:6)
7.16	778.53	NQ	36:6	PC(14:0/22:6, 18:3/18:3)
7.17	744.54	NQ	33:2	PC(15:1/18:1)
7.51	734.62	NQ	34:0	PC(O-16:0/O-18:0)
7.57	754.53	3.22	34:4	PC(14:0/20:4)
7.57	738.54	1.04	34:4	PC(P-16:0/18:4)
7.80	780.55	53.99	36:5	PC(16:0/20:5)
7.88	806.56	9.32	38:6	PC(16:0/22:6)
7.93	790.57	NQ	38:6	PC(P-18:1/20:5)
8.04	756.55	1.62	34:3	PC(16:1/18:2)
8.13	794.56	0.96	37:5	PC(15:1/22:4)
8.23	832.58	1.36	40:7	PC(18:2/22:5)
8.52	766.57	0.95	36:4/36:5	PC (O-16:0/20:5. O-16:1/20:4 P-18:1/18:3)
8.61	732.55	1.33	32:1	PC (14:0/18:1, 16:0/16:1)
8.76	758.57	2.69	34:2	PC(16:1/18:1)
8.74	788.61	NQ	36:1	PC(18:0/18:1)
8.90	792.58	1.11	38:5	PC(P-18:1/20:4)

All species were detected as the $[M+H]^+$

Lyso-PC lyso-phosphatidylcholine, *PC* phosphatidylcholine, *CN* carbon number, *DB* double bonds, *NQ* not quantifiable

being the species (14:0/16:0/18:1), (16:0/16:1/16:1), (16:0/18:1/18:1), and (16:0/16:0/18:1) the most abundant with amounts of about 8, 6, 5.5, and 4.8 %, respectively. The combinations of these FA are also in agreement with the large presence of 14:0, 16:0, 16:1, and 18:1 in the F1 FAME profile.

DAG Molecular Species

A total of 22 DAG different molecular species were identified in fraction F2, and as well as in TAG, none of them was saturated (Table 5) due to the major unsaturated profile (64 %) of this fraction. The range of DAG was from CN32 to CN44 and the most abundant content was for the groups CN36 (17 %), CN38 (20 %), CN40 (34 %), and CN42 (16 %). It is remarkable that the major relative content was for those DAGs that contained the n-3 FA C20:5 (EPA) in its composition as (16:0/20:5), (18:1/20:5), (20:5/22:6), and specially the DAG (20:5/20:5) which showed the highest content of 22.62 %.

Phospholipid Molecular Species

As before, the identification of molecular species of PL present in F3 was carried out with UPLC/QToF-MS (Table 6). A total of three lyso-PC containing the FAs 16:0, 18:1, and 18:2 were identified although they almost reached 5 % of the total PL content. Winther et al. (2010) reported that they found seven lyso-PC species (16:0, 16:1, 17:0, 18:1, 20:5, 21:1, and 22:6) while Le Grandois et al. (2009) did not find any. Twenty-eight different molecular species of PC were observed in the present study ranging from CN32 to CN44, all being unsaturated species in agreement with the studies cited before (Le Grandois et al. 2009; Winther et al. 2010), although they reported the identification of 21 and 51 different species, respectively. The CN36:5 group contained the most abundant specie PC (16:0/20:5) which reached the 54 % of the total F3 fraction. It is remarkable that the n-3 PC (20:5/22:6) and (20:5/20:5) species reached a relative content of 6.5 %. These observations were also in agreement with the values provided by

Le Grandois et al. (2009) who quantified the PC (16:0/18:1), (16:0/20:5), and (16:0/22:6) as the most abundant with a total of 50 % after the analysis of a commercial KO by LC-ESI-MS2. On the other hand, Winther et al. (2010) showed that these PC species were the most present in KO based in their relative intensity measured by NPLC-ESI-MS. A highlight is that most of these PC species contain EPA, DHA, or both. These results confirm the high n-3 LCPUFA composition content in the PL from KO. The present UPLC/QToF-MS analysis could also identify six different molecular species of SM (18:1/20:0), (18:1/21:0), (16:1/24:1), (18:1/22:1), (18:1/22:0), and (17:1/24:1). Other authors (Zhou et al. 2012b) analyzed the SM species in KO with LC-ESI-MS2, but they could not found any. The same authors in a different work reported the presence of eight different species of PE (Zhou et al. 2012a). In the present study, PE was also detected but in an amount less than 0.1 % of total PL.

Conclusions

In conclusion, KO is a highly unsaturated product with a high content of EPA and DHA, half of which are located in phospholipids, mostly phosphatidylcholine. SFA was mainly bound to TAG, but a trend was observed in which the amount of unsaturated TAG increased with increasing CN. The DAG+MAG+FFA molecules contained similar concentrations of SFA and PUFA (due to its high content of EPA) and a minor amount of MUFA, which resulted in that a quarter of the possible molecular species were saturated. PL fraction was highly polyunsaturated due the content of EPA and DHA, which appeared in a great part of the 37 molecular species of phospholipids identified. These data suggest that KO but specially the isolated fraction F3 could be used as a valuable functional ingredient or nutraceutical.

Acknowledgments This work has been financially supported by the Spanish Ministry of Science and Innovation AGL 2011-26713.

Conflict of Interest María Pilar Castro-Gómez declares that she has no conflict of interest. Francisca Holgado declares that she has no conflict of interest. Luis Miguel Rodríguez-Alcalá declares that he has no conflict of interest. Olimpio Montero declares that he has no conflict of interest. Javier Fontecha declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

References

Ali-Nehari A, Chun B-S (2011) Characterization of purified phospholipids from krill (*Euphausia superba*) residues deoiled by supercritical carbon dioxide. Korean J of Chem Eng 29:918–924

Araujo P, Zhu H, Breivik J, Hjelle J, Zeng Y (2014) Determination and Structural Elucidation of Triacylglycerols in Krill Oil by Chromatographic Techniques. Lipids 49:163–172

Balk EM, Lichtenstein AH, Chung M, Kupelnick B, Chew P, Lau J (2006) Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: A systematic review. Atherosclerosis 189:19–30

Barrento S, Marques A, Teixeira B, Mendes R, Bandarra N, Vaz-Pires P, Nunes ML (2010) Chemical composition, cholesterol, fatty acid and amino acid in two populations of brown crab Cancer pagurus: Ecological and human health implications. J Food Compos Anal 23:716–725

Batetta B, Griinari M, Carta G, Murru E, Ligresti A, Cordeddu L, Giordano E, Sanna F, Bisogno T, Uda S, Collu M, Bruheim I, Di Marzo V, Banni S (2009) Endocannabinoids May Mediate the Ability of (n-3) Fatty Acids to Reduce Ectopic Fat and Inflammatory Mediators in Obese Zucker Rats. J Nutr 139:1495–1501

Berge K, Musa-Veloso K, Harwood M, Hoem N, Burri L (2014) Krill oil supplementation lowers serum triglycerides without increasing low-density lipoprotein cholesterol in adults with borderline high or high triglyceride levels. Nutr Res 34:126–133

Breslow JL (2006) n–3 Fatty acids and cardiovascular disease. Am J Clin Nutr 83:S1477–1482S

Bunea R, El Farrah K, Deutsch L (2004) Evaluation of the effects of Neptune Krill Oil on the clinical course of hyperlipidemia. Altern Med Rev: J Clin Ther 9:420–428

Castro-Gomez MP, Rodriguez-Alcala LM, Calvo MV, Romero J, Mendiola JA, Ibanez E, Fontecha J (2014) Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques. J Dairy Sci 97:6719–6728

Davidson MH, Stein EA, Bays HE, Maki KC, Doyle RT, Shalwitz RA, Ballantyne CM, Ginsberg HN (2007) Efficacy and tolerability of adding prescription Omega-3 fatty acids 4 g/d to Simvastatin 40 mg/d in hypertriglyceridemic patients: An 8-week, randomized, double-blind, placebo-controlled study. Clin Ther 29:1354–1367

Deutsch L (2007) Evaluation of the Effect of Neptune Krill Oil on Chronic Inflammation and Arthritic Symptoms. J Am Coll Nutr 26:39–48

Di Marzo V, Griinari M, Carta G, Murru E, Ligresti A, Cordeddu L, Giordano E, Bisogno T, Collu M, Betetta B, Uda S, Berge K, Banni S (2010) Dietary krill oil increases docosahexaenoic acid and reduces 2-arachidonoylglycerol but not N-acylethanolamine levels in the brain of obese Zucker rats. Int Dairy J 20:231–235

Eslick GD, Howe PRC, Smith C, Priest R, Bensoussan A (2009) Benefits of fish oil supplementation in hyperlipidemia: a systematic review and meta-analysis. Int J Cardiol 136:4–16

Fontecha J, Mayo I, Toledano G, Juarez M (2006) Triacylglycerol composition of protected designation of origin cheeses during ripening. Authenticity of milk fat. J Dairy Sci 89:882–887

Fraga MJ, Fontecha J, Lozada L, Juárez M (1998) Silver Ion Adsorption Thin Layer Chromatography and Capillary Gas Chromatography in the Study of the Composition of Milk Fat Triglycerides. J Agric Food Chem 46:1836–1843

Fraga MJ, Fontecha J, Lozada L, Martínez-Castro I, Juárez M (2000) Composition of the sterol fraction of caprine milk fat by gas chromatography and mass spectrometry. J Dairy Res 67:437–441

Gigliotti JC, Davenport MP, Beamer SK, Tou JC, Jaczynski J (2011) Extraction and characterisation of lipids from Antarctic krill (*Euphausia superba*). Food Chem 125:1028–1036

Harris WS, Kris-Etherton PM, Harris KA (2008) Intakes of long-chain omega-3 fatty acid associated with reduced risk for death from coronary heart disease in healthy adults. Curr Atheroscler Rep 10:503–509

Hussein G, Sankawa U, Goto H, Matsumoto K, Watanabe H (2006) Astaxanthin, a carotenoid with potential in human health and nutrition. J Nat Prod 69:443–449

Ikeuchi M, Koyama T, Takahashi J, Yazawa K (2007) Effects of astaxanthin in obese mice fed a high-fat diet. Biosci Biotechnol Biochem 71:893–899

ISO IS (2002) Milk fat-Preparation of fatty acid methyl esters ISO 15884-IDF:182:2002

Ju S-J, Harvey HR (2004) Lipids as markers of nutritional condition and diet in the Antarctic krill *Euphausia superba* and *Euphausia crystallorophias* during austral winter. Deep Sea Res Part II 51: 2199–2214

Ju S-J, Kang H-K, Kim W, Harvey HR (2009) Comparative lipid dynamics of euphausiids from the Antarctic and Northeast Pacific Oceans. Mar Biol 156:1459–1473

Kassis NM, Gigliotti JC, Beamer SK, Tou JC, Jaczynski J (2012) Characterization of lipids and antioxidant capacity of novel nutraceutical egg products developed with omega-3-rich oils. J Sci Food Agric 92:66–73

Kolakowska A, Kolakowski E, Szczygielski M (1994) Winter season krill (*Euphausia superba* D.) as a source of n-3 polyunsaturated fatty acids. Food Nahrung 38:128–134

Le Grandois J, Marchioni E, Zhao M, Giuffrida F, Ennahar S, Bindler F (2009) Investigation of Natural Phosphatidylcholine Sources: Separation and Identification by Liquid Chromatography−Electrospray Ionization−Tandem Mass Spectrometry (LC−ESI−MS2) of Molecular Species. J Agric Food Chem 57:6014–6020

Maki KC, Reeves MS, Farmer M, Griinari M, Berge K, Vik H, Hubacher R, Rains TM (2009) Krill oil supplementation increases plasma concentrations of eicosapentaenoic and docosahexaenoic acids in overweight and obese men and women. Nutr Res 29:609–615

Phleger CF, Nichols PD, Virtue P (1998) Lipids and trophodynamics of Antarctic zooplankton. Comp Biochem Physiol Part B: Biochem Mol Biol 120:311–323

Rodríguez-Alcalá LM, Fontecha J (2007) Hot topic: Fatty acid and conjugated linoleic acid (CLA) isomer composition of commercial CLA-fortified dairy products: Evaluation after processing and storage. J Dairy Sci 90:2083–2090

Saether O, Ellingsen TE, Mohr V (1986) Lipids of North Atlantic krill. J Lipid Res 27:274–285

Sampalis F, Bunea R, Pelland MF, Kowalski O, Duguet N, Dupuis S (2003) Evaluation of the effects of Neptune Krill Oil on the management of premenstrual syndrome and dysmenorrhea. Altern Med Rev: J Clin Ther 8:171–179

Schuchardt JP, Schneider I, Meyer H, Neubronner J, von Schacky C, Hahn A (2011) Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations–a comparative bioavailability study of fish oil vs. krill oil. Lipids Health Dis 10:145

Suzuki T, Shibata N (1990) The utilization of Antarctic krill for human food. Food Rev Int 6:119–147

Svetlova NI, Golovnya RV, Zhuravleva IL, Grigorieva DN, Samusenko AL (1985) Gas chromatographic investigation of volatile nitrogen containing bases of Antarctic krill *Euphausia superba*. Dana Die Nahrung 29:143–151

Tandy S, Chung R, Wat E, Kamili A, Berge K, Griinari M, Cohn J (2009) Dietary krill oil supplementation reduces hepatic steatosis, glycemia and hypercholesterolemia in high-fat fed mice. J Agric Food Chem 57:9339–9345

Tou J, Jaczynski J, Chen YC (2007) Special Articles - Krill for Human Consumption: Nutritional Value and Potential Health Benefits. Nutr Rev 65:63–77

Tsape K, Sinanoglou VJ, Miniadis-Meimaroglou S (2010) Comparative analysis of the fatty acid and sterol profiles of widely consumed Mediterranean crustacean species. Food Chem 122:292–299

Ulven S, Kirkhus B, Lamglait A, Basu S, Elind E, Haider T, Berge K, Vik H, Pedersen JI (2011) Metabolic Effects of Krill Oil are Essentially Similar to Those of Fish Oil but at Lower Dose of EPA and DHA, in Healthy Volunteers. Lipids 46:37–46

Wijendran V, Huang MC, Diau GY, Boehm G, Nathanielsz PW, Brenna JT (2002) Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. Pediatr Res 51:265–272

Winther B, Hoem N, Berge K, Reubsaet L (2010) Elucidation of phosphatidylcholine composition in krill oil extracted from *Euphausia superba*. Lipids 46:25–36

Zhou L, Zhao M, Ennahar S, Bindler F, Marchioni E (2012a) Determination of phosphatidylethanolamine molecular species in various food matrices by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS). Anal Bioanal Chem 403:291–300

Zhou L, Zhao M, Ennahar S, Bindler F, Marchioni E (2012b) Liquid Chromatography–Tandem Mass Spectrometry for the Determination of Sphingomyelin Species from Calf Brain, Ox Liver, Egg Yolk, and Krill Oil. J Agric Food Chem 60:293–298

2. Estudio de leche enriquecida en fosfo- y esfingolípidos, procedente de rumiantes con dietas suplementadas con aceites ricos en ácidos grasos poliinsaturados.

2.1. Qualitative and quantitative analysis of phospholipids and sphingolipids in ewes´ milk fat with enhanced nutritional quality through diet supplementation.

Qualitative and quantitative analysis of phospholipids and sphingolipids in ewes' milk fat with enhanced nutritional quality through diet supplementation

Pilar Castro-Gómez, Luis Miguel Rodríguez-Alcalá, María Visitación Calvo and Javier Fontecha

Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Madrid, Spain 28049

*Corresponding author: j.fontecha@csic.es

ABSTRACT

Milk fat polar lipids (PL) as phospho- and sphingolipids are located in the milk fat globule membrane, which have been attributed positive effects on cancer, cardiovascular disease, metabolic syndrome or cognitive activity. Many studies have observed the positive changes occurred in fatty acids profile after ruminant diet supplementation with unsaturated oil, however the assessment of the effect on PL variations are scarce. Therefore the main objective was to evaluate the changes occurred in the PL of ewes' milk as consequence of the ruminant diet supplementation with sunflower oil (SO) with the aim of improving milk nutritional value. As expected the dietary supplementation resulted in marked changes in milk FA profile increasing the cis-9, trans-11 CLA (rumenic acid) content and also the n-3 PUFA, mainly alpha-linolenic acid. There was also a remarkable increase in the contents of the total PL especially of sphingomyelin (from 7 to 21% on total PL). Higher PUFA proportions were also found in the phospholipids of the supplemented milk than in control milk. However, the FA composition changes observed in sphingomyelin after the supplementation did not differ significantly among milk samples. In conclusion, the sunflower oil addition to ewes diet not only improved the total FA profile in ewe milk but also the phospho- and sphingolipids distribution and the FA composition of the membrane lipids.

INTRODUCTION

It is increasingly apparent that some of the milk fat components, uniquely present in milk fat, may have beneficial effects on human health maintenance and disease prevention.

Milk is a complex food made up of numerous components, which are very important from a nutritional point of view. Among them it is remarkable the milk fat whose compounds, mainly triacylglycerides (TAG) (98% of total fat), are into globules surrounded by a protein and phospholipid membrane namely milk fat globule (MFGM). Due the high concentration of saturated fatty acids (SFA) and its relation with cardiovascular diseases development, the dairy fat image has been widely devaluated (Mills et al., 2011). However, recent studies have not only refuted these ideas because lack of evidences (O'Keeffe and St-Onge, 2013, Ravnskov, 2014) but even highlighted the biological activity that other components promote on human health (German and Dillard, 2006, Steijns, 2008, Parodi, 2009). Some fatty acids (FA) as short FA (as butyric acid) or other as oleic or conjugated linoleic acid (CLA), as well as other lipid components as the PL (phospho- and sphingolipids), have been attributed favorable effects in cardiovascular and mental diseases or as anticarcinogenic agent (Huth et al., 2006, El-Loly, 2011, Kullenberg et al., 2012). With the objective of increase these compound proportions in milk fat, some studies have reported that after a cattle diet supplementation with unsaturated oils, there are a reduction of SFA and an increment of beneficial monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) as the CLA (Mele et al., 2006, Gómez-Cortés et al., 2008, Hervás et al., 2008).

Polar lipids are minor constituents of milk lipids, but have been suggested to have health-promoting properties and beneficial effects on humans (Kullenberg et al., 2012), however the changes occurred in the distribution and composition of phospho- and sphingolipid after the ruminant supplementation diet has not been thoroughly studied.

In the present study, the identification and composition of the main milk lipid constituents as total TAG, DAG and MAG groups and the phospho- and sphingolipids as well as their fatty acid composition was determined. The inclusion of cholesterol in our composition analysis and its variation because the

ruminant supplementation diet on milk fat composition might also account for the differences between the results of the present study and those of previous studies (Lopez et al., 2008, Ménard et al., 2010).

Therefore the aim of this work was to evaluate the changes occurred in the neutral and polar lipid fractions (mainly phospho- and sphingolipids) occurring in ewes milk fat as a consequence of the diet supplementation with sunflower oil (SO).

MATERIALS AND METHODS

Samples

Twenty-four Spanish Assaf ewes were distributed in 2 lots of 12 animals, balanced for milk production and allocated at random to two experimental treatments, one was used as the control (Control) and the other received the diet supplemented with sunflower oil (SO). The diets consisted of a high concentrate total mixed ration based on alfalfa hay and concentrate supplemented with 0 (Control) or 60 g sunflower oil/kg DM (SO). The experiment lasted for a total of 5 weeks in mid-lactation and all detailed information about different diets and milk FA composition are previous study (Hervás et al.,(2008).

Chemicals and reagents

All solvents were at least HPLC grade and MS grade when available. Chloroform, hexane, methanol, isooctane, isopropanol, ammonium hydroxide, arsenic formate and acetronitile were purchased from LABSCAN (Dublín, reland). Potassium hydroxide and sodium carbonate were obtained from PANREAC (Barcelona, Spain). Formic acid (98%) and triethylamine (99.5%), the TAG standards trinanoin and tritridecanoin, the free fatty acids (FFA) standars pelargonic (C9), tridecanoic (C13), myristic (C14), palmitic (C16), estearic (C18), araquidonic (AA, 20:4), eicosapentanoic acid (EPA, 20:5) and

docosahexapentanoic acid (DHA, 22:6), the sterols 5α-cholestane, cholesterol (CHOL) and cholesterol ester (CE), the mono- (MAG) and diacylglyceride (DAG) monostearin and diolein, the PL: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), sphingomyelin (SM) phosphatidylcholine (PC) and N-oleoylethanolamine, as well as the matrix 9-aminoacridine were purchased to Sigma (Bellefonte, PA, USA). The reference samples: butter fat BCR-159 (EU Commissions; Brussels, Belgium) were purchased from Fedelco Inc. (Madrid, Spain), omega-3 Ropufa (Derbyshire, England) from DSM and DHA oil from MartekTM-S (Martek Bioscience Corporation, Columbia, MD, US) were used. The 2,5-dihidroxibenzoic and the calibrant HCCA were obtained from Bruker (Bremen, Germany).

Milk fat extraction

The milk fat extraction was carried out using dichloromethane-methanol procedure as in Castro-Gomez et al. (2014). Briefly, a volume of 3mL of sample was placed in 50-mL centrifuge tube with 1 mg of previously added trinonanoin as internal standard and 22.5 mL of a dichloromethane-methanol solution (2:1, vol/vol) was then added. The mixture was shaken mechanically for 30 min and centrifuged at 6,600 × *g* for 5 min at 4°C. As much of the upper organic solvent fraction as possible was carefully removed with a pipette. The sediment was washed with 18 mL of a dichloromethane-methanol solution (2:1, vol/vol) and, after shaking for 1 min, the sample was, again centrifuged. The removed organic solvent was combined with that previously collected and 4.5 mL of a 0.9% solution of sodium chloride was added and mixed mechanically for 1 min before the tubes were stored overnight at 4°C. Afterwards, It was again centrifuged and the bottom dichloromethane layer filtered through a syringe PVDF filter with a 0.45 µm pore size (CAPTIVA Econo Filter, Agilent Technologies Inc.) containing approximately 1.5 g of anhydrous sodium sulfate. Finally, the extract was concentrated by removing dichloromethane in a rotatory evaporator and dried under a gentle stream of nitrogen. The extracted fat was weighted before/after in amber vials flushed with nitrogen and stored at -35ºC until chromatographic analysis.

Phospho- and Sphingolipids isolation by TLC

Milk lipid classes were separated from TLC separation system (CAMAG, Muttenz, Switzerland) as Christie (1985). Briefly, the milk fat was extracted using a method modified from Folch (Castro-Gómez et al., 2014a). Lipid samples (60mg/mL) spiked with C17 as internal standard (1.51 mg/mL) were prepared in chloroform-methanol (2:1) and 60 µL applied on a lane at 1.5 cm from the 10 X 20 cm pre-coated silica gel 60 plate bottom edge. Standard lipid mixtures from Larodan TLC-34 and TLC-40 (Solna, Sweden) were prepared (20mg/mL) and 4 µL applied at the lower left-hand corner of the plate. The plates were developed in a chamber containing a solvent system hexane:diethyl ether:formic acid (70:30:2) and chromatographed at room temperature in the range of 20–25°C in a closed tank allowing the solvent to reach the TLC top edge for approximately 25 min. The plate was removed and set to dry before being sprayed with 2′,7′-dichlorofluorescein (DCF) solution (1:1 methanol:water) and set in a chamber containing 25 % ammonium hydroxide for 5 min. Lipid classes were then observed under UV light (Fig. 2 A). Afterwards, phospholipid fractions were scraped into individual 15-mL tubes extracted in chloroform and again applied as before to the 10 X 20 cm TLC plate. The plate was now developed in a solvent system chloroform:methanol:petroleum ether:Acetic acid:boric acid (40:20:30:10:1.8). The solvent was freshly prepared and the boric acid was dissolved in the methanol-acetic acid before addition of the other components. Standard phospholipids from Larodan were prepared (20 mg/mL) and 2 µL each applied at the lower left-hand corner of the plate. The solvent was allowed to run to the top of the plate, and phospholipids were separated and extracted individually by TLC scrapings containing each phospholipid band (see Fig. 2) and dissolved with Toluene and stored until analysis by GC-MS as below.

Fatty acid methyl esters determination of phospholipids and polar lipid fractions by GC-MS

The fatty acid methyl esters (FAME) of each PL were prepared as in Castro-Gómez et al. (2014) with slight modifications. Briefly, the FAME were separated

using a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2-µm film thickness; Agilent Technologies Inc., Palo Alto, CA) coupled to an Agilent chromatograph (model 6890N; Agilent Technologies Inc.) equipped with a mass spectrometry detector. The method temperature was programmed at 7°C/min to 170°C, held at 170°C for 55 min, and then temperature programmed at 10°C/min to 230°C and held at 230°C for 33 min. The injector temperature was set at 250°C. Helium was the carrier gas with a column inlet pressure of 206.9 kPa. The mass spectrometry detector conditions were as follows: transfer line temperature: 250°C, source temperature: 230°C, quad temperature: 150°C, electron impact ionization: 70eV and the range from 50 to 500 m/z was scanned. For identification of the peaks, the National Institute of Standards and Technology (NIST, Gaithersburg, MD) library and mass spectra of the standards used in our laboratory were used. The injection volume was 1 µL and the split ratio used was 1:25. Response factors were calculated using an anhydrous milk fat (reference butterfat BCR-164) and tritridecanoin as internal standard (200 µL; 1.3 mg/mL) was used. Assays carried out in triplicate.

Lipid classes composition by HPLC-Evaporative Light Scattering Detection

Separation of lipid classes was accomplished with a HPLC Agilent Technologies, model 1260 (Palo Alto, CA, USA) coupled to an ELSD detector (SEDERE. SEDEX 85 model, Alfortville Cedex, France) using pre-filtered compressed air as the nebulizing gas at pressure of 350 KPa, temperature of 60ºC and the gain was set at 3. Two columns Zorvax Rx-SIL column (Agilent Technologies, Palo Alto, CA, USA) of 250 mm × 4.5 mm and 5 µm particle size, were used in series with a precolumn of the same packing which were equilibrated at 40ºC. The injection volume was 50 µL at concentration of 50 mg/mL. Solvent gradient was as detailed in Castro-Gómez et al. (2014). Analyses were carried out with solvents freshly prepared. Samples and standards were analyzed under the same conditions. Assays were carried out in triplicate.

Triacylglycerides and cholesterol determination by GC-FID

TAG and CHOL and analysis of milk fat was performed following Fontecha et al. (2005). The samples were injected (30 mg/mL) in a CLARUS 400 gas chromatograph (Perkin Elmer, Beaconsfield, UK) fitted with a RTX-65 TAG fused silica capillary column Crossbond® (65% diphenyl:35% dimethyl polysiloxane) (30m x 0.25 mm ID x 0.1 μm) (Restek Corpotration, Bellefonte, PA, USA). Analysis for CHOL content, as well as different acyl carbon number (CN) TAG were carried out as follow: 120ºC held 30s, 10ºC/min to 220ºC held 30s, 6ºC/min to 350ºC held 30min. Injector and FID temperatures were 355ºC and 370ºC, respectively. Helium was used as carrier gas at 172.37 KPa and the injection volume was 0.5 μL. The reference butterfat BCR-519 and trinanoin were used as internal standards (100 μL; 1 mg/mL). Assays carried out in triplicate.

Phospho- and sphingolipid species determination by MALDI-TOF

The PL species determination was carried out following Fuchs et al (2010). Solution of 1mg/mL of each isolated PL were prepared to mix matrix:sample in ratio 2:1. Then 0.5μL of this mixture was directly applied to an MTP Anchor Chip 800/384 (Bruker Daltonics GmbH, Bremen, Germany). Qualitative determination was carried out in a Bruker Autoflex Speed (Bruker Daltonic GmbH, Bremen, Germany) MALDI-TOF, operated in positive-ion reflector mode. During acquisition, mass range was limited to 400-1100 Da to prevent possible detector saturation with ions from the matrix (2,5-dihidroxybenzoic acid). Laser power was kept constant at 70% and set to 1500 shots for analysis. Mass spectrometric data were analyzed using Bruker Daltonics flexAnalysis and Lipid Mass Spectra Prediction software (LIPID MAPS consortium). The Na+ and H+ adducts were established for the PL species detection. Each PL fraction was analyzed at list once.

Statistical analysis

Statistical analysis of difference was assessed by Mann-Whitney U-test. Statistical significance ($P<0.05$) was determined using two tailed Mann-Whitney U-test. All analyses were performed using the SPSS Statistics software (v19.0 for Windows; IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

Lipid classes distribution and composition

It is well known that the amount and composition of milk fat depend on several factors, such as breed, lactation stage, genotype and the ruminants diet. Table 1 and Figure 1 show the lipid classes distribution and chromatographic profiles of CtM and SppM. The ewes diet supplementation with SO rich in unsaturated FA resulted in higher milk fat content (4.8 vs. 5.3) and in higher n-3 PUFA and CLA as previously reported by Hervas et al., (2008). Dietary supplementation also resulted in an increase in TAG and PL lipid classes observed in SppM at the expense of a decreases in other fractions as DAG and Chol ($P<0.01$) that could be due to the rise of the PL synthesis in the mammary gland cells as reported by Barbano and Sherbon (1981). On the other hand, the significant decrease observed of the CHOL content of SppM (0.93 vs 0.37%) have not been reported before and could be related to the differences of CHOL endogenous synthesis among ruminant due to the PUFA supplemented diet (Gorban and Izzeldin, 1999). No significant changes were observed in other minor lipid classes as FFA, MAG, CE, glucosylceramide and lactosylceramide between studied milks. In terms of total PL content, SppM showed an increment of 12% of total PL in comparison to the CtM content although this difference was not significant. Similar result was reported by Lopez et al. (2008) whose observed and increment of 16% of total PL on cow milk when the diet was supplemented with linseed. These authors attributed this rise of PL to the lower size but high number of milk fat globules in supplemented milk. The individual components of the PL fraction, i.e., phospho- and sphingolipid, showed

significant differences in their content ($p<0.05$) because the diet supplementation. The significant increase of SM content in SppM which is almost three-fold higher than the CtM (from 7.07% in CtM to 19.72% in SppM on total PL) could be considered of great importance from the human health point of view mainly due to the numerous beneficial effects attributed to this sphingolipid (Kuchta et al., 2012, Pralhada Rao et al., 2013). This increment is agreement with the increase of around ~30% for the SM of cows supplemented diet reported by Lopez et al. (2008). The content of the other phospholipids present in this PL fraction decreased slightly as PI (~4% vs. ~3%) and PS (~3% vs. ~2%) and PE (~42% vs. ~30%) with exception of PC that did not change.

TAG and CHOL composition

Milk fat consists primarily of TAGs and its composition together with the CHOL level is of great interest due to their role in nutrition and cardiovascular diseases.

In this study, 16 peaks of TAG were discernible on the chromatograms obtained from the milk fat samples, corresponding to TAG structures with the acyl carbon number (CN) from 24 to 54 (and the CHOL peak that eluted close to the CN 26) in agreement with previously reported by Fontecha et al. (2005) and Castro-Gomez et al. (2014) in ovine milk. Table 2, presents the mean and SD values for the TAG from the CtM and SppM. Although the total TAG content determined by HPLC-ELSD did not show differences among the studied samples (Table 1), the percentage of the TAG groups by CN was dependent of their fatty acid composition. Therefore, the SppM provided a significant increase of high molecular weight TAG from CN 48 to CN 54 accounted for a threefold higher than CtM. These variations in TAG recorded were consistent with the higher long chain PUFA content for the SppM as reported by Hervás et al. (2008). Furthermore, it is supported by the described preference of FA with 18 CN to incorporate to long chain TAG (CN 48 to CN 54) (Fontecha et al., 2000, Gastaldi et al., 2011). Consequently, the SppM presented significant decrease of a low molecular weight TAG groups range from TAG 24 to TAG 36 and from TAG 42 to TAG 44 in comparison with CtM. These TAG groups, are classified

as short and medium chain TAG (TAG 24-TAG C40) and are mainly composed of FA from C4 to C12 which are unique for milk fat products.
Related to the CHOL content, in agreement with reported above, the GC-FID result showed that in SppM there was a significantly lower content than in CtM (1.07 vs. 0.41) on total fat. This is probably due to the reduction of endogenous CHOL synthesis in the animal consequence of the sunflower oil supplement.

Phospho- and sphingolipids composition

The bidirectional TLC allowed elute compounds from neutral to more polar lipids. As can be observed in Figure 2, primarily, a total group of non-polar components was separated. However, the isolation of PL was carried out individually and in the following order: PE, PC, PS, PI and SM. When each PL was isolated from the TLC plaque, their FAME composition was analysed by GC-MS.
The knowledge of FA composition of PL is of wide importance due the reported higher FA absorption observed than when they are linked to TAG (Schuchardt et al., 2011, Ulven et al., 2011). Despite the milk fat is the main source of short FA in the diet, the PL of both milks only had linked FA with 14 or more CN. This is in according with Martini et al. (2013) in ewes MFGM whose neither found short FA bounded to phospho- and sphingolipids. The phospholipids (PE, PI, PS and PC) of SpM showed a diminution of total SFA when they are compared with the CtM. This diminution, mainly due the decrease of the FA C14:0, C16:0 and C18:0, suggested that these FA have the preference to be bounded in neutral lipids, as TAG, instead of phospholipids. The FA with 20 or more CN were found in high amount linked to SM. Significant increments of some FA of this sphingolipids, as C22:0 (from 11.06% in CtM to15.94% in SpM on total FA) and C24:0 (from 13.36% in CtM to 21.21% in SpM on total FA) after the supplementation was balanced by the decrease of C23:0 from 29.86% in CtM to 17.85% in SpM on total FA. These changes resulted in a similar total SFA content in the SM of both samples. The main saturated profile observed in SM in both milks (~88%) is in according with the 90% of SFA (overall with FA which CN ≥20) reported by previous studies in similar samples (Bitman and Wood, 1990, Fong et al., 2007, Lopez et al., 2008). In terms of total MUFA, it was

increased in all the PL of SpM, mainly due the increment of C18:1 9t, C18:1 10t and C18:1 11t (TVA). This behaviour after the supplementation was also observed by Lopez et al. (2008) in cow MFGM FA composition after a linseed oil supplementation. The increment of TVA is remarkable due its role as precursor of rumenic acid (RA) (C18:2 9c, t11t) with known beneficial effects (Oh et al., 2014). The main PUFA in all the samples was the C18:2 9c, 12c which showed higher concentration in SpM than in CtM. This increment resulted in a high content of n6-FA in SpM while the variation of n3-FA was less pronounced. Related to the functional RA (Pariza, 2004), it had a significant increase in SpM PE (from 0.13 in CtM to 2.88% in SpM, on total FA) and even appeared *de novo* in PI, PS and PC after the supplementation. As described above, this increment of functional FA in SpM is remarkable from a nutritional point of view due the reported better absorption of FA linked to PL than to TAG. The FAME composition of all the PL in CtM is in according with those described by Fong et al. (2007) in PL isolated from MFGM. However, despite the study of Lopez et al. (2008) reported a total FA profile of total PL in control and supplemented cow milks, the study of each PL composition have not been reported before.

PL species composition

The identification of the different phospho- and sphingolipid groups (CN:double bounds (DB)) was carried out with MALDI-ToF. Furthermore, the identification of the most probable specie in each group was based on the PL FAME composition (Table 3). As expected, the changes in each PL FA composition after the diet supplementation gave rise to different molecular species in milks. The PE provided 16 different species in CtM and 9 in SpM. While the 25% of the PE species in CtM were saturated, the SpM mainly showed unsaturated (Table 4), among other causes, because the new synthesis of the PUFA species PE (14:0/18:4), PE (18:2/20:0) and PE (18:1/20:3). This is in according with the lower SFA and higher unsaturated FA profile observed in SpM in comparison with CtM. Although the study of Fong et al. (2007) found similar amount of PE species (14) in cow MFGM, they were grouped from C32:0 to C36:4 while in the present study they were distributed from C30:0 to C44:5.

Related to PI and PS (Table 5), both PL showed 7 different species in CtM. On a hand, after the supplementation, the PI provided only 4 species due the disappearance of C15:0 and C16:1 in FA profile, however the increment of total C18:2 FA caused the synthesis of the specie PI (C18:2/C18:4). On the other hand, the PS provided 8 species in SpM after the lack of PS (14:0/16:1) (due C16:1 was not present in SpM PS) and the synthesis of PS (18:1/24:1) and PS (20:2/24:1) due the rise of total C18:1 and the apparition of C24:1 in SpM (Table 3). Despite the high unsaturated profile of PI and PS after the supplementation, both PL showed similar proportion of unsaturated species in CtM and SpM. The study of Fong et al. (2007) in cow MFGM showed more species of PI and PS (11 and 8 respectively) with different composition than those observed in CtM results probably due the different origin and composition of the analyzed samples. In terms of PC, this group showed 27 species in CtM and 19 in SpM (Table 6). Although the number of species was low in SpM, the species PC (18:1/20:0) and PC (18:2 /22:0) were newly synthetized in SpM due the increment of the total C18:1 and C18:2 c9,c12 in this PL. As occurred with total SFA content in PC after the supplementation, the concentration of saturated species decreased from ~30% in CtM to ~20% in SpM. The study mentioned above (Fong et al., 2007) described only 17 PC species in MFGM from C28:0 to C36:5 while the present study distributed 27 species from C28:0 to C46:0 in CtM. The differences related to cited study, as in the PL above described, could be due the use of the milk from other ruminant or the different analytical technique used to PL species determination. Finally, both milks showed the same species of SM, however the SpM had one more specie (15 species in total) than CtM due the synthesis of SM with 16:1 (Table 7). This is supported by the apparition *de novo* of this FA in SM after the supplementation (Table 3). In according with different studies (Jensen, 2002, Lopez et al., 2008), the SM profile in both milks were mainly saturated, being >50% of SM species in this study. The study of Malmsten et al. (1994), carried out in cow SM, also reported this sphingolipid distributed from C14:0 to 24:1 (as in the present study), however only 11 different species were identified.

In conclusion, the supplementation of ewe feed with sunflower oil, causes not only beneficial changes in total fatty acids, it also modifies lipid classes, overall polar lipids. The total phospho- and sphingolipids concentration is not widely modified by the supplementation; however their distribution and composition are improved with an increment of bioactive fatty acids linked to them.

REFERENCES

Barbano, D. M. and J. W. Sherbon. 1981. Polyunsaturated Protected Lipid: Effect on Milk Phospholipids. J. Dairy Sci. 64(11):2170-2174.

Bitman, J. and D. L. Wood. 1990. Changes in Milk Fat Phospholipids During Lactation. J. Dairy Sci. 73(5):1208-1216.

Castro-Gomez, M. P., L. M. Rodriguez-Alcala, M. V. Calvo, J. Romero, J. A. Mendiola, E. Ibanez, and J. Fontecha. 2014. Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques. J Dairy Sci. 97(11):6719-6728.

Castro-Gómez, P., J. Fontecha, and L. M. Rodríguez-Alcalá. 2014. A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples. Talanta 128(0):518-523.

Fong, B. Y., C. S. Norris, and A. K. H. MacGibbon. 2007. Protein and lipid composition of bovine milk-fat-globule membrane. Int. Dairy J. 17(4):275-288.

Fontecha, J., H. Goudjil, J. J. Rios, M. J. Fraga, and M. Juarez. 2005. Identity of the major triacylglycerols in ovine milk fat. Int. Dairy J. 15(12):1217-1224.

Fontecha, J., J. J. Rios, L. Lozada, M. J. Fraga, and M. Juarez. 2000. Composition of goat's milk fat triglycerides analysed by silver ion adsorption-TLC and GC-MS. Int. Dairy J. 10(1/2):119-128.

Gastaldi, D., C. Medana, V. Giancotti, R. Aigotti, F. Dal Bello, and C. Baiocchi. 2011. HPLC-APCI analysis of triacylglycerols in milk fat from different sources. Eur. J. Lipid Sci. Technol. 113(2):197-207.

Gorban, A. M. S. and O. M. Izzeldin. 1999. Study on cholesteryl ester fatty acids in camel and cow milk lipid. J. Food Technol. 34(3):229-234.

Hervás, G., P. Luna, A. R. Mantecón, N. Castañares, M. A. De La Fuente, M. Juárez, and P. Frutos. 2008. Effect of diet supplementation with sunflower oil on milk production, fatty acid profile and ruminal fermentation in lactating dairy ewes. J. Dairy Res. 75(4):399-405.

Jensen, R. G. 2002. The composition of bovine milk lipids: January 1995 to December 2000. J. Dairy Sci. 85(2):295-350.

Kuchta, A. M., P. M. Kelly, C. Stanton, and R. A. Devery. 2012. Milk fat globule membrane – a source of polar lipids for colon health? A review. Int. J. Dairy Technol. 65(3):315-333.

Lopez, C., V. Briard-Bion, O. Menard, F. Rousseau, P. Pradel, and J.-M. Besle. 2008. Phospholipid, Sphingolipid, and Fatty Acid Compositions of the Milk Fat Globule Membrane are Modified by Diet. J. Agric. Food Chem. 56(13):5226-5236.

Malmsten, M., B. Bergenståhl, L. Nyberg, and G. Odham. 1994. Sphingomyelin from milk-characterization of liquid crystalline, liposome and emulsion properties. J. Am. Oil Chem. Soc. 71(9):1021-1026.

Martini, M., I. Altomonte, and F. Salari. 2013. Evaluation of the fatty acid profile from the core and membrane of fat globules in ewe's milk during lactation. LWT-Food Sci. Technol. 50(1):253-258.

Ménard, O., S. Ahmad, F. Rousseau, V. Briard-Bion, F. Gaucheron, and C. Lopez. 2010. Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. Food Chem. 120(2):544-551.

Oh, J.-J., J.-S. Lee, J.-N. Lim, T. Wang, S.-H. Kim, and H.-G. Lee. 2014. Trans vaccenic acid (trans-11 18:1), a precursor of cis-9, trans-11-conjugated linoleic acid, exerts a direct anti-carcinogenic function in T47D breast carcinoma cells. Food Sci. Biotechnol. 23(2):641-646.

Pariza, M. W. 2004. Perspective on the safety and effectivenes of conjugated linoleic acid. Am. J. Clin. Nutr. 79(6, Suppl.):1132S-1136S.

Pralhada Rao, R., N. Vaidyanathan, M. Rengasamy, A. Mammen Oommen, N. Somaiya, and M. R. Jagannath. 2013. Sphingolipid Metabolic Pathway: An Overview of Major Roles Played in Human Diseases. J. Lipids 2013:12.

Schuchardt, J. P., I. Schneider, H. Meyer, J. Neubronner, C. von Schacky, and A. Hahn. 2011. Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations--a comparative bioavailability study of fish oil vs. krill oil. Lipids Health Dis. 10:145.

Ulven, S., B. Kirkhus, A. Lamglait, S. Basu, E. Elind, T. Haider, K. Berge, H. Vik, and J. Pedersen. 2011. Metabolic Effects of Krill Oil are Essentially Similar to Those of Fish Oil but at Lower Dose of EPA and DHA, in Healthy Volunteers. Lipids 46(1):37-46.

Table 1. Lipid classes composition (%, *w/w*) of control (CtM) and supplemented milk (SppM) determined by HPLC-ELSD

Lipid classes	CtM Mean±SD	SppM Mean±SD
CE	<0.1	<0.1
TAG	97.75 ± 0.08	98.83 ± 0.05
DAG	1.12 ± 0.03	0.59 ± 0.03*
CHOL	0.93 ± 0.04	0.37 ± 0.02*
FFA	<0.1	<0.1
MAG	<0.1	<0.1
GLUCER	<0.1	<0.1
LACCER	<0.1	<0.1
PL	0.16 ± 0.01	0.18 ± 0.01
g/100 g of PL		
PE	41.97 ± 0.69	29.53 ± 0.81*
PI	4.01 ± 0.16	3.32 ± 0.04*
PS	3.04 ± 0.16	1.74 ± 0.20*
PC	43.92 ± 0.58	45.69 ± 0.26
SM	7.07 ± 0.19	19.72 ± 1.49*

CE: cholesterol esters; TAG: triacylglycerides; DAG: diacylglycerides; CHOL: cholesterol; MAG: monoglycerides; FFA: free fatty acid; GLUCER: glucosylceramides; LACCER: lactosylceramides; PL: polar lipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin; Asterisk means significant differences within the same row ($p<0.05$).

Table 2. Mean values of triacylglycerides content by carbon number (%, w/w) of control (CtM) and supplemented milk (SppM) determined by GC-FID.

TAG CN (%)	CtM	SppM
TAG 24	<0.1	<0.1
TAG 26	1.62 ± 0.04	0.83 ± 0.06*
TAG 28	2.00 ± 0.04	0.84 ± 0.06*
TAG 30	5.12 ± 0.11	2.16 ± 0.19*
TAG 32	7.61 ± 0.06	3.74 ± 0.05*
TAG 34	10.62 ± 0.22	6.45 ± 0.22*
TAG 36	14.2 ± 0.1	10.91 ± 0.17*
TAG 38	15.66 ± 0.04	16.37 ± 0.23
TAG 40	12.75 ± 0.07	15.65 ± 0.24
TAG 42	9.61 ± 0.21	6.84 ± 0.27*
TAG 44	7.6 ± 0.01	5.78 ± 0.24*
TAG 46	5.26 ± 0.07	5.57 ± 0.19
TAG 48	3.54 ± 0.18	6.27 ± 0.28*
TAG 50	2.51 ± 0.04	6.99 ± 0.34*
TAG 52	1.54 ± 0.12	7.13 ± 0.28*
TAG 54	0.36 ± 0.07	4.49 ± 0.07*

TAG: triacylglycerides; CN: carbon number;
*Asterisk means significant differences within the same row ($p<0.05$).

Table 3. Comparison of polar lipid FAME composition (%; g FA/100g total FA) of milk fat obtained from control (CtM) and supplemented (SppM) milk determined by GC-MS.

	PE			PI			PS			PC			SM		
FAME (%)	CtM	SppM	SEM	CtM	SppM	SEM	CtM	SppM	SEM	CtM	SppM	SEM	CtM	SppM	SEM
C14:0	0.59	0.29*	0.15	0.68	-	0.34	0.92	0.78	0.07	2.65	0.13*	1.26	2.14	2.09*	0.03
C15:0	1.09	0.23*	0.43	0.70	-	0.35	-	-	-	0.74	0.48*	0.13	0.31	0.32	-
C16:0	10.50	9.49*	0.51	8.76	6.51*	1.12	6.23	4.89*	0.67	33.49	27.14*	3.17	23.23	22.41*	0.41
C16:1 n9	0.64	0.96*	0.16	0.46	-	0.23	0.57	-	0.28	0.20	1.37*	0.58	-	0.27	0.14
C18:0	15.98	9.63*	3.18	27.96	26.68	0.64	34.20	31.03*	1.58	15.34	10.94*	2.20	5.63	6.21*	0.29
C18:1 9t	0.80	1.56*	0.38	0.47	1.00*	0.26	0.84	1.19*	0.18	0.88	1.45*	0.29	0.11	0.11	-
C18:1 10t	0.41	4.60*	2.10	1.05	8.43*	3.69	-	2.80	1.40	0.56	7.05*	3.25	0.10	0.62*	0.26
C18:1 11t	1.29	6.46*	2.58	0.96	6.96*	3.00	1.24	6.11*	2.43	1.57	8.12*	3.27	0.12	0.71*	0.29
C18:1 9c	49.66	44.99*	2.33	36.02	25.36*	5.33	27.31	27.30	0.01	31.80	26.46*	2.67	1.74	2.30*	0.28
C18:2 9c,12c	10.55	12.91*	1.18	7.86	12.14*	2.14	5.78	7.28*	0.75	5.40	9.43*	2.02	0.31	0.82*	0.25
C18:3 n6	0.24	0.18*	0.03	0.43	0.30	0.06	0.81	1.41*	0.30	0.31	0.27	0.02	0.55	0.44	0.06
C18:3 n3	0.41	0.33	0.04	0.28	0.22	0.03	-	0.24	0.12	0.24	0.26	0.01	-	0.15	0.07
C18:2 9c,11t	0.13	2.88*	1.38	-	0.55	0.28	-	0.57	0.29	-	1.23	0.61	-	-	-
C18:4	0.23	1.21*	0.49	0.85	0.96	0.05	1.48	1.53	0.03	0.22	0.36*	0.07	0.65	0.57	0.04
C20	0.68	0.71	0.01	1.17	1.07	0.05	2.44	2.25	0.09	1.15	1.27*	0.06	2.27	2.14*	0.07
C20:2 n9	3.46	0.55*	1.46	9.24	3.13*	3.05	14.39	4.91*	4.74	3.12	1.01*	1.06	5.94	3.59*	1.17
C20:4 n6	1.17	1.20	0.02	1.57	4.64*	1.53	0.96	2.54*	0.79	0.74	0.84	0.05	-	-	-
C22:0	0.97	1.00	0.01	1.55	1.58	0.01	2.85	3.18*	0.16	1.19	1.73*	0.27	11.06	15.94*	2.44
C23:0	0.26	0.27	-	-	0.47	0.23	-	0.96	0.48	0.42	0.48	0.03	29.86	17.85*	6.00
C24:0	-	-	-	-	-	-	-	-	-	-	-	-	13.36	21.21*	3.92
C24:1	0.94	0.56*	0.19	-	-	-	-	0.74	0.37	-	-	-	2.62	2.28*	0.17
∑ SFA	30.08	21.61*	4.24	40.81	36.30*	2.26	46.63	43.37*	1.63	54.97	42.16*	6.41	87.86	88.16*	0.15
∑ MUFA	53.74	59.13*	2.70	38.96	41.75*	1.40	29.96	38.15*	4.10	35.01	44.45*	4.72	4.68	6.28*	0.80
∑ PUFA	16.18	19.26*	1.54	20.23	21.95*	0.86	23.41	18.48*	2.46	10.02	13.39*	1.68	7.45	5.57*	0.94
∑ n6 PUFA	11.96	14.29*	1.16	9.85	17.08*	3.61	7.55	11.23*	1.84	6.44	10.54*	2.05	0.87	1.26*	0.20
∑ n3 PUFA	0.64	1.55*	0.46	1.13	1.18	0.03	1.48	1.77	0.14	0.46	0.61*	0.08	0.65	0.71	0.03

SFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin; SEM: standard error of mean; Asterisk means significant differences among milks within the same polar lipid ($p<0.05$).

Table 4. Phosphatidylethanolamine species in control and supplemented milk by MALDI-TOF.

	CtM		SppM	
CN:DB	**Attribution**	***m/z* (adduct)**	**Attribution**	***m/z* (aduct)**
30:0	(14:0/16:0, 15:0/15:0)	686.533(Na^+)/664.562(H^+)	(14:0/16:0, 15:0/15:0)	686.529 (Na^+)/664.55 ($^+$)
32:1	(14:0/18:1,16:0/16:1)	712.681(Na^+)	(14:0/18:1, 16:0/16:1)	712.680(Na^+)
32:3	(14:0/18:3)	708.506 (Na^+)	(14:0/18:3)	686.499 (H^+)
32:4			(14:0/18:4)	684.507 (H^+)
33:0	(15:0/18:0)	728.666 (Na^+)	(15:0/18:0)	728.659 (Na^+)
33:1	(15:0/18:1)	704.541 (H^+)		
33:2	(15:0/18:2)	724.433 (Na^+)	(15:0/18:2)	724.439 (Na^+)
34:0	(14:0/20:0, 16:0/18:0)	742.659 (Na^+)		
34:4	(14:0/20:4, 16:0/18:4, 16:1/18:3)	712.680 (H^+)	(14:0/20:4, 16:0/18:4, 16:1/18:3)	712.680 (H^+)
36:0	(14:0/22:0, 16:0/20:0, 18:0/18:0)	770.624 (Na^+)		
36:1	(16:1/20:0, 18:0/18:1)	746.552 (H^+)		
36:2	(16:0/20:2, 18:0/18:2, 18:1/18:1)	766.626 (Na^+)		
36:3	(16:1/20:2, 18:0/18:3, 18:1/18:2)	742.659 (H^+)		
38:2			(18:0/20:2, 18:2/20:0)	772.57 (H^+)
38:3	(18:1/20:2, 18:3/20:0)	770.624 (H^+)		
38:5	(18:1/20:4, 18:3/20:2)	766.629 (H^+)		
41:1			(18:1/23:0)	816.573 (H^+)
42:5	(18:4/24:1)	844.641 (Na^+)		
44:5	(20:4/24:1)	872.686 (Na^+)		

(Na^+) sodium adduct; (H^+): hydrogen adduct; CN: carbon number; DB: double bound; CtM; control milk; SppM: supplemented milk. Underline specie for the most probable fatty acid composition based on the polar lipids fatty acid content

Table 5. Phosphatidylinositol and phosphatidylserine species in control milk (CtM) and supplemented milk (SppM) by MALDI-TOF.

	CtM		SppM	
CN:DB	**Attribution**	***m/z* (adduct)**	**Attribution**	***m/z* (adduct)**
33:3	PI(15:0/18:3)	841.583 (Na^+)		
34:1	PI(16:0/18:1, 16:1/18:0)	859.595 (Na^+)	PI(16:0/18:1)	859.582 (Na^+)
34:2	PI(14:0/20:2, 16:0/18:2, 16:1/18:1)	835.371 (H^+)		
35:2	PI(15:0/20:2)	871.709 (Na^+)		
36:4	PI(16:0/20:4, 18:0/18:4, 18:1/18:3, 18:2/18:2)	859.595 (H^+)	PI(16:0/20:4, 18:0/18:4, 18:1/18:3, 18:2/18:2)	859.596 (H^+)
36:6			PI(18:2/18:4, 18:3/18:3)	855.503 (H^+)
40:2	PI(18:2/22:0, 20:0/20:2)	919.71 (H^+)	PI(18:2/22:0, 20:0/20:2)	919.673 (H^+)
42:2	PI(20:2/22:0)	947.551 (H^+)		
30:1	PS(14:0/16:1)	728.610 (Na^+)/706.655 (H^+)		
32:1	PS(14:0/18:1, 16:0/16:1)	756.624 (Na^+)/734.647 (H^+)	PS(14:0/18:1)	756.636 (Na^+)/734.643 (H^+)
32:4	PS(14:0/18:4)	728.610 (H^+)	PS(14:0/18:4)	728.619 (H^+)
34:4	PS(14:0/20:4, 16:0/18:4, 16:1/18:3)	756.624 (H^+)	PS(14:0/20:4, 16:0/18:4)	756.633 (H^+)
38:0	PS(16:0/22:0, 18:0/20:0)	842.594 (Na^+)	PS(16:0/22:0, 18:0/20:0)	842.610 (Na^+)
38:2	PS(18:0/20:2, 18:2/20:0)	816.623 (H^+)	PS(18:0/20:2, 18:2/20:0)	816.650 (H^+)
40:3	PS(18:3/22:0)	842.594 (H^+)	PS(18:3/22:0)	842.610 (H^+)
42:2			PS(18:1/24:1, 20:2/22:0)	872.801 (H^+)
44:3			PS(20:2/24:1)	920.859 (Na^+)

(Na^+) sodium adduct; (H^+): hydrogen adduct; PI: phosphatidylinositol; PS: phosphatidylserine; CN: carbon number; DB: double bound; Underline specie for the most probable fatty acid composition based on the polar lipids fatty acid content

Table 6. Phosphatidylcholine species in control milk (CtM) and supplemented milk (SppM) by MALDI-TOF.

	CtM		SppM	
CN:CB	**Attribution**	***m/z* (adduct)**	**Attribution**	***m/z* (adduct)**
28:0	(14:0/14:0)	678.637 (H^+)		
30:0	(14:0/16:0, 15:0/15:0)	706.655 (H^+)		
32:0	(14:0/18:0, 16:0/16:0)	756.693 (Na^+)/734.725 (H^+)	(14:0/18:0, 16:0/16:0)	756.681 (Na^+)/734.715(H^+)
32:1	(14:0/18:1, 16:0/16:1)	732.664 (H^+)		
32:4	(14:0/18:4)	748.717 (H^+)		
33:0	(15:0/18:0)	748.717 (H^+)		
33:1	(15:0/18:1)	746.671 (H^+)		
33:4	(15:0/18:4)	762.746 (Na^+)	(15:0/18:4)	762.758 (Na^+)
34:0	(14:0/20:0, 16:0/18:0)	784.723 (Na^+)/762.746 (H^+)	(14:0/20:0, 16:0/18:0)	784.732 (Na^+)/762.758 (H^+)
34:1	(16:0/18:1, 16:1/18:0)	782.676 (Na^+)/760.680 (H^+)	(16:0/18:1, 16:1/18:0)	782.673 (Na^+)/760.704(H^+)
34:2	(14:0/20:2, 16:0/18:2, 16:1/18:1)	780.657 (Na^+)/758.690 (H^+)	(14:0/20:2, 16:0/18:2, 16:1/18:1)	780.646 (Na^+)/758.688 (H^+)
34:3	(16:0/18:3, 16:1/18:2)	756.69 (H^+)	(16:0/18:3, 16:1/18:2)	756.680 (H^+)
34:4	(14:0/20:4, 16:0/18:4, 16:1/18:3)	776.772 (Na^+)		
34:5	(16:1/18:4)	774.724 (Na^+)		
35:0	(15:0/20:0)	798.667 (Na^+)/776.772 (H^+)	(15:0/20:0)	798.672 (Na^+)
35:2	(15:0/20:2)	772.715 (H^+)		
35:4	(15:0/20:4)	790.743 (Na^+)	(15:0/20:4)	790.743 (Na^+)
36:0	(14:0/22:0, 16:0/20:0, 18:0/18:0)	790.743 (H^+)	(14:0/22:0, 16:0/20:0, 18:0/18:0)	790.743 (H^+)
36:1	(16:1/20:0, 18:0/18:1)	788.738 (H^+)	(16:1/20:0, 18:0/18:1)	788.747 (H^+)
36:2	(16:0/20:2, 18:0/18:2, 18:1/18:1)	808.684 (Na^+)/786.723 (H^+)	(16:0/20:2, 18:0/18:2, 18:1/18:1)	808.671 (Na^+)/786.723 (H^+)
36:3	(16:1/20:2, 18:0/18:3, 18:1/18:2)	806.73 (Na^+)/784.723 (H^+)	(16:1/20:2, 18:0/18:3, 18:1/18:2)	806.639 (Na^+)/784.731 (H^+)
36:4	(16:0/20:4, 18:0/18:4, 18:1/18:3, 18:2/18:2)	782.676 (H^+)	(16:0/20:4, 18:0/18:4, 18:1/18:3, 18:2/18:2)	782.671 (H^+)
36:5	(16:1/20:4, 18:1/18:4, 18:2/18:3)	802.706 (Na^+)/780.657 (H^+)	(16:1/20:4, 18:1/18:4, 18:2/18:3)	780.648 (H^+)
36:6	(18:2/18:4, 18:3/18:3)	800.698 (Na^+)	(18:2/18:4, 18:3/18:3)	800.700 (Na^+)
38:1			(16:1/22:0, 18:1/20:0)	816.536 (H^+)
38:5	(18:1/20:4, 18:3/20:2)	808.684 (H^+)	(18:1/20:4, 18:3/20:2)	808.690 (H^+)
38:6	(18:2/20:4, 18:4/20:2)	806.730 (H^+)	(18:2/20:4, 18:4/20:2)	806.729 (H^+)
40:2			(18:2/22:0, 20:0/20:2)	842.556 (H^+)
46:0	(23:0/23:0)	952.677 (Na^+)		

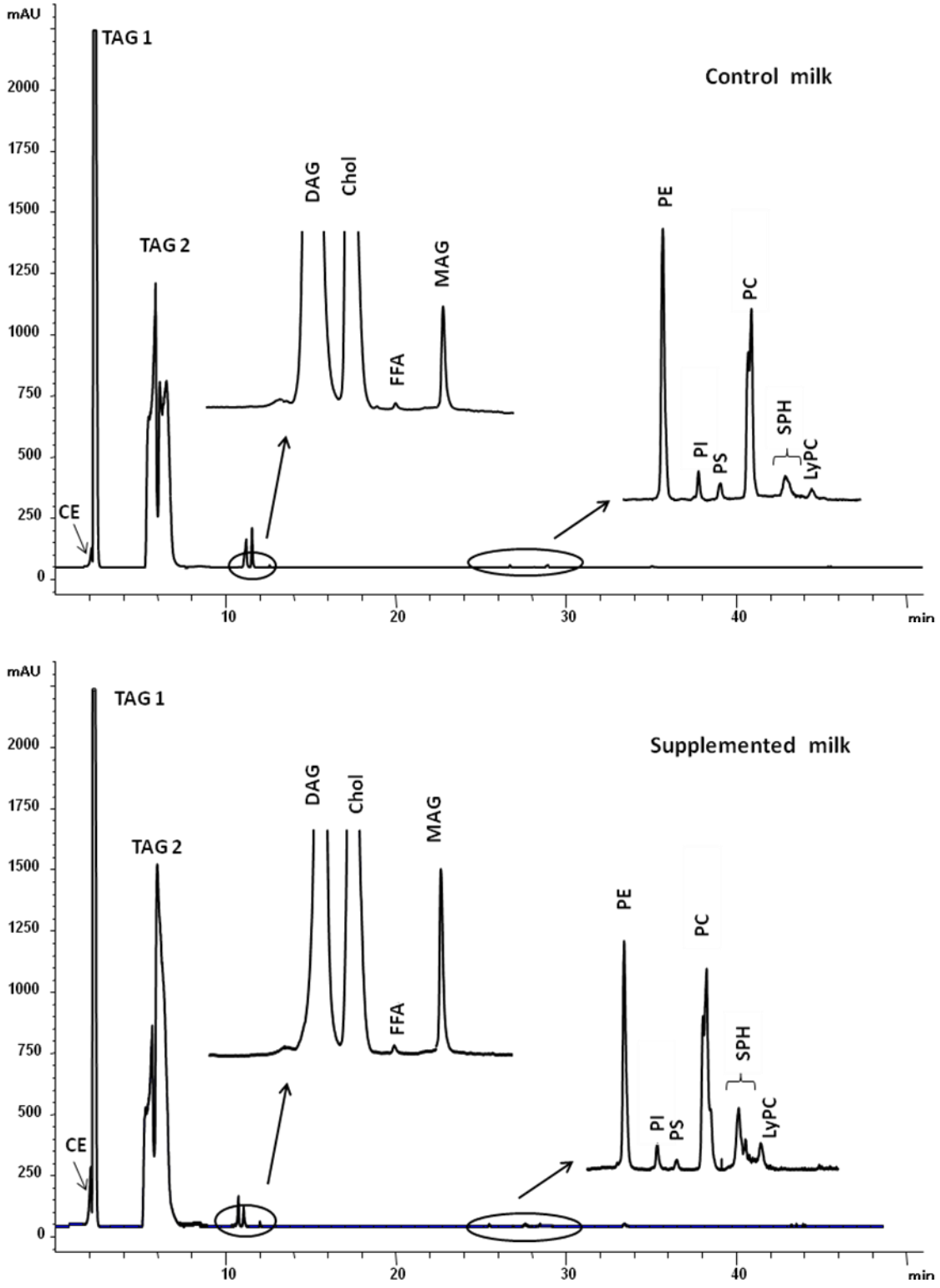
(Na^+) sodium adduct; (H^+): hydrogen adduct; CN: carbon number; DB: double bound; Underline specie for the most probable fatty acid composition based on the polar lipids fatty acid content

Table 7: Sphingomyelin species in control milk (CtM) and supplemented milk (SppM) by MALDI-TOF.

	CtM	SppM
CN:DB	**m/z (adduct)**	**m/z (adduct)**
14:0	697.827 (Na^+)	697.812 (Na^+)
15:0	711.740 (Na^+)	711.740 (Na^+)/789.519 (H^+)
16:0	725.675 (Na^+)/703.687 (H^+)	725.64 (Na^+)/703.679 (H^+)
16:1		723.45 (Na^+)/701.566 (H^+)
18:0	753.677 (Na^+)	731.698 (H^+)
18:1	729.650 (H^+)	729.645 (H^+)
18:2	727.551 (H^+)	727.569 (H^+)
18:3	725.675 (H^+)	725.649 (H^+)
18:4	745.635 (H^+)	745.632 (H^+)
20:0	759.685(H^+)	759.675 (H^+)
20:2	777.609 (Na^+)	777.590 (Na^+)
22:0	809.798 (Na^+)	787.789 (H^+)
23:0	823.752 (Na^+)	823.564 (Na^+)
24:0	837.825 (Na^+)/815.641 (H^+)	815.659 (H^+)
24:1	835.761(Na^+)	813.492 (H^+)

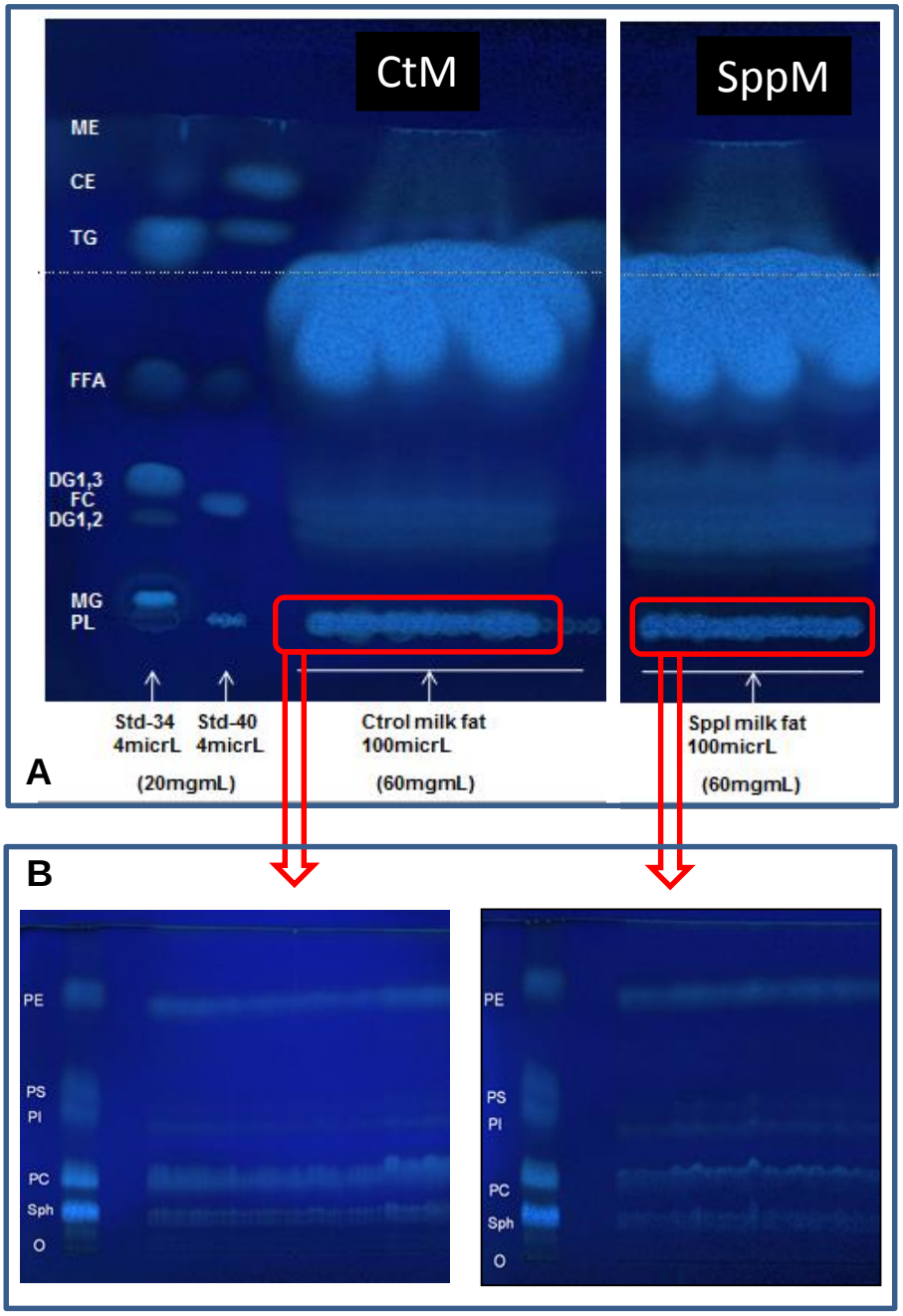
(Na^+) sodium adduct; (H^+): hydrogen adduct; CN: carbon number; DB: double bound;

FIG 1.



CE: cholesterol ester; TAG: triacylglycerides; DAG: diacylglicerides; Chol: cholesterol; FFA: free fatty acid; MAG: monoacylglicerides; PE: phosphatidylethanolamine; PI: phosphoatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SPH: sphingomyelin; LyPC: lyso-phosphatidylcholine.

FIG.2


CtM
SppM
ME
CE
TG
FFA
DG1,3
FC
DG1,2
MG
PL
Std-34
4micrL
(20mgmL)
Std-40
4micrL
Ctrol milk fat
100micrL
(60mgmL)
Sppl milk fat
100micrL
(60mgmL)
A
B
PE
PS
PI
PC
Sph
O
PE
PS
PI
PC
Sph
O

3. Estudio de la actividad biológica y bioaccesibilidad de fracciones y extractos lipídicos ricos en fosfo- y esfingolípidos obtenidos de mazada mediante ensayos *in vitro* y estudios en humanos.

3.1. Antiproliferative activity of buttermilk lipid fractions isolated using food grade and non-food grade solvents on human cancer cell lines.

Elsevier Editorial System(tm) for Journal of Functional Foods
Manuscript Draft

Manuscript Number:

Title: Antiproliferative activity of buttermilk lipid fractions isolated using food grade and non-food grade solvents on human cancer cell lines

Article Type: Full Length Article

Keywords: Buttermilk; Polar lipids; Phospholipids; Human cancer cells;
Food grade extraction.

Corresponding Author: Dr. JAVIER FONTECHA, Ph.D.

Corresponding Author's Institution: CIAL (CSIC)

First Author: Pilar Castro-Gómez, PhD STUDENT

Order of Authors: Pilar Castro-Gómez, PhD STUDENT; LUIS M Rodríguez-Alcalá, PhD; Karin Maia M Monteiro , PhD; Ana Lúcia T RUIZ, PhD; João E Carvalho, PhD; JAVIER FONTECHA, Ph.D.

Editor-in-Chief
Journal of Functional Foods

Madrid, 11 may, 2015

Dear Editor,

Enclosed please find our Manuscript entitled "Antiproliferative activity of buttermilk lipid fractions isolated using food grade and non-food grade solvents on human cancer cell lines" for your consideration to be published in the Journal of Functional Foods.

Buttermilk, a by-product obtained during the production of butter, contains high amounts of remaining milk fat globule membrane (MFGM) which has been considered a bioactive ingredient for its immunomodulatory, antimicrobial and anticarcinogenic capacity. Nevertheless, to our knowledge, previous studies using buttermilk lipid extracts to assess its antiproliferative activity against human cancer cell lines, has not been reported before.

Therefore the purpose of this study was to evaluate the in vitro bioactivity and the antiproliferative effect of the buttermilk and its obtained lipid fractions, from two extraction methods using food grade and non-food grade solvents. Yields were determined for recovery of different lipids and chemical composition of the extract fractions identified by HPLC procedures. The composition of each isolated lipid fraction and its relation with the effectiveness in inducing inhibition of 9 human cancer cell lines proliferation have been investigated.

Sincerely,

Dr. Javier Fontecha.
Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM)
Department of Lipids
C/ Nicolás Cabrera 9, Campus de la Universidad Autónoma de Madrid (UAM)
28049, Madrid, Spain.
E mail: j.fontecha@csic.es

HIGHLIGHTS

- Buttermilk is a rich source of milk fat globule membrane polar lipids that may contribute as a possible nutraceutical.

- Bioactive lipid components from buttermilk obtained using food grade solvents retain their antitumor activity

- The presence of phospho- and sphingolipids in the isolate buttermilk lipid extracts seems to be crucial for its antiproliferative activity.

- Lipid extracts from buttermilk using food grade solvents could be used as functional ingredients in human foods.

Antiproliferative activity of buttermilk lipid fractions isolated using food grade and non-food grade solvents on human cancer cell lines

Pilar Castro-Gómez [a], Luis Miguel Rodríguez-Alcalá [a], Karin Maia Monteiro [b,c], Ana Lúcia T. G. Ruiz [b] , João Ernesto Carvalho [b,d], Javier Fontecha [a*]

[a] Institute of Food Science Research (CIAL; CSIC-UAM). Group of lipids. Universidad Autónoma de Madrid, Calle Nicolás Cabrera 9, 28049 Madrid, Spain

[b] Chemical, Biological and Agricultural Pluridisciplinary Research Center, CPQBA, University of Campinas, Rua Alexandre Cazelatto, 999, Vila Betel, Paulínia, SP 13148-218, Brazil

[c] Faculty of Medical Sciences, University of Campinas, Cidade Universitária Zeferino Vaz, Campinas, SP 13081-970, Brazil

[d] Chemical, Biological and Agricultural Pluridisciplinary Research Center, CPQBA. Faculty of Pharmaceutical Sciences, University of Campinas, Rua Alexandre Cazelatto, 999, Vila Betel, Paulínia, SP 13148-218, Brazil

* Corresponding author: Dr. Javier Fontecha.

E-mail: j.fontecha@csic.es.

Phone: +34 910017935

Address: Institute of Food Science Research (CIAL; CSIC-UAM). Group of lipids. Universidad Autónoma de Madrid, Calle Nicolás Cabrera 9, 28049 Madrid, Spain

Abbreviations:

MFGM: milk fat globule membrane; TAG: triacylglycerols; DAG: diacylglycerols; MAG: monoacylglycerols; FFA: free fatty acids; CHOL: cholesterol; CE: cholesterol ester; GLUCER: glucosylceramides; LACCER: lactosylceramides; PL: polar lipids; PI: phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin GLUCER; BM: buttermilk; TL: total lipid; FG: food grade method; NFG: non-food grade method; FBS: fetal bovine serum; FC: flash chromatography; ELSD: evaporative light scattering detector; DMSO: dimetylsulfoxide; TCA: trichloroacetic acid; TGI: total growth inhibition; TG_{50}: inhibition 50% of growth.

ABSTRACT

Buttermilk, a by-product obtained during the production of butter, contains high amounts of remaining milk fat globule membrane (MFGM) which has been considered a bioactive ingredient for its immunomodulatory, antimicrobial, antiviral, and anticarcinogenic capacity. Besides glycolipids and membrane proteins, buttermilk is a major source of polar lipids from MFGM (mainly phospho- and sphingolipids).

These bioactivities could be due to the whole MFGM effect or to the isolated polar lipid components In order to assess the antiproliferative activity of the MFGM polar lipids, the selection of the suitable extraction and fractionation methods could be crucial in bioactivity maintenance. Therefore the aim of this study was to evaluate the in vitro antiproliferative effects in 9 human cancer cell lines of the whole buttermilk and its lipid fractions, obtained using food grade or non-food grade solvents.

Only fractions isolated with food grade solvents were cell growth inhibitors. Furthermore the phospho- and sphingolipids presence was important to have antiproliferative activity against colon and ovary human cancer cells lines at < 250 µg/mL.

Keywords:

Buttermilk

Polar lipids

Phospholipids

Human cancer cells

Food grade extraction

1. Introduction

The fat in milk occurs as an oil-in-water emulsion with a unique stabilizing lipoprotein membrane, referred to as the milk fat globule membrane (MFGM). The globule core is mainly composed of triacylglycerols (TAG) (98%) including a large number of esterified fatty acids. The milk fat is protected against both chemical attach or physical destabilization by the MFGM (Danthine, Blecker, Paquot, Innocente, & Deroanne, 2000) mostly composed of proteins associated to membrane as well as polar lipids (PL) mainly phospho- and sphingolipids. However, although the MFGM has a unique trilayer structure formed by an inner layer from endoplasmic reticulum and involved with an outer bilayer from the mammary cell (Mather, 2000). It is remarkable that the PL account for 0.5-1% of total milk lipids (Rombaut, Camp, & Dewettinck, 2006). Despite this low amount, this fraction is important because the structure acquired from cellular membranes provide to MFGM an exclusive PL composition when it is compared with other commercial PL sources (Kuchta, Kelly, Stanton, & Devery, 2012). However, some milk by-products such as buttermilk (BM) and, especially, its MFGM fraction contain up to 40% (in weight) of PL, of which around 30% is phosphatidylethanolamine (PE), 7% is phosphatidylinositol (PI), 5% is phosphatidylserine (PS), 31% is phosphatidylcholine (PC), and 20% is sphingomyelin (SM) (Rodriguez-Alcalá & Fontecha, 2010).

Studies carried out with PL from different sources are being actively investigated for their role in human physiology and health. Even though there is a wide array of diseases whose outcome can be potentially influenced by PLs intake modulation, most research is centered on some cancers and neurological pathologies and liver diseases (Kullenberg, Taylor, Schneider, & Massing, 2012).

However, the anticarcinogenic effect of MFGM has not been comprehensively studied and it has been attributed both to membrane proteins and PL (Spitsberg, 2005). In order to carry out studies about the antitumor effects of the PL of BM, the fat extraction and the PL isolation procedures could be a critical step if it has to be used as a bioactive component for subsequent in vitro or in vivo experiments. Previous studies carried out in our laboratory suggested that the use of some organic solvents extensively employed to lipid extraction and/or fractionation procedures could lead to compound bioactivity losses (Castro-Gómez et al., 2013).

In this study, the antiproliferative effect of different BM lipid fractions isolated using either food grade (FG) or non-food grade (NFG) solvents have been screened against 9 human cancer cells lines. We have also investigated the composition of each isolated lipid fraction and its relation with the inhibited cell proliferation.

2. Materials and Methods

2.1. Samples and Reagents

BM powder samples were from Reny-Picot (Asturias, Spain). All solvents were HPLC grade and MS grade when available. Chloroform, dichloromethane, hexane, methanol, isooctane, isopropanol, acetone, diethyl ether and ethanol (95%) were purchased from LABSCAN (Dublin, Ireland). Sea sand, potassium hydroxide and sodium carbonate were from PANREAC (Barcelona, Spain). Formic acid (98%) and triethylamine (99.5%), tritridecanoin, pelargonic (C9), cholesterol (CHOL) and cholesterol ester (CE), monostearin, diolein, and the phospholipids: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), were purchased to Sigma-Aldrich (Bellefonte, PA, USA). The fetal bovine serum (FBS) was from Gibco (Gibco BRl, Gaithersburgm MD, USA).

2.2. Total fat extraction by using a pressurized liquid system

Pressurized liquid extraction (PLE) procedure was carried out to isolate the total lipids from powder BM following the optimized conditions described by Castro-Gómez et al. (2014) with a PLE (Dionex Corp., Sunnyvale, CA) using either food grade (FG) or non-food grade (NFG) solvents. Briefly, 2 g of powder BM was mixed with 2 g of sea sand and loaded into a stainless steel extraction cell covered with filters on both sides. For FG lipid extraction procedure, ethanol was used as the only organic solvent during 4 static cycles of 5 minutes at 10.3 MPa of pressure and 60ºC. For NFG lipid extraction procedure, dichloromethane-methanol solution (2:1; v/v) as in Castro-Gómez et al. (2014) was used. The obtained BM lipid extracts were concentrated by removing the organic solvent in a vacuum rotary evaporator (Strike 202 model; Steroglass S.R.L., Perugia, Italy) and dried under a gentle stream of nitrogen. Then lipid extracts were

weighed and stored in amber vials, exposed to a stream of nitrogen, and frozen at -40°C until analysis. Each extraction was performed in triplicate.

2.3. Lipid fractionation by Flash Chromatography

The obtained BM lipid extracts were fractionated by using a preparative Reveleris® Flash System Chromatography (FC) (Grace, Deerfield, IL, USA) equipped with an evaporative light scattering detector (ELSD). Different lipid fractionation was carried out depending if FG or NFG solvents were used. For FG fractionation procedure, BM fat was dissolved in 4 mL methanol (50 mg/mL) and loaded onto a preconditioned 4g C18 silica cartridge (Grace Reveleris, Deerfield, USA). The elution solvent program consisted of methanol and then ethanol. Each step was maintained during 5 min at a flow rate of 7 mL/min. Pressure during fractionation was below 100 psi. The two lipid fractions collected (F1, F2) were evaporated under nitrogen stream, weighed, and kept at −40 °C until further analysis. For NFG fractionation procedure, BM fat was dissolved in 4 mL hexane (50 mg/mL) and loaded onto a preconditioned 4g normal silica cartridge. The elution solvent program consisted of hexane/diethyl ether (98:2), hexane/diethyl ether (95:5), and finally methanol. Two lipid fractions were also collected (F1, F2) as above that were evaporated and kept frozen until further analysis. In all cases assays were carried out in triplicate.

2.4. Lipid Class Composition by HPLC-ELSD

Separation of lipid classes was accomplished with a HPLC Agilent Technologies, model 1200 (Palo Alto, CA, USA) coupled to an ELSD detector (SEDERE, SEDEX 85 model, Alfortville Cedex, France) using prefiltered compressed air as the nebulizing gas at pressure of 3.5 bar, temperature of 60 °C, and the gain set at 3. Two columns of Zorbax Rx-SIL column (Agilent Technologies, Palo Alto, CA, USA) of 250 mm× 4.5 mm and 5-µm particle size were used in series along with a precolumn of the same packing. Samples of BM fat as well as the isolated BM fat fractions were prepared at 5 mg/mL, and the injection volume was 50 µL. Solvent gradient was as detailed in Castro-Gómez, Holgado, Rodríguez-Alcalá, Montero and Fontecha (2015). Analyses were carried out with solvents freshly prepared. Lipid standards were analyzed under the same conditions and used for further identification. Assays were carried out in triplicate.

2.5. Antiproliferative Activity Assay

The in vitro antiproliferative assay was performed according to Monk et al. (1991). The human tumor cell lines were kindly donated by Frederick Cancer Research & Development Center, National Cancer Institute (NCI) and HaCat cell lines by Prof. Dr. Ricardo Della Coletta (FOP/UNICAMP) (Table 1). All cell lines were maintained in 25 cm3 (Costar/Corning) bottles with 5 mL of culture medium RPMI 1640 supplemented with 5% FBS and incubated at 37°C, in humid atmosphere with 5% CO2. A volume of 100µL per well of each cellular suspension, at the concentration showed in table 1, were inoculated in each compartment of 96-well plates in RPMI/5% FBS, and penicillin: streptomycin (1000U/mL:1000µg/mL, 1%) medium. They were incubated at 37ºC in a humid atmosphere with 5% CO2 during 24h. An amount of 5 mg of each sample was dissolved in 50µL of dimetylsulfoxide (DMSO) followed by 950 µL of medium RPMI/5% FBS. These solutions were diluted in culture medium to reach 0.25, 2.5, 25 and 250 µg/mL and applied on cell lines (100µL per well). Doxorubicin (0.025 - 25 µg/mL) was used as a positive control. DMSO final concentration (up to 0.25%) did not affect cell viability. After 48 h of incubation, cells were fixed on the plate with 50µL per well of trichloroacetic acid (TCA) (50%) and incubated again 1 h at 4ºC. The plaques were washed 4 times with distillated water to remove TCA, culture medium, FBS and secondary metabolites and were maintained at room temperature until they were dry. Then, cells were stained with 50 µL/well of sulforhodamine B 0,4% (w/v) on acetic acid (1%) during 20 min at room temperature. Again, they were washed 4 times with acetic acid (1%) and dry at room temperature. A volume of 150µL/well of Trizma Base (10µM, pH 10.5) was added to solubilize the binding stain. The spectrophotometric read was made in VersaMaxTM Absorbance Microplate Reader (California, USA) at 540 nm. A plaque with all cellular lines was submitted to the same process without the addition of sample to know the quantity of cells present on the moment that the sample was added (To). Three measurements were obtained: at the beginning of incubation (To) and 48 h post-incubation without tested (T1) and with tested samples (T). Cell proliferation was determined according to the equation 100 x [(T- To)/ (T1-To)]. Cytostatic effect was observed when $T1>T\geq T0$ while cytocidal effect occurred when $T <$ To. The sample concentration required to reach total growth inhibition (TGI) was determined through nonlinear regression analysis using the software Origin 8.0

(OriginLab Corporation, Inc. Northampton, MA, USA). The experiments were done in triplicate.

2.6. Statistical analysis

All results are expressed as means and standard deviations (n=3 for each BM fat and isolated fraction). An exploratory analysis of data was performed to test normal distribution and homogeneity of variance. Besides, total BM fat and isolated BM lipid classes composition obtained by the FG and NFG procedure comparison were carried out with a non-parametric proof of two independent samples. The analyses were performed using the SPSS Statistics software (v19.0 for Windows; IBM Corp., Armonk, NY). The level of significance was fixed at $p<0.05$.

3. Results and discussion

3.1. Total Lipid Yields and Lipid Extract Fractionations of Buttermilk

As indicated before, PLE procedure was carried out to isolate the total lipids from 2g of powder BM following Castro-Gómez et al. (2014). Total lipid (TL) yields using food grade (FG) or non-food grade (NFG) solvents ranged from 5.8 % to 7.2 % and no statistically significant differences were shown ($p<0.05$) due to the solvents used. These results in TL were in agreement with those reported by Sodini, Morin, Olabi and Jimenez-Flores (2006) for two commercials BM (5.7% and 7.2 %) obtained by using chloroform: methanol as extraction procedure. These results were also in accordance with Rombaut, Camp and Dewettinck (2006) whose reported a BM fat content of 6.14% using a thermocalcic aggregation extraction process. Morin, Jimenez-Flores and Pouliot, (2007) reported a TL content slightly high (8.29 %) maybe due to the different BM composition and because the use of sodium citrate precipitation as extraction method.

The TL extracted either by FG or NFG solvents were fractionated using flash chromatography (FC). Following this procedure that was proved to be very robust, reliable, and repeatable, we were able to isolate groups of lipid classes of related polarity. Therefore BM fat was fractionated into two fractions F1 and F2 with a quantity of lipid recovered close to 100 % of the original BM fat weight.

Previous studies of other authors have reported the isolation of some BM or MFGM components with different techniques as thin layer chromatography, electrophoresis or solid phase extraction (Caboni, Menotta, & Lercker, 1996; Fong, Norris, & MacGibbon, 2007) as well as ultrafiltration and supercritical fluid extraction (Costa, Elias-Argote, Jiménez-Flores, & Gigante, 2010; Konrad, Kleinschmidt, & Lorenz, 2013). However some difficulties as low purity or low recovery or toxic solvents needed have been reported. To go beyond these limitations, FC is a valuable option to obtain high amounts of pure lipid fractions with the possibility to use FG solvents. Nevertheless, to our knowledge, previous BM fractionation with FC has not been reported before.

3.2. Characterization of Buttermilk Lipid Extracts by HPLC-ELSD

Fig. 1 and Fig. 2 show the BM lipid profile and the characterization of the lipid composition of the fraction F1 and F2 extracts obtained by FC using FG and NFG solvents and analyzed by HPLC-ELSD. BM fat extracted out both with FG and NFG solvents showed a similar percentage of TAG content (77 vs. 76). Nevertheless, the distribution in other lipid classes was significant different ($p<0.01$) such as DAG (7 vs. 2.8) and PL (14.5 vs 20) (table 1 and 2).

With respect to the lipid extracts obtained with FG solvents, two lipid fractions that reached the 73.9±2.31% and 26.1±0.79% of the total BM fat were achieved (F1 and F2 respectively). Although most of the polar lipids (PL) were in the F1, where phospho- and sphingolipids were the major components (62%) some neutral lipids (NL) consisting of DAGs and TAGs and some other compounds that include sterols and free fatty acids (CHOL+FFA) were also present (Table 2). However F2 contained mainly neutral lipids comprised in TAGs (82%) and DAGs and only an small amount of PL (8.6%) in the form of PC and SM where present (Table 2).

Fig. 2 shows the BM and the lipid extract F1 and F2 profiles obtained using NFG solvents. The F1 fraction represents the 75.8±1.82% and the F2 the 24.2±1.07% of the total BM fat. In this case, the F1 eluted with the first NFG solvent (mixture of hexane/diethyl ether 98:2) consisted of NL almost completely separated (91% TAG and 8% DAG; Table 3) from the polar lipids that eluted in the F2 (94% PL) with hexane/diethyl ether (95:5), and contained a mixture of phospholipids where the major phospholipid was PE (Table 3). PC and SM were present in this F2 lipid fraction but in a very small amount because their strong binding affinity on this 4g silica cartridge.

Nevertheless previous studies from our research group demonstrated that PC and SM can be obtained in the F2 lipid extract with the same NFG solvents mixtures but using a 0.5g SPE cartridge with different brand of silica (Castro-Gómez et al., 2013).

The distribution of the BM lipid classes obtained with NFG solvents was in agreement with the previously reported data by Rodriguez-Alcalá and Fontecha (2010). The total content of PL extracted with NFG found in this study was also in agreement with Rombaut, Camp and Dewettinck (2005) although different phospholipid distribution was reported (PE 42.9%, PI 8.9%, PS 8.6%, PC 19.1% and 12.8% for SM). The data of PL obtained with FG solvents was in agreement with Costa, Elias-Argote, Jiménez-Flores and Gigante, (2010) that reported a total PL of 12.40% with similar phospholipid distribution (17%, 46% and 21.7% for PE, PC and SM respectively).

There are some previous studies reporting the PL isolation from BM with processes as ultrafiltration (Konrad, Kleinschmidt, & Lorenz, 2013) or ultrafiltration combined with supercritical fluid extraction (Costa, Elias-Argote, Jiménez-Flores, & Gigante, 2010) that reported four-fold increase in PL or an increase of 14% of the PL content in whey buttermilk, respectively. These results could be compared with the 4-5 fold increase in PL obtained in F1 and F2 isolated in this study with FG and NFG methods respectively. However, as indicated before, the fractionation of BM using FC has not been reported before.

3.3. Antiproliferation activity of Buttermilk sample and lipid extracts in human tumor cell lines

The antiproliferative activity was accessed using the methodology described by Developmental Therapeutics Program NCI/NIH (http://dtp.nci.nih.gov/; Monks et al. 1991) prioritizing the evaluation of a sample in various human tumor cell lines in order to obtain a profile of antiproliferative activity. Some studies suggest that the use of MFGM acts as a preventive cancer agent, mainly due to some of its proteins effect on breast and colon cancer cells in vitro (Dewettinck et al., 2008; Spitsberg, 2005; Zanabria, Tellez, Griffiths, & Corredig, 2013). Nevertheless, few studies have been done specifically to examine the antitumor effects of the MFGM lipids (around 50% content). Therefore in this study three samples of BM were prepared and their growth inhibition activity was assayed (Fig. 3). The whole BM powder samples which contains proteins and lipids in its composition did not show antiproliferative activity in none of

the 9 human cancer cells lines (figure 3 B) assayed. Only the BM fat extract obtained using FG solvents showed antiproliferative activity against K562 (leukemia) with a growth inhibition higher than 50% (GI50) when the sample concentration in the culture medium was 250 µg/mL (figure 3 C). However cancer cell lines proliferation was not inhibited by the BM fat extract obtained by using NFG solvents (Fig. 3 D). Although higher activity could be expected of the NFG-BM lipid extracts because its significant high PL content than the same FG-BM lipid extracts (Table 2 and 3), but did not occur because the isolation of lipids extracts using organic solvents may be negatively affect their bioactivity as we presumed in previous research work (Castro-Gómez et al., 2013). Same results were found with respect to the isolated lipid fractions F1 and F2 (Fig. 4). When NFG solvents were used in the extraction procedures none of the lipid extracts provide antiproliferative activity in any of the nine tumor cell lines assessed (Fig 4 C and D). However, both F1 and F2 fractions isolated with FG procedure, showed important antiproliferative activity. FG-F1 isolated fraction, with a highly enriched content in PL (>60% of total lipids) was a very antitumor active fraction that showed a total growth inhibition (TGI) against NCI-ADR/RES (ovary cancer cells with multidrug resistance) at 96.7 µg/mL and HT29 (colon cancer cells) at lower concentration than 250 µg/mL in the culture cell. This F1 also reached the TG50 inhibition in the cellular growth against 786-0 (kidney cancer cells) and K562 (leukemia cancer cells) at concentrations between 25 and 250 µg/mL (Fig 4 A). The result of the antiproliferative effect of FG-F1 against colon cancer cells, is in agreement with the bioactive antitumor effect previously reported by Kullenberg, Taylor, Schneider and Massing (2012) for PL obtained from different sources. Also Dial, Doyen and Lichtenberger (2006) observed that in colon cancer cell lines (SW-480) treated with soybean PC in combination with a non-steroidal anti-inflammatory drug resulted in a reduction of cancer cell growth. Other studies reported that the administration of PL from marine source (mainly PC bounded with n-3 FA) inhibited the growth of colon cancer cells (Caco-2) and it was also supported by in vivo studies increasing apoptosis in rats (Fukunaga, Hossain, & Takahashi, 2008). Furthermore, Snow et al. (2010) obtained the same results reducing the incidence of aberrant crypt foci (colon cancer) when whole MFGM was added to the Fischer 344 rats diet.

The F2 fraction isolated with food grade solvents also showed a TG50 activity against NCI-ADR/RES (ovary multidrug resistant) at concentrations below 250 µg/mL (Fig.4 B). Although this fraction presented a moderately low PL content (9%) the proportion of

SM and PC was very high. The anticarcinogenic effect of dairy SM on cancer cells have been widely reported because they are essential messengers in the growth control, differentiation and apoptosis in human adenoma cell lines (Kuchta, Kelly, Stanton, & Devery, 2012). SM fractions isolated from bovine milk have been reported to have preventive effects against colon cancer in ICR mice (Zhang, Li, Gao, & Duan, 2008). Antitumor activities of PL fractions against 786-0 (kidney cancer cell) and K562 (leukemia cancer cell) are reported here for the first time.

4. Conclussions

In conclusion, the use of FG solvents for the extraction or isolation of the BM or MFGM lipid components is a critical issue to maintain its antitumor activity. The presence of phospho- and sphingolipids in this lipid extracts obtained by FG solvents seems crucial to have antiproliferative activity against HT-29 (colon) and NCI-ADR/RES (ovary) human cancer cells. This study confirms that besides proteins, also the polar lipids associated to MFGM, show antitumor activity. However, further experimental animal studies are needed to elucidate the precise mechanisms of the anticarcinogenic effects of the isolated lipid extracts from BM using different food grade solvents.

Acknowledgments

This work was financially supported by the projects AGL2011-26713 and the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brasil).

REFERENCES

Caboni, M., Menotta, S., & Lercker, G. (1996). Separation and analysis of phospholipids in different foods with a light-scattering detector. *Journal of the American Oil Chemists' Society, 73*, 1561-1566.

Castro-Gomez, M. P., Rodriguez-Alcala, L. M., Calvo, M. V., Romero, J., Mendiola, J. A., Ibanez, E., & Fontecha, J. (2014). Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques. *Jornal of Dairy Science, 97*, 6719-6728.

Castro-Gómez, M.P., Holgado, F., Rodríguez-Alcalá, L.M., Montero, O., & Fontecha, J. (2015). Comprehensive study of the lipid classes of krill oil by fractionation and identification of triacylglycerols, diacylglycerols, and phospholipid molecular

species by using UPLC/QToF-MS. *Food Analytical Methods*, DOI 10.1007/s12161-015-0150-6.

Castro-Gómez, P., Rodríguez Alcalá, L. M., Calvo, M. V., Holgado, F., Carvalho, J. E., & Fontecha, F. J. (2013). Antiproliferative activity of buttermilk, milk fat globule membrane and isolated lipid fractions on ten human cancer cell lines. *Annals of Nutrition and Metabolism*, *63*, 1623.

Costa, M. R., Elias-Argote, X. E., Jiménez-Flores, R., & Gigante, M. L. (2010). Use of ultrafiltration and supercritical fluid extraction to obtain a whey buttermilk powder enriched in milk fat globule membrane phospholipids. *International Dairy Journal*, *20*, 598-602.

Danthine, S., Blecker, C., Paquot, M., Innocente, N., & Deroanne, C. (2000). Progress in milk fat globule membrane research: A review. Évolution des connaissances sur la membrane du globule gras du lait : Synthèse bibliographique. *Lait*, *80*, 209-222.

Dewettinck, K., Rombaut, R., Thienpont, N., Le, T. T., Messens, K., & Van Camp, J. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal*, *18*, 436-457.

Dial, E. J., Doyen, J. R., & Lichtenberger, L. M. (2006). Phosphatidylcholine-associated nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit DNA synthesis and the growth of colon cancer cells in vitro. *Cancer Chemotherapy and Pharmacology*, *57*, 295-300.

Fong, B. Y., Norris, C. S., & MacGibbon, A. K. H. (2007). Protein and lipid composition of bovine milk-fat-globule membrane. *International Dairy Journal*, *17*, 275-288.

Fukunaga, K., Hossain, Z., & Takahashi, K. (2008). Marine phosphatidylcholine suppresses 1,2-dimethylhydrazine-induced colon carcinogenesis in rats by inducing apoptosis. *Nutrition Research*, *28*, 635-640.

Konrad, G., Kleinschmidt, T., & Lorenz, C. (2013). Ultrafiltration of whey buttermilk to obtain a phospholipid concentrate. *International Dairy Journal*, *30*, 39-44.

Kuchta, A. M., Kelly, P. M., Stanton, C., & Devery, R. A. (2012). Milk fat globule membrane – a source of polar lipids for colon health? A review. *International Journal of Dairy Technology*, *65*, 315-333.

Kullenberg, D., Taylor, L. A., Schneider, M., & Massing, U. (2012). Health effects of dietary phospholipids. *Lipids in Health and Disease*, *11*, 3.

Mather, I. H. (2000). A review and proposed nomenclature for major proteins of the milk-fat globule membrane. *Journal of Dairy Science*, *83*, 203-247.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, j., Cronise, P., Viagro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., & Boyd, M. (1991). Feasibility of a High-Flux Anticancer Drug Screen Using a Diverse Panel of Cultured Human Tumor Cell Lines. *Journal of the National Cancer Institute*, *83*, 757-766.

Morin, P., Jimenez-Flores, R., & Pouliot, Y. (2007). Effect of processing on the composition and microstructure of buttermilk and its milk fat globule membranes. *International Dairy Journal*, *17*, 1179-1187.

Rodríguez-Alcalá, L. M., & Fontecha, J. (2010). Major lipid classes separation of buttermilk, and cows, goats and ewes milk by high performance liquid chromatography with an evaporative light scattering detector focused on the phospholipid fraction. *Journal of Chromatography A*, *1217*, 3063-3066.

Rombaut, R., Camp, J. V., & Dewettinck, K. (2005). Analysis of Phospho- and Sphingolipids in Dairy Products by a New HPLC Method. *Journal of Dairy Science*, *88*, 482-488.

Rombaut, R., Camp, J. V., & Dewettinck, K. (2006). Phospho- and sphingolipid distribution during processing of milk, butter and whey. *International Journal of Food Science & Technology*, *41*, 435-443.

Snow, D. R., Jimenez-Flores, R., Ward, R. E., Cambell, J., Young, M. J., Nemere, I., & Hintze, K. J. (2010). Dietary Milk Fat Globule Membrane Reduces the Incidence of Aberrant Crypt Foci in Fischer-344 Rats. *Journal of Agricultural and Food Chemistry*, *58*, 2157-2163.

Sodini, I., Morin, P., Olabi, A., & Jimenez-Flores, R. (2006). Compositional and Functional Properties of Buttermilk: A Comparison Between Sweet, Sour, and Whey Buttermilk. *Journal of Dairy Science*, *89*, 525-536.

Spitsberg, V. L. (2005). Invited review: Bovine milk fat globule membrane as a potential nutraceutical. *Journal of Dairy Science*, *88*, 2289-2294.

Zanabria, R., Tellez, A. M., Griffiths, M., & Corredig, M. (2013). Milk fat globule membrane isolate induces apoptosis in HT-29 human colon cancer cells. *Food & Function*, *4*, 222-230.

Zhang, P., Li, B., Gao, S., & Duan, R. D. (2008). Dietary sphingomyelin inhibits colonic tumorigenesis with an up-regulation of alkaline sphingomyelinase expression in ICR mice. *Anticancer Research*, *28*, 3631-3635.

Table 1 - Human cancer cell lines used to assess the antiproliferative activity.

Human cell lines	Organ/Desease	Embryonic origin	Inoculation density (10^4 cel/mL)
UACC-62	Skin/melanoma	Ectoderm	4,0
MCF-7	Breast/adecarcinoma	Ectoderm	6,0
NCI-ADR/RES	Ovary/adenocarinoma	Ectoderm	5,0
786-0	Kidney/adenocarcinoma	Mesoderm	5,0
NCI-H460	Lung/carcinoma	Endoderm	4,0
OVCAR-3	Ovary/adenocarcinoma	Mesoderm	7,0
HT-29	Colon/adenocarcinoma	Endoderm	5,0
K562	Bone marrow/Myeloid leukemia	Mesenchyme	6,0
HaCaT	Skin (keratinocyte)/no tumor	Ectoderm	4,0

Table 2 - Lipid classes distribution (%, w/w) of buttermilk fat (BM), fraction 1 (F1) and fraction 2 (F2) isolated using food grade (FG) solvents analyzed by HPLC-ELSD.

	BM	F1	F2
TAG	77.58 ± 1.07	5.08 ± 0.05	81.69 ± 2.12
DAG	6.96 ± 0.15	22.35 ± 1.35	9.05 ± 0.30
CHOL+FFA	0.72 ± 0.03	8.6 ± 0.53	0.53 ± 0.08
MAG	<0.1	<0.1	<0.1
GLUCER	<0.1	<0.1	<0.1
LACCER	<0.1	<0.1	<0.1
PL	14.46 ± 2.53	62.17 ± 0.20	8.63 ± 0.99
g/100 g PL			
PE	28.98 ± 3.91	40.1 ± 1.98	<0.1
PI	0.91 ± 0.01	3.27 ± 0.13	n.d.
PS	1.21 ± 0.15	4.01 ± 0.22	n.d.
PC	47.42 ± 3.62	41.04 ± 2.2	43.56 ± 3.67
SM	21.48 ± 0.53	11.58 ± 0.87	55.81 ± 3.48

TAG: triacylglycerols; DAG: diacylglycerols; CHOL: cholesterol; FFA: free fatty acids; MAG: monoacylglycerols; GLUCER: glucosylceramides; LACCER: lactosylceramides; PL: polar lipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: hosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin

Table 3 - Lipid classes distribution (%, w/w) of buttermilk fat (BM), fraction 1 (F1) and fraction 2 (F2) isolated using non-food grade (FG) solvents analyzed by HPLC-ELSD.

	BM	F1	F2
TAG	76.36 ± 0.76	90.68 ± 4.19	1.22 ± 0.20
DAG	2.82 ± 0.01	8.26 ± 0.83	0.94 ± 0.09
CHOL+FFA	0.51 ± 0.19	0.9 ± 0.02	0.3 ± 0.01
MAG	<0.1	<0.1	<0.1
GLUCER	<0.1	<0.1	<0.1
LACCER	<0.1	<0.1	<0.1
PL	19.92 ± 0.53	0.11 ± 0.01	94.05 ± 3.58
g/100 g PL			
PE	18.55 ± 1.10	86.19 ± 5.48	64.97 ± 2.12
PI	0.25 ± 0.01	n.d.	15.21 ± 0.88
PS	2.41 ± 0.88	13.81 ± 2.67	18.56 ± 0.92
PC	57.19 ± 0.16	n.d.	0.78 ± 0.02
SM	21.5 ± 0.46	n.d.	0.35 ± 0.02

TAG: triacylglycerols; DAG: diacylglycerols; CHOL: cholesterol; FFA: free fatty acids; MAG: monoacylglycerols; GLUCER: glucosylceramides; LACCER: lactosylceramides; PL: polar lipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: hosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin

Fig 1.

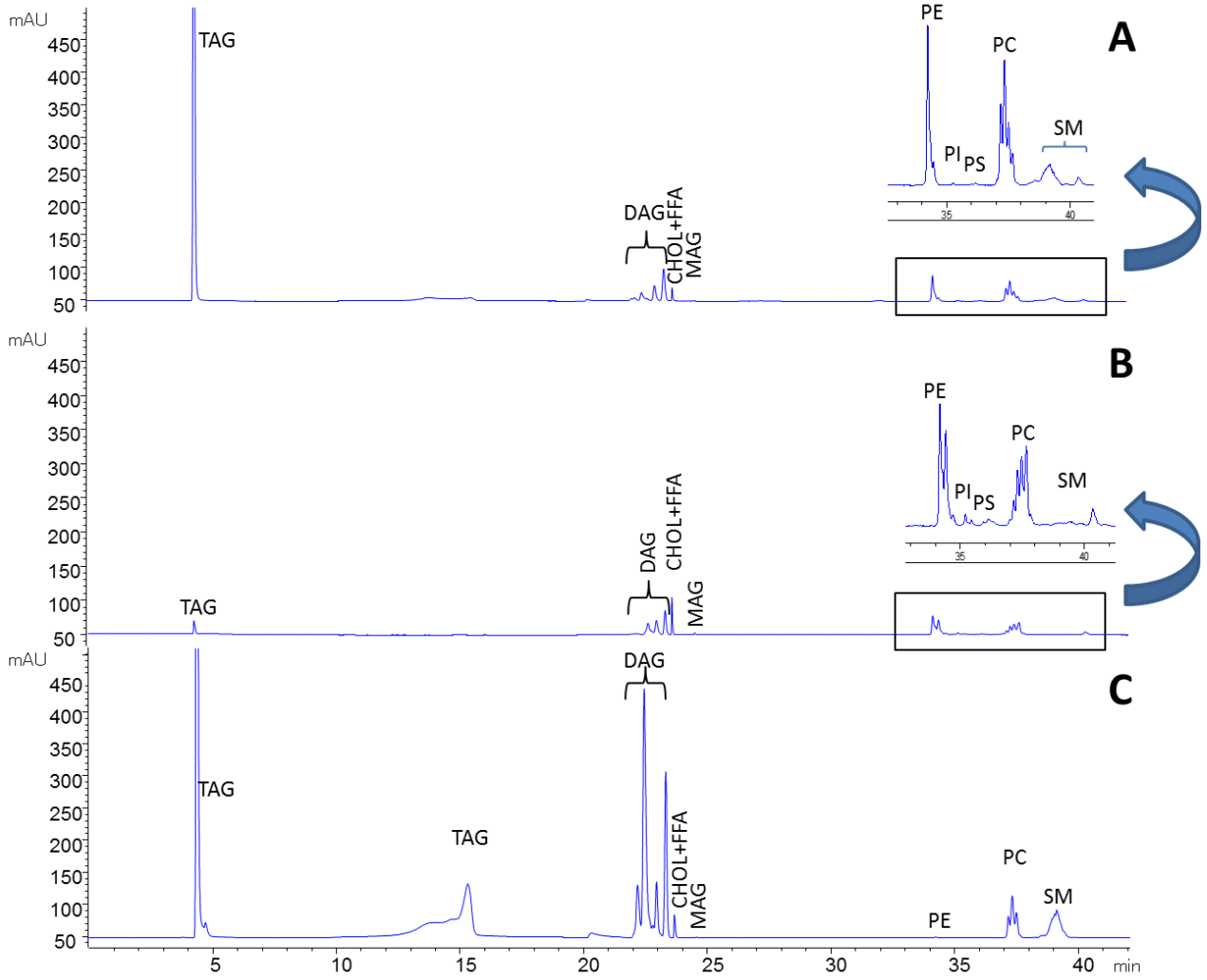

mAU
450
400
350
300
250
200
150
100
50
TAG
DAG
CHOL+FFA
MAG
PE
PC
SM
PI
PS
35
40
A
B
C
5
10
15
20
25
30
35
40
min

Fig 2.

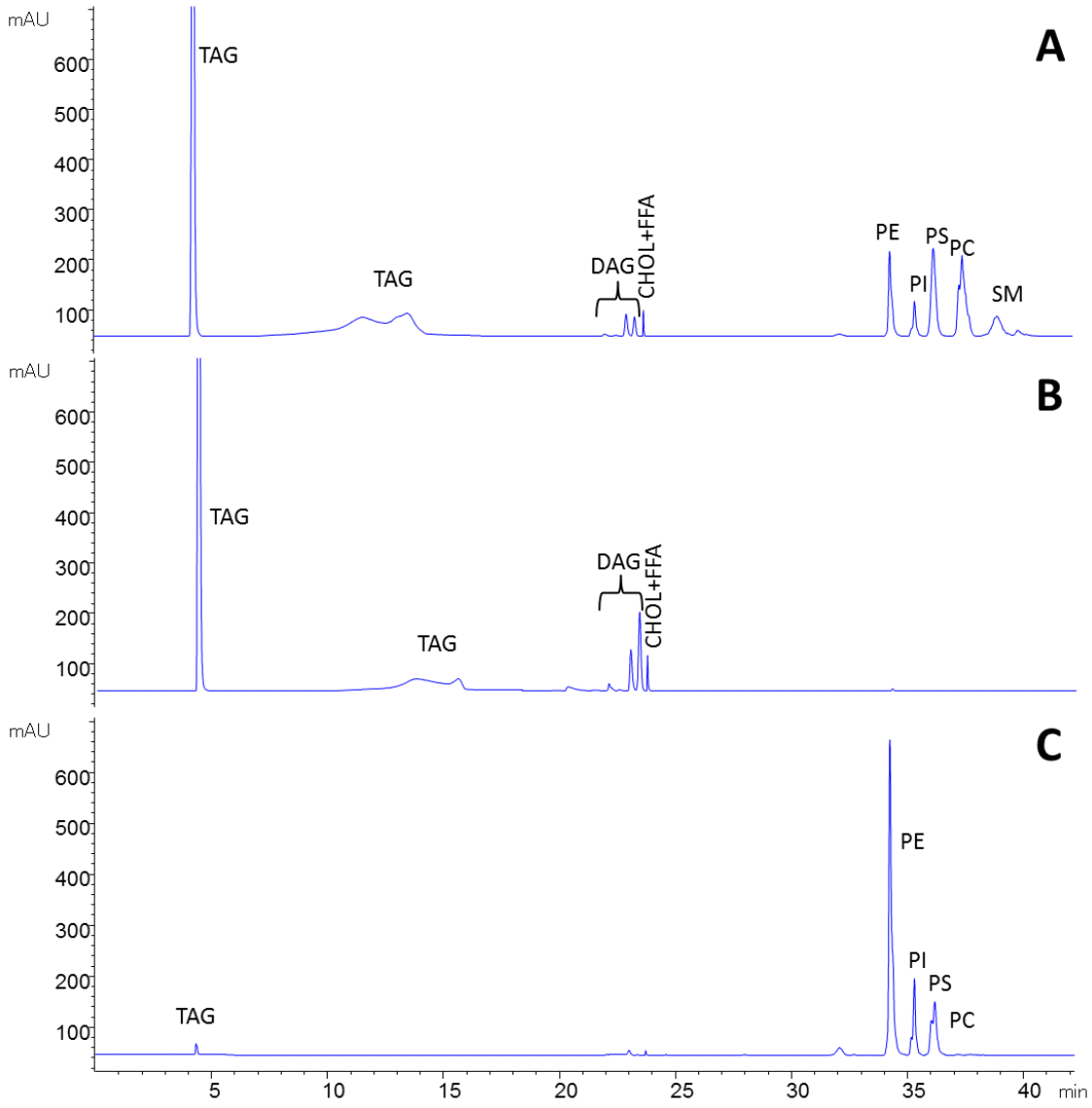

mAU
600
500
400
300
200
100
A
TAG
TAG
DAG
CHOL+FFA
PE
PS
PC
PI
SM
B
TAG
TAG
DAG
CHOL+FFA
C
TAG
PE
PI
PS
PC
5
10
15
20
25
30
35
40
min

Fig 3.

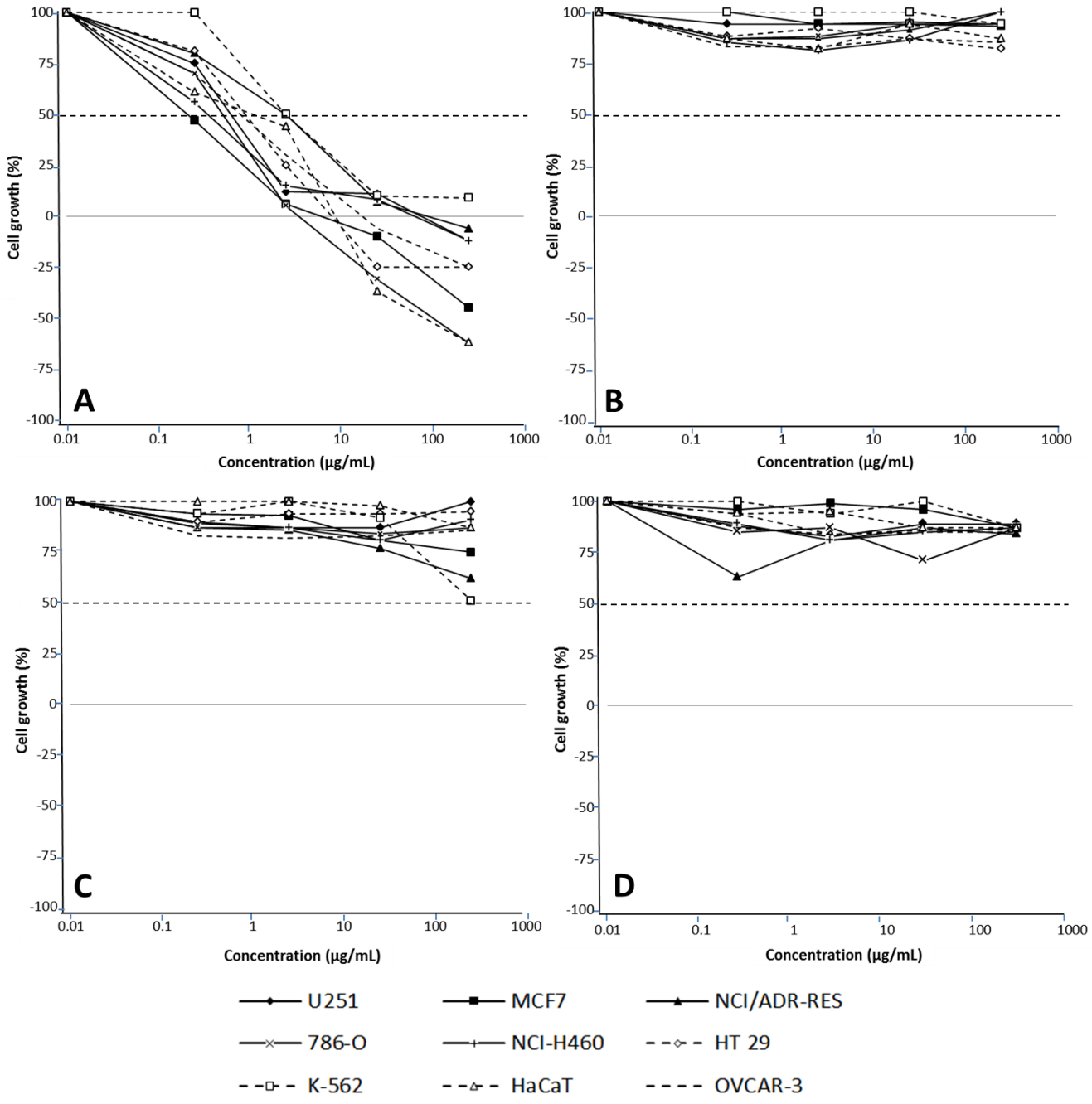
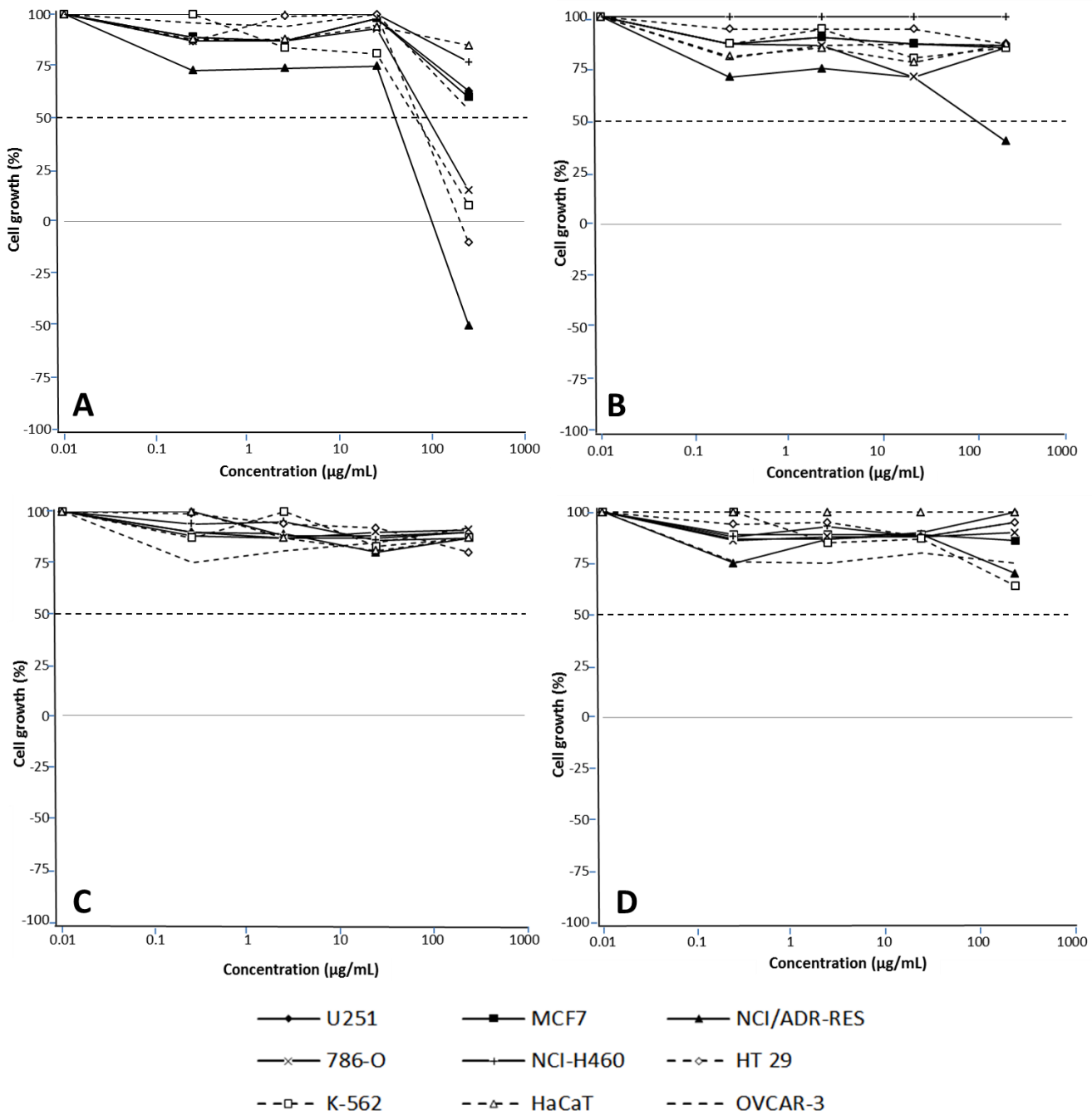

A
B
C
D
Cell growth (%)
Concentration (µg/mL)
100
75
50
25
0
-25
-50
-75
-100
0.01
0.1
1
10
100
1000
U251
MCF7
NCI/ADR-RES
786-O
NCI-H460
HT 29
K-562
HaCaT
OVCAR-3

Fig. 4.


A
B
C
D
Cell growth (%)
Concentration (µg/mL)
100
75
50
25
0
-25
-50
-75
-100
0.01
0.1
1
10
100
1000
U251
MCF7
NCI/ADR-RES
786-O
NCI-H460
HT 29
K-562
HaCaT
OVCAR-3

3.2. Postprandial variations on plasma lipid classes and fatty acids content after consumption of skimmed milk enriched with buttermilk in healthy subjects

Postprandial variations on plasma lipid classes and fatty acids content after consumption of skimmed milk enriched with buttermilk in healthy subjects

M.P. Castro-Gómez[a], L.M. Rodríguez-Alcalá[a], I. Guillén-Guillén[b] , F.J. López-Román[b], J. Fontecha[a*]

[a]Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM). Universidad Autónoma de Madrid, Calle Nicolás Cabrera 9, 28049, Madrid, Spain.
[b]Cátedra de Fisiología del Ejercicio. Departamento de Ciencias de la Salud. Universidad Católica San Antonio de Murcia, Guadalupe, Murcia, Spain.

[*] Corresponding autor e-mail: j.fontecha@csic.es

Abstract

The bioactive compounds of milk fat are differently distributed during the dairy products manufacture. The buttermilk, a by-product of the butter making process, contains several of these compounds, highlighting the polar lipids, specially phospho- and sphingolipids which form the milk fat globule membrane. Although, these compounds have been reported to promote beneficial functions on human health, it is not widely studied the way that each lipid classes and fatty acids are absorbed. The intake of a skim milk enriched with buttermilk and the monitoring of triacylglycerides, cholesterol, phospholipids and fatty acid during the following 5 hours, showed that glycerolipids and major bioactive fatty acids reach a maximum concentration 2-3 hour after the intake followed by a decrease to basal concentration. However, the polar lipids displayed a extended increment up to 5 hours, which is supported with the higher absorption of the fatty acids linked to phospholipids than triacylglycerides.

Introduction

The dairy fat has been widely studied due the numerous and complex components which are forming part of the milk fat globule. Although during the last years some studies have focused theirs investigations in devaluing it because its high content in saturated fatty acids (SFA) and its relation with cardiovascular diseases, recent studies discarded it because lack of evidences (O'Keeffe and St-Onge, 2013, Ravnskov, 2014). Furthermore, this fat showed some components which have been described with beneficial effects in human health. The buttermilk (BM), a byproduct obtained from the butter production and with high content in milk fat globule membranes (MFGM), contains most of these beneficial compounds. The MFGM are rich in polar lipids (PL), as phospho- and sphingolipids, which are associated with beneficial effects against stress, depression, cholesterolemia, gastrointestinal and age diseases as Alzheimer, inflammatory responses and as therapeutic in some cancers as colon (Simons and Vaz, 2004, Rombaut and Dewettinck, 2006, El-Loly, 2011, Kullenberg et al., 2012). Furthermore, this fat also contains different fatty acids (FA) distributed in PL, triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG) or as free fatty acids (FFA), which can provided beneficial effects on human health. The anticarcinogenic activity of the short fatty acid C4:0 or the conjugated linoleic acid (CLA) and its precursor trans-vaccenic acid (TVA), as well as, the antibacterial and antiviral effect of FA from C6:0 to C10:0 have been reported. Moreover, benefits on cardiovascular diseases of polyunsaturated fatty acid (PUFA) C18:2 c9, c12 and C18:3 c9, c12 or the reduction of cholesterol (CHOL) carried out by C18:0 and C18:1 c9 (Fontecha et al., 2011) have been also supported by some research works. The exhaustive characterization and knowledge of these bioactive lipid are of high importance from a human health point of view, however has not been widely reported how each of these different and functional compounds are absorbed by intestinal cells and transported to blood to carry out theirs functions in cells and tissues after the consumption of a food with high concentration of them.

The aim of the present study was to increase the knowledge of the different lipid classes and FA absorption in plasma, with particular attention in functional lipids, during 5 hours after the intake of a skim milk enriched with BM.

Materials and methods

Reagents

All solvents were at least HPLC grade and MS grade when available. Chloroform, dichloromethane, hexane, methanol, isooctane, isopropanol and sulfuric acid (95%) were purchased from LABSCAN (Dublín, Ireland). Potassium hydroxide and sodium carbonate were obtained from PANREAC (Barcelona, Spain). Formic acid (98%) and triethylamine (99.5%), the TAG standard tritridecanoin, the FFA standards pelargonic (C9), tridecanoic (C13), myristic (C14), palmitic (C16), estearic (C18), araquidonic (Aa, 20:4), eicosapentanoic acid (EPA, 20:5) and docosahexapentanoic acid (DHA, 22:6), the sterols 5α-cholestane, CHOL and cholesterol ester (CE), the MAG and DAG monostearin and diolein, the PL: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM) were purchased to Sigma (Bellefonte, PA, USA). The reference samples: butter fat BCR-164 (EU Commissions; Brussels, Belgium) was purchased from Fedelco Inc. (Madrid, Spain).

Study design

The study protocol was reviewed and approved by the Ethics Committee of the Universidad Católica San Antonio (Murcia, Spain). The trial was carried out in accordance with Good Clinical Practices and was conducted in accordance with the Declaration of Helsinki (1964 and subsequent revisions). Volunteers were fully informed of the aims and purposes of the study, and written informed consent was obtained from all participants.

Twelve healthy of both sexes, aged between 50 and 65 were recruited to the oral consumption of 400 mL of the tested drink from the former study carried out at Department of Health Science at the Universidad Católica San Antonio de Murcia (Murcia, Spain). The data of age, weight, height and BMI are showed in table 1. The volunteers had not metabolic, cardiovascular or obesity diseases, as well as, none of them received medicament treatment which could alter blood lipid levels. They also did a physical examination, including vital signs The eligibility was assessed through the screening visit performed 2 weeks before the start of the study. A total of 20 volunteers were assessed for eligibility, and 12 volunteers (5 men and 7 women) were selected based on these criteria.

Test product

The drink assayed to check the absorption of each of lipid classes and FA was a skim milk enriched with BM in concentrations of 12.5 g powder BM/100mL of drink kindly donated by CAPSA (Asturias, Spain). This drink reach a caloric value of 46 Kcal/100mL and it had a total lipid content of 0.6 g/100 mL, proteins 3.8 g/100 mL and carbohydrates 6.4 g/100 mL. The composition in lipid classes showed a PL content of ~13% where de phospho- and sphingolipids classes were distributed in PC, PE, PS, SM and PI from the highest to lowest content. Likewise, in terms of neutral lipids, only TAG as the major lipid class, DAG and CHOL+FFA could be quantified (table 2). On the other hand, the FAME content showed a main saturated profile (~72%) due the high content of C14:0, C16:0 and C18:0. It was followed by monounsaturated fatty acids (MUFA) content (~26%) mainly due to C18:1 as well as the C18:2 c9, c12 was more than the half of total PUFA (~3%) (Table 3).

Intervention

The volunteers were attended at the department of Physiology at the Universidad Católica San Antonio. At the screening visit, all subjects were informed about the study orally, and after checking inclusion and exclusion criteria, patients were selected. On study day, venous blood samples were drawn with a cannula (ABOCATH, Abbott Laboratories, IL, USA) that was

inserted into a vein for blood sampling and fasting blood samples (baseline blood sample) for clinical laboratory test. The cannula was regularly flushed with normal saline solution to maintain patency between the blood samples.
Before the test drink administration, a venous blood sample was drawn for baseline blood measurements. Next, volunteers received 400 mL of test product, as well as a standardized breakfast (two slices of bread with tomato and turkey ham, and a coffee or orange juice). The test drink was consumed under supervision to ensure that all drink test and breakfast were eaten. Blood samples were collected at 1-h intervals, being the first blood sampling before the product intake (baseline), and subsequently, postprandial blood samples were drawn after they finished the product intake (time 0), every hour for 5 h. Subjects were not allowed to eat or drink anything more except tap water during the 5 h of the study.

Fat extraction

The lipid extraction method for plasma and the drink was carried out as Castro-Gomez et al. (2014) but with slight modification. A volume of 1mL of sample was placed in 50-mL centrifuge tubes and eight milliliters of a dichloromethane-methanol solution (2:1, vol/vol) was then added to each tube. The mixture was shaken mechanically for 30 min and centrifuged at 6,600 × *g* for 5 min at 4°C. As much of the upper organic solvent fraction as possible was carefully removed with a pipette. The sediment was washed with 6 mL of a dichloromethane-methanol solution (2:1, vol/vol) and, after shaking for 1 min, the sample was, again centrifuged at 6,600 × *g* for 5 min at 4°C. The removed organic solvent was combined with that previously collected and 1.5 mL of a 0.9% solution of sodium chloride was added and mixed mechanically for 1 min before the tubes were stored overnight at 4°C. Afterward, they were again centrifuged at 6,600 × *g* for 5 min at 4°C and the bottom dichloromethane layer was collected and filtered through a PVDF filters of 45 µm of diameter (CAPTIVA Econo Filter, Agilent Technologies Inc.) containing approximately 1.5 g of anhydrous sodium sulfate. Finally, the extract was concentrated by removing dichloromethane under a gentle stream of nitrogen. The extracted fat

was weighted before and after in amber vials flushed with nitrogen and stored at −35°C until chromatographic analysis.

Lipid classes composition by HPLC-ELSD-Evaporative Light Scattering Detection

Separation of lipid classes of plasmas and the drink was accomplished in an HPLC system (model 1260; Agilent Technologies Inc.) coupled with an evaporative light scattering detector (SEDEX 85 model; Sedere SAS, Alfortville Cedex, France) using prefiltered compressed air as the nebulizing gas at a pressure of 350 kPa at 60°C; the gain was set at 3. Two columns in series (250 × 4.5 mm Zorbax Rx-SIL column with 5-µm particle diameter; Agilent Technologies Inc.) and a precolumn with the same packing were used. Before analysis, samples were dissolved in CH_2Cl_2 at 5 mg/mL and 50 µL was injected after column equilibration at 40°C. The solvent gradient was as detailed in Castro-Gómez et al. (2014).

FA Determination and Quantification

Fatty acid methyl esters of plasmas and drink were separated using a CPSil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2-µm film thickness; Agilent Technologies Inc., Palo Alto, CA) in an Agilent chromatograph (model 6890N; Agilent Technologies Inc.) equipped with a mass spectrometry detector. The analysis were carried out as in Castro-Gómez et al. (2014). The temperature programmed at 7°C/min to 170°C, held at 170°C for 55 min, and then temperature programmed at 10°C/min to 230°C and held at 230°C for 33 min. The injector temperature was set at 250°C. Helium was the carrier gas with a column inlet pressure of 206.9 kPa. The mass spectrometry detector conditions were as follows: transfer line temperature: 250°C, source temperature: 230°C, quad temperature: 150°C, electron impact ionization: 70eV and the range from 50 to 500 m/z was scanned. For identification of the peaks, the National Institute of Standards and Technology (NIST, Gaithersburg, MD) library and mass spectra of the standards used in our laboratory were used. The injection volume was 1 µL and the split ratio used was 1:10. Response

factors were calculated using an anhydrous milk fat reference butterfat BCR-164) and tritridecanoate as internal standard (200 µL; 1.3 mg/mL) was used.

Statistical analysis

All results are expressed as means and standard deviations (n = 12 volunteers for each time). An exploratory analysis of data was performed to test normal distribution and homogeneity of variance. Thus, during the check of fat yield, lipid classes and FAME composition in each time, the results where compared using the Kruskal-Wallis test. All analyses were performed using the SPSS Statistics software (v19.0 for Windows; IBM Corp., Armonk, NY). The level of significance was fixed at $P < 0.05$.

Results and discussion

Plasma lipid content during the study

The concentration of total plasma lipid content in each time can be observed in figure 1. Although significant differences were not detected between times due the data dispersion that occurs when biological samples are assessed, could be observed a clear tendency. During the first hour, the lipid content in plasma was maintained in a concentration of 6.74 mg fat/mL±1.61 just after the consumption and 6.71 mg fat/mL ±1.30 an hour later. After this, a light increase could be observed 2 h after the intake (7.07 mg/mL ±1.80) followed of the maximum peak found at 3 h which value reach 9.05 mg/mL±2.47. When this point was achieved, the lipid concentration tendency during the last two hours was decreased until attain the initial values (6.79 mg/mL ±1.10 for t 4h and 6.57 mg/mL ±2.05 for t 5h). These data suggest that the maximum fat absorption occurs 3 hours after the ingestion and because the cells lipid absorption, only two more hours are needed to reach the basal values in plasma. Although the evolution of total fat concentration in plasma has been lightly reported, this same tendency was also observed by Tholstrup et al. (1998) in healthy men

after the consumption of a diet rich in milk fat. Furthermore, the initial concentration of lipid in plasma (0 h) is in according with several studies as Dougherty et al. (1987) and Roseboom et al. (2000), whose reported values from 5.96 to 6.15 mg/mL and from 6.94 to 7.21 mg/mL respectively in fasting normal men.

Lipid classes composition during the study

The concentrations of the neutral lipid classes and total PL (g/100g fat), as well as phospho- and sphingolipids (g/100g PL) 5 h after the intake, are showed in figure 2. Although the concentration of each lipid class is the sum of those which are linked in chylomicrons (QM) (from the diet) and in very low density lipoproteins (VLDL) (synthesized in liver), the changes of each lipid class suggest that not all the lipid classes are absorbed and synthetized in the same way or at the same time (Agren et al., 2006). However, it is well known that during the first hours after the intake, the plasma lipid contents come mainly from the diet. Although the neutral lipid classes TAG, DAG and MAG did not provided significant differences among times because the data dispersion, they showed a remarkable tendency. It is important to highlight the wide variation of TAG during the checked time probably due they are the highest component in the drink (Table 2). The TAG and DAG showed an increment reaching a maximum peak of concentration 2 hour after the ingestion. After reaching this point, the concentrations declined to reach the initial content. The TAG results are in agreement with Goltz et al. (2012) and Vilarrasa et al. (2014) whose reported a major peak concentration of TAG in healthy human adults between 2 and 4 hour after intake diets with different amount of butter (in the first study) and palm, canola or soy oil (in the second study). In the same way, Olefsky et al. (1976) observed a major TAG concentration 2 hour after the ingestion which was attributed to the TAG linked to QM and consequently which come from the diet. Cited study also observed after 5 hours of the intake, a second increment of the TAG peak which in this case came from the VLDL synthetized in the liver. The DAG results are in according with a study of Vogelberg and Gries (1970) whose also observed the highest concentration of DAG 2 hour after the diet consumption followed by a decrease to initial concentration. This same

tendency of TAG and DAG could be because while the synthesis of TAG linked to QM are being carried out in the epithelial intestinal cells, in blood, the lipoprotein lipase acts on the QM which have been released before. This enzyme releases FFA from the TAG linked to QM to be absorbed by the cells of different tissues. During this lack of FA from TAG, the DAG are being created which then will be also hydrolyzed to release more FFA for cells (Michalski, 2009). In terms of MAG from diet, the transportation is not carried out by the QM because due theirs minor size, they can pass from the intestine lumen to the blood torrent thought intestinal cells without the need of a transporter. In this study, the amount of MAG increased in plasma from the intake also to 2-3 hours, followed by a decrease until 5 hours. Due the almost inexistent amount of MAG in consumed drink (<0.1%, Table 1), this variation could be explained with the same argument than with DAG. When the DAG has been attained from TAG, the lipoprotein lipase continues acting releasing more FFA for cells. Finally, cited enzyme separates the last FA from MAG and the remaining glycerol is brought to the liver where will be used to synthetize new TAG linked to VLDL. Although, the information about MAG have not been widely reported, the present results are not in according with a study which showed a light diminution of MAG from the intake to 2 hour followed by an increment to 4 hours after the consume of a food with 42% of fat (Vogelberg and Gries, 1970) may be due the different diet. Other neutral compound, the CE, maintained very similar concentration during all the study. The CE in QM comes from the des-esterification and re-esterification of CE, as well as, esterification of some dietary CHOL in the intestinal cell to be included in the QM structure before flow to blood torrent. The absence of changes could be due to the low amount of CE and free CHOL provided by the drink (<1%, Table 2). The CE results in plasma are in according with Cohn et al. (1988) whose observed a maintenance of CE concentration during 12 hour after the intake of diet with soy oil also poor in CHOL and CE. The same results were also reported by Sutherland et al. (2007) during 6 hours after the administration of a shake milk with 70% of fat to health volunteers. Respect the total non-esterified CHOL, beyond the CHOL from diet is not efficiently absorbed and the changes in plasma could be negligible (Castro and Fielding, 1985), the concentration is maintained in the time because the maximum peak of CHOL linked to QM and VLDL observed after 4

hours is compensated with the minimum peak of CHOL linked to low density lipoproteins (LDL) and high density lipoproteins (HDL) observed at the same time (Tholstrup et al., 2001). In terms of FFA, some studies (Wolever and Mehling, 2003, Torronen et al., 2012, Vors et al., 2013) reported a light diminution of FFA until 2 h after the intake of a fat diet followed by an increment to initial concentration. Based in the CHOL maintenance, these results are in according with the present study where the group of FFA+CHOL also showed a slight tendency of diminution to 2 h followed by an increment. The increment of FFA from 2 h to 5 h could be due the release of FA provided by the TAG, DAG and MAG hydrolysis just before been consumed by the cells. The PL come to the enterocytes following a similar way than glycerolipids. They enter in the intestinal cell after a hydrolysis carried out by a pancreatic enzyme in the intestinal lumen. When all the hydrolyzed components are in the intestinal cell, they are newly esterified in PL structure to be included in the external part of the QM. However, the FA released from PL can be also used to create TAG which will be in the core of the QM (Nilsson and Duan, 2006, Cohn et al., 2010). When QM are flowed to lymph and then to blood, the phospholipase hydrolyzes the PL being rapidly absorbed by the cells. However, part of the PL is not hydrolyzed but some of them are transferred to HDL and brought to liver to be linked to VLDL (Tall et al., 1983). The total PL results of the present study showed a light tendency of increment over time from 5.45% (t0) to 5.94% (t5) which supposed an increase of 6%. These increment have been also reported by some studies as Cohn et al. (1988) whose observed an increase of total PL in human plasma until 6 hours after the intake of a diet rich in fat. The same tendency was reported by Simonsson et al. (1982) and Castro and Fielding (1985) in human plasma after the ingestion of high fat diets. Each PL followed similar tendency. The PC and the SM were the major and had an increment from 92.92 to 93.59% and 4.45 to 5.94% on total PL from 0h to 5h respectively, however, significant differences could not be detected among time either due the data dispersion. These results were also observed during 5 hours by Simonsson et al. (1982) in PC when diet rich in yolk was provided. Agren et al. (2006), also reported an increment of SM after 4-5 hours of consumption of a drink with more than 20% of fat. Furthermore, the cited studies, also observed that during the first 5 hours, the changes of PC and SM are attributed to the diet

because during this time the PC and SM in blood torrent are linked to QM and not to VLDL (constant the first 5 hours). This suggest that these PL, components with beneficial effects in cancer and neurological effects among other (Rombaut and Dewettinck, 2006), have been provided and absorbed from the drink. The PE and PI contents also have an increment of 13% and 78% showing range values of 0.53-0.60% and 0.19-0.34% from 0 h to 5 h respectively. Although changes of PE and PI content in human plasma after the intake have not been reported before, the concentration in fasting of each PL is in according with those observed by Nilsson and Duan. (2006) whose also reported the absence of PI in human plasma. On the other hand, the increase and high maintenance of PL in plasma during more time, support the theory that FA linked to PL are better absorbed that when they are linked to TAG (Di Marzo et al., 2010), due the higher time in plasma provided that PL been hydrolyzed by the phospholipases during more time and in consequence released more FA to be absorbed by the cells.

Plasma FA composition during the study

The plasma FA composition at every hour after intake is showed in table 4. The total FA composition provided an increment from 0h to 3h where reached the maximum FA concentration (320.37 mg/g fat). 3 hours after the ingestion, the FA concentration tends to decrease until reaching the initial concentration at 5h (216.86 mg/ g fat). This behavior was also observed in the plasma total lipids described previously where the major concentration of lipids was also observed 3 h after the intake (Fig. 1) and as well as with the trend of neutral lipid fraction observed by HPLC-ELSD (Fig. 2). As expected, the same tendency was also observed in the most of FA present in plasma samples. The FA which showed the highest concentration were C16:0 and C18:2 *c*9,*c*12 showing also the maximum concentration 3 hours after the intake (88.62 mg and 90.07 mg/g fat respectively) followed to a decrease. Other major FA, were C18:0 and C18:1*c*9, showing maximum values of 48.82 and 63.04 mg/g fat at the same time, respectively. It is remarkable, that C18:0, C18:1*c*9 and C18:2 *c*9,*c*12 with high concentration in plasma after the absorption, have been attributed with beneficial effects in cardiovascular diseases and reduction of plasmatic CHOL

(Fontecha et al., 2011). These major FAME present in the plasma were in according with Castro-Gómez et al. (2014) whose also observed C16:0, C18:0, C18:1 *c*9 and C18:2 *c*9,*c*12 as the major in a standard plasma sample. The results of Vors et al. (2013) in normal-weight subjects fed with milk fat, are in agreement with those observed in C16:0 and C18:1 *c*9 in the present study. Cited study also showed an increment from the ingestion to 3-4 hour after, however, it did not observed a posterior decrease but maintenance in both FA. Furthermore, Abia et al. (1999) also observed the highest concentration of C16:0 4 h after a meal enriched with olive oil intake followed by a light decrease to 6h as in the present study. Furthermore, with respect C18:1*c*9, this last study showed a highest peak 2 h after the intake observing then a decrease to time 4h. The trend of latter FA is also in according with Malpuech-Brugere et al. (2010) whose although the maximum peak was 5 hour after the intake, it was followed by a decrease to initial concentrations as in the present study. The difference in the time of the maximum peak could be due to the subject in cited study were obese and fed with doses of sunflower. The results reported for C18:0 and C18:2 *c*9,*c*12 are in according with the tendency reported by Abia et al. (1999) whose showed an increment from 2 hours to 4 hours followed by a decrease to 6 hours. It is remarkable that the high increment of C16:0, C18:0 and C18:1 *c*9 in plasma samples could be due these FA, which also were the major FA in the assayed drink, have been absorbed (table 3). However, the C18:2 *c*9,*c*12, other major FA in plasma, showed a low concentration in the provided drink (<2% on total FAME), as well as, C20:4 which showed contents above 10 mg/g fat in plasma (5 and 7% on total FAME), while it was <0.5% in the drink (table 2). The other FA contents provided concentration over 1 mg/g fat, except C14:0 which showed concentrations between 1.07 and 2.37 mg/g fat may be due it was also one of the major FA in the drink. It should be also noted that despite the drink provided short and medium FA (from C6:0 to C10:0) with anticarcinogenic, antibacterial and antiviral effects (Fontecha et al., 2011), only FA with 12 or more carbon number could be detected in plasma. Due all the FA behaved similarly in terms of absorption, the sum of SFA, MUFA and PUFA provided the same tendency. This was also observed by Jans et al. (2012) whose observed an increment of SFA, MUFA and PUFA from fasting to 4 hour after the intake of high fat meal as occurred in the present study. The study of

Koutsari et al. (2004) also observed a maximum peak of concentration of SFA and MUFA 2-3 h after the ingestion of a breakfast with 43% of fat content. As occurred in this study, the content after these hours was decreasing to initial concentration.

In conclusion, the consumption of a skim milk enriched with buttermilk showed that lipid classes and fatty acids are differently absorbed. Polar lipids, with beneficial effects on human health, are increased from the intake to 5 or more hours being supported by the theory of the better absorption of fatty acid linked to polar lipids than triglycerides because the higher exposition time to lipases in plasma. However, the major neutral lipids, as well as, total plasma lipids showed theirs maximum peak of absorption at 3 hour followed by a decreased to initial concentration. The same tendency was showed by all the FAME, especially those with functional properties in human health.

References

Abia, R., J. S. Perona, Y. M. Pacheco, E. Montero, F. J. G. Muriana, and V. Ruiz-Gutiérrez. 1999. Postprandial Triacylglycerols from Dietary Virgin Olive Oil Are Selectively Cleared in Humans. Journal of Nutrition 129(12):2184-2191.

Agren, J. J., M. Hallikainen, H. Vidgren, T. A. Miettinen, and H. Gylling. 2006. Postprandial lipemic response and lipoprotein composition in subjects with low or high cholesterol absorption efficiency. Clinica Chimica acta; International Journal of Clinical Chemistry 366(1-2):309-315.

Castro-Gomez, M. P., L. M. Rodriguez-Alcala, M. V. Calvo, J. Romero, J. A. Mendiola, E. Ibanez, and J. Fontecha. 2014. Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques. Journal of Dairy Science 97(11):6719-6728.

Castro-Gómez, P., J. Fontecha, and L. M. Rodríguez-Alcalá. 2014. A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples. Talanta 128(0):518-523.

Castro, G. R. and C. J. Fielding. 1985. Effects of postprandial lipemia on plasma cholesterol metabolism. The Journal of Clinical Investigation 75(3):874-882.

Cohn, J., A. Kamili, E. Wat, R. W. Chung, and S. Tandy. 2010. Dietary Phospholipids and Intestinal Cholesterol Absorption. Nutrients 2(2):116-127.

Cohn, J. S., J. R. McNamara, S. D. Cohn, J. M. Ordovas, and E. J. Schaefer. 1988. Postprandial plasma lipoprotein changes in human subjects of different ages. Journal of Lipid Research 29(4):469-479.

Di Marzo, V., M. Griinari, G. Carta, E. Murru, A. Ligresti, L. Cordeddu, E. Giordano, T. Bisogno, M. Collu, and B. Batetta. 2010. Dietary krill oil increases docosahexaenoic acid and reduces 2-arachidonoylglycerol but not N-acylethanolamine levels in the brain of obese Zucker rats. International Dairy Journal 20(4):231 - 235.

Dougherty, R. M., C. Galli, A. Ferro-Luzzi, and J. M. Iacono. 1987. Lipid and phospholipid fatty acid composition of plasma, red blood cells, and platelets and how they are affected by dietary lipids: a study of normal subjects from Italy, Finland, and the USA. American Journal of Clinical Nutrition 45(2):443-455.

El-Loly, M. M. 2011. Composition, properties and nutritional aspects of milk fat globule membrane - a Review. Polish Journal of Food and Nutrition Science 61(1):7-32.

Fontecha, J., L. M. Rodriguez-Alcala, M. V. Calvo, and M. Juarez. 2011. Bioactive Milk Lipids. Current Nutrition & Food Science 7(3):155-159.

Goltz, S. R., W. W. Campbell, C. Chitchumroonchokchai, M. L. Failla, and M. G. Ferruzzi. 2012. Meal triacylglycerol profile modulates postprandial absorption of carotenoids in humans. Molecular Nutrition & Food Research 56(6):866-877.

Jans, A., E. Konings, G. H. Goossens, F. G. Bouwman, C. C. Moors, M. V. Boekschoten, L. A. Afman, M. Müller, E. C. Mariman, and E. E. Blaak. 2012. PUFAs acutely affect triacylglycerol-derived skeletal muscle fatty acid uptake and increase postprandial insulin sensitivity. American Journal of Clinical Nutrition 95(4):825-836.

Koutsari, C., A. Zagana, I. Tzoras, L. S. Sidossis, and A. L. Matalas. 2004. Gender influence on plasma triacylglycerol response to meals with different monounsaturated and saturated fatty acid content. European Journal of Clinical Nutrition 58(3):495-502.

Kullenberg, D., L. A. Taylor, M. Schneider, and U. Massing. 2012. Health effects of dietary phospholipids. Lipids in Health and Disease 11:3.

Malpuech-Brugere, C., R. P. Mensink, O. Loreau, A. Maret, C. E. Fernie, T. S. Lassel, J. M. Chardigny, C. M. Scrimgeour, J. L. Sebedio, and B. Beaufrere. 2010. Comparison of postprandial oleic acid, 9c,11t CLA and 10t,12c CLA oxidation in healthy moderately overweight subjects. Lipids 45(11):1047-1051.

Michalski, M.-C. 2009. Specific molecular and colloidal structures of milk fat affecting lipolysis, absorption and postprandial lipemia. European Journal of Lipid Science and Technology 111(5):413-431.

Nilsson, A. and R. D. Duan. 2006. Absorption and lipoprotein transport of sphingomyelin. Journal of Lipid Research 47(1):154-171.

O'Keeffe, M. and M. P. St-Onge. 2013. Saturated Fat and Cardiovascular Disease: A Review of Current Evidence. Current Cardiovascular Risk Reports 7(2):154-162.

Olefsky, J. M., P. Crapo, and G. M. Reaven. 1976. Postprandial plasma triglyceride and cholesterol responses to a low-fat meal. American Journal of Clinical Nutrition 29(5):535-539.

Ravnskov, U. 2014. Lack of evidence that saturated fat causes cardiovascular disease. Vol. 348.

Rombaut, R. and K. Dewettinck. 2006. Properties, analysis and purification of milk polar lipids. International Dairy Journal 16(11):1362-1373.

Roseboom, T. J., J. H. van der Meulen, C. Osmond, D. J. Barker, A. C. Ravelli, and O. P. Bleker. 2000. Plasma lipid profiles in adults after prenatal exposure to the Dutch famine. American Journal of Clinical Nutrition 72(5):1101-1106.

Simons, K. and W. L. C. Vaz. 2004. MODEL SYSTEMS, LIPID RAFTS, AND CELL MEMBRANES1. Annual Review of Biophysics and Biomolecular Structure 33(1):269-295.

Simonsson, P., A. Nilsson, and B. Akesson. 1982. Postprandial effects of dietary phosphatidylcholine on plasma lipoproteins in man American Journal of Clinical Nutrition 35(1):36-41.

Sutherland, W. H., S. A. de Jong, and R. J. Walker. 2007. Effect of dietary cholesterol and fat on cell cholesterol transfer to postprandial plasma in hyperlipidemic men. Lipids 42(10):901-911.

Tall, A. R., C. B. Blum, and S. M. Grundy. 1983. Incorporation of radioactive phospholipid into subclasses of high-density lipoproteins. The American Journal of Physiology 244(5):E513-516.

Tholstrup, T., B. Sandström, A. Bysted, and G. Hølmer. 2001. Effect of 6 dietary fatty acids on the postprandial lipid profile, plasma fatty acids, lipoprotein lipase, and cholesterol ester transfer activities in healthy young men. American Journal of Clinical Nutrition 73(2):198-208.

Tholstrup, T., B. Sandström, J. Hermansen, and G. Hølmer. 1998. Effect of modified dairy fat on postprandial and fasting plasma lipids and lipoproteins in healthy young men. Lipids 33(1):11-21.

Torronen, R., M. Kolehmainen, E. Sarkkinen, H. Mykkanen, and L. Niskanen. 2012. Postprandial glucose, insulin, and free fatty acid responses to sucrose consumed with blackcurrants and lingonberries in healthy women. American Journal of Clinical Nutrition 96(3):527-533.

Vilarrasa, E., A. Tres, L. Bayes-Garcia, T. Parella, E. Esteve-Garcia, and A. C. Barroeta. 2014. Re-esterified palm oils, compared to native palm oil, do not alter fat absorption, postprandial lipemia or growth performance in broiler chicks. Lipids 49(8):795-805.

Vogelberg, K. H. and F. A. Gries. 1970. Tri-, Di- und Monoglyceride im menschlichen Serum und ihre Beziehung zum Lipoproteidspektrum. Klin Wochenschr 48(4):227-231.

Vors, C., G. Pineau, L. Gabert, J. Drai, C. Louche-Pelissier, C. Defoort, D. Lairon, M. Desage, S. Danthine, S. Lambert-Porcheron, H. Vidal, M. Laville, and M. C. Michalski. 2013. Modulating absorption and postprandial handling of dietary fatty acids by structuring fat in the meal: a randomized crossover clinical trial. American Journal of Clinical Nutrition 97(1):23-36.

Wolever, T. M. and C. Mehling. 2003. Long-term effect of varying the source or amount of dietary carbohydrate on postprandial plasma glucose, insulin, triacylglycerol, and free fatty acid concentrations in subjects with impaired glucose tolerance American Journal of Clinical Nutrition. 77(3):612-621.

Table 1. Age, weight, height and BMI data of subjects (*n* = 12)

	Age (years)	Weight (kg)	Height (cm)	BMI
Woman	65	63.5	158	25
	52	70.5	168	24
	57	52.2	161	20
	59	56.6	168	20
	60	59.2	169	20
	62	64.6	160	25
	62	63.6	162	24
Men	66	78.5	171	26
	55	67.5	168	23
	59	84.5	180	26
	62	75.5	175	24
	64	77	178	24

BMI: body mass index

Table 2. Mean values and statistical deviation of lipid classes analysis including phospholipid content determined by HPLC-ELSD of the skim milk enriched with buttermilk.

Lipid class	**(g/100g of fat)**
CE	<0.1
TAG	84.00 ± 3.26
DAG	2.64 ± 2.45
CHOL+FFA	0.65 ± 0.54
MAG	<0.1
GLUCER	<0.1
LACCER	<0.1
PL	12.51 ± 1.54
% of PL	
PE	24.1 ± 1.48
PI	8.0 ± 0.56
PS	19.9 ± 1.24
PC	34.4 ± 0.51
SM	13.7 ± 0.45

CE: cholesterol esters; TAG: triacylglycerols; DAG: diacylglycerols; CHOL: cholesterol; MAG: monoglycerols; GLUCER: glucosylceramides; LACCER: lactosylceramides; PL: polar lipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin. (Data is for triplicate).

Table 3. Mean values of FAME composition of the skim milk enriched with buttermilk determined by GC-MS.

FAME	(g/100g ± SD)		
C6:0	0.91	±	0.06
C8:0	0.92	±	0.01
C10:0	2.34	±	0.08
C12:0	3.10	±	0.07
C14:0i	0.09	±	0.02
C14:0	11.68	±	0.38
C15:0ai	0.18	±	0.01
C14:1	0.66	±	0.08
C15:0i	0.39	±	0.02
C15:0	1.15	±	0.05
C16:0i	0.30	±	0.06
C16:0	39.43	±	1.17
C16:1	1.43	±	0.15
C17:0i	0.34	±	0.03
C17:0	0.40	±	0.05
C17:1	0.12	±	0.03
C18:0	11.93	±	0.43
C18:t11	0.72	±	0.03
C18:1c9	22.53	±	0.70
C18:1c11	0.30	±	0.01
C18:2	1.55	±	0.27
C18:3	0.21	±	0.03
C18:2c9t11	0.27	±	0.06
C20:2	0.20	±	0.06
C20:3	0.26	±	0.07
C20:4	0.36	±	0.14
Σ SFA	71.86	±	0.17
Σ MUFA	25.56	±	0.24
Σ PUFA	2.60	±	0.10

ai:anteiso; i:iso; c:cis; t:trans; ΣSFA:Total saturated fatty acids; ΣMUFA:Total monounsaturated fatty acids; ΣPUFA: Total polyunsaturated fatty acids. The fatty acid content are expressed mean values ± SD (n=12).

Table 4. Mean values of the human plasma FAME evolution over time (mg/g fat) after the intake of the skim milk enriched with buttermilk determined by GC-MS (n=12).

FAME	0h	1h	2h	3h	4h	5h
C12	0.56 ± 0.27	0.60 ± 0.29	0.70 ± 0.42	1.04 ± 1.25	0.54 ± 0.17	0.54 ± 0.34
C14	1.11 ± 0.43	1.20 ± 0.42	1.51 ± 1.00	2.39 ± 2.85	1.12 ± 0.49	1.07 ± 0.39
C14:1	0.09 ± 0.04	0.10 ± 0.05	0.11 ± 0.08	0.10 ± 0.06	0.08 ± 0.02	0.07 ± 0.04
C15	0.23 ± 0.11	0.30 ± 0.11	0.28 ± 0.13	0.52 ± 0.65	0.26 ± 0.08	0.34 ± 0.17
C16 DMA	0.91 ± 0.43	0.89 ± 0.55	0.87 ± 0.43	0.95 ± 0.48	0.66 ± 0.33	0.61 ± 0.39
C16i	0.07 ± 0.03	0.09 ± 0.07	0.07 ± 0.03	0.09 ± 0.11	0.06 ± 0.01	0.05 ± 0.02
C16	55.24 ± 14.51	57.96 ± 14.75	65.37 ± 16.41	88.62 ± 34.66	59.47 ± 23.07	56.79 ± 10.27
C17ai	0.10 ± 0.05	0.13 ± 0.05	0.15 ± 0.09	0.19 ± 0.15	0.12 ± 0.03	0.12 ± 0.04
C17i	0.18 ± 0.05	0.19 ± 0.05	0.20 ± 0.08	0.24 ± 0.09	0.17 ± 0.07	0.17 ± 0.02
C16:1	0.64 ± 0.28	0.60 ± 0.27	0.67 ± 0.27	0.91 ± 0.51	0.67 ± 0.50	0.53 ± 0.15
C17	0.27 ± 0.08	0.28 ± 0.09	0.33 ± 0.11	0.43 ± 0.29	0.28 ± 0.10	0.26 ± 0.09
C18 DMA	0.62 ± 0.28	0.62 ± 0.28	0.69 ± 0.30	0.80 ± 0.57	0.47 ± 0.29	0.54 ± 0.33
C18:1 DMA	0.18 ± 0.07	0.19 ± 0.08	0.15 ± 0.07	0.20 ± 0.09	0.13 ± 0.06	0.14 ± 0.05
C18	30.53 ± 6.64	31.38 ± 8.62	35.94 ± 6.74	48.82 ± 18.34	32.87 ± 9.28	30.50 ± 6.90
C18:1 c9	40.23 ± 13.56	41.49 ± 17.45	46.34 ± 14.39	63.04 ± 16.23	46.37 ± 14.30	43.36 ± 5.82
C18:1 c11	0.48 ± 0.16	0.55 ± 0.19	0.53 ± 0.22	0.69 ± 0.24	0.45 ± 0.18	0.52 ± 0.13
C18:2 c9,c12	70.52 ± 17.28	75.57 ± 30.43	76.02 ± 28.37	90.07 ± 17.40	70.51 ± 9.07	66.69 ± 6.89
C20	0.12 ± 0.04	0.16 ± 0.06	0.15 ± 0.06	0.22 ± 0.18	0.11 ± 0.03	0.13 ± 0.05
γ C18:3	0.20 ± 0.06	0.22 ± 0.05	0.23 ± 0.11	0.31 ± 0.17	0.20 ± 0.11	0.18 ± 0.04
α C18:3	0.16 ± 0.03	0.17 ± 0.05	0.18 ± 0.04	0.19 ± 0.06	0.15 ± 0.03	0.14 ± 0.02
C20:2	0.20 ± 0.08	0.17 ± 0.06	0.23 ± 0.09	0.22 ± 0.14	0.19 ± 0.10	0.17 ± 0.08
C20:3 n3	0.41 ± 0.11	0.42 ± 0.11	0.42 ± 0.12	0.56 ± 0.14	0.38 ± 0.13	0.31 ± 0.01
C20:4	12.96 ± 1.51	14.67 ± 3.84	15.40 ± 2.51	18.71 ± 0.54	16.15 ± 2.13	12.73 ± 6.92
EPA	0.24 ± 0.14	0.25 ± 0.14	0.23 ± 0.15	0.30 ± 0.13	0.15 ± 0.04	0.20 ± 0.14
DPA	0.11 ± 0.02	0.15 ± 0.06	0.11 ± 0.03	0.15 ± 0.05	0.08 ± 0.03	0.10 ± 0.02
DHA	0.48 ± 0.17	0.50 ± 0.20	0.46 ± 0.15	0.59 ± 0.22	0.47 ± 0.07	0.45 ± 0.18
Σ SFA	89.96 ± 18.32	93.80 ± 18.75	106.25 ± 16.43	144.32 ± 28.79	96.12 ± 11.64	91.12 ± 15.41
Σ MUFA	41.62 ± 14.04	42.92 ± 10.06	47.80 ± 11.88	64.94 ± 11.03	47.70 ± 19.51	44.61 ± 11.03
Σ PUFA	85.29 ± 13.26	92.10 ± 19.50	93.29 ± 26.45	111.11 ± 16.33	88.29 ± 9.89	80.95 ± 17.13

DMA: dimethyl acetal; ai: anteiso; i: iso;; c: cis double bond; t: trans double bond; n3: omega3 fatty acid; EPA: eicosapentanoicacid; DPA: docosapentanoicacid; DHA: docosahexanoic. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Figure 1. Evolution over time of the total lipids (mg/mL) in human plasma after the intake of the skim milk enriched with buttermilk (n=12).

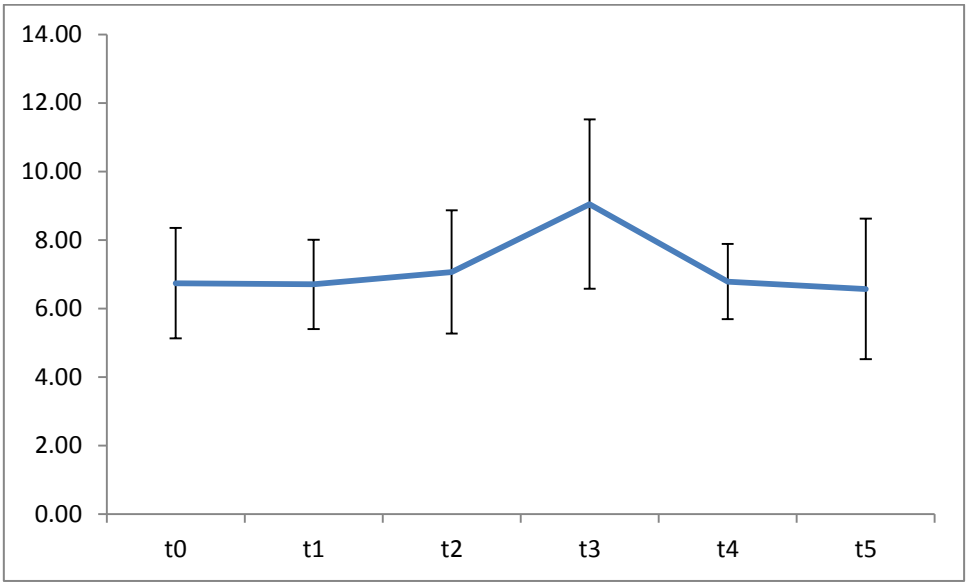
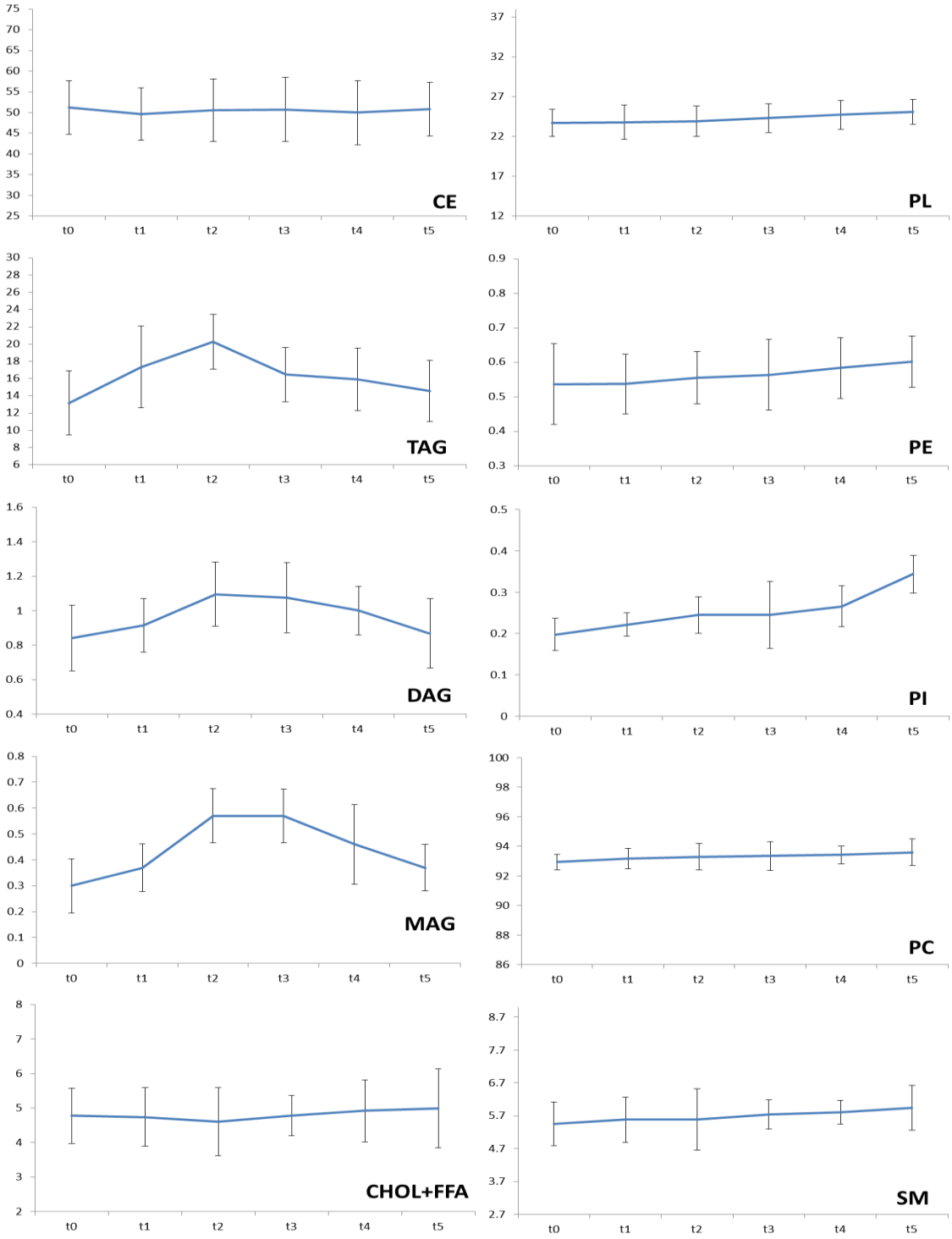


Figure 2. Evolution over time observed in the different lipids classes (g/100g fat) as well as phospho- and sphingolipids (g/100g PL) in human plasma after the intake of the skim milk enriched with buttermilk determined by HPLC-ELSD (n=12).



CE: cholesterol esters; TAG: triacylglycerols; DAG: diacylglycerols; CHOL: cholesterol; MAG: monoglycerols; PL: polar lipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin

4. DISCUSIÓN GENERAL

La consecución de la presente Tesis Doctoral, cuyo objetivo general es el desarrollo de estrategias que mejoren el valor nutricional de la mazada mediante el enriquecimiento en lípidos polares de la MGFM (fuente de fosfo- y esfingolípidos) para su empleo como ingrediente bioactivo, ha dado lugar a una serie de resultados que se discuten a continuación de acuerdo a los 3 objetivos parciales propuestos.

1. Desarrollo, optimización y validación de métodos analíticos y técnicas cromatográficas para el aislamiento de fracciones ricas en fosfo- y esfingolípidos y su posterior caracterización.

La obtención de extractos y fracciones lipídicas para su uso como ingredientes funcionales o nutraceúticos, hacen necesario el desarrollo de diversas metodologías y técnicas que permitan extraer, fraccionar y analizar su composición en profundidad.

1.1. Extracción lipídica completa de leche mediante líquidos presurizados.

Los resultados discutidos en este apartado forman parte de la publicación "Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques", Journal of Dairy Science, 97 (2014) 6719-6728 (Páginas 117-126).

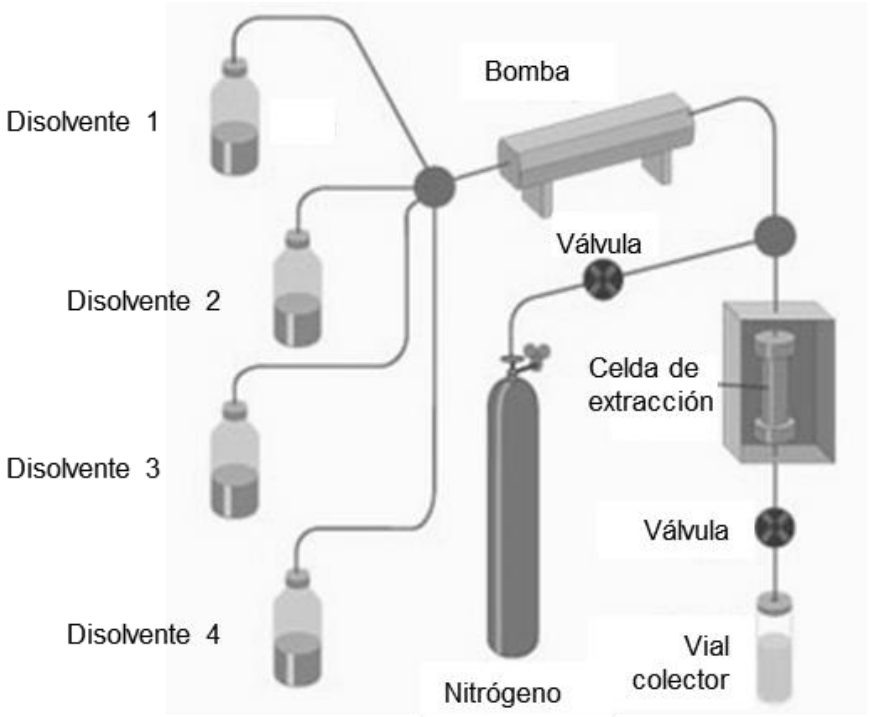
La grasa láctea es una matriz compleja que contiene una gran variedad de FA y clases lipídicas que hacen necesaria la utilización de técnicas específicas que permitan la caracterización exhaustiva de su composición. La grasa de la leche está compuesta mayoritariamente por TAG (~98%), sin embargo existen otros compuestos que aunque presentes en menor concentración, como los PL (<1%), poseen gran importancia desde el punto de vista de la salud. Para el estudio de todos ellos resulta crítica la elección de un método de extracción que no sea selectivo, de forma que extraiga con la misma eficacia todos los componentes lipídicos.

El empleo de la mezcla de cloroformo/metanol (2:1) (en proporción 20:1 con la muestra), es la técnica más efectiva para la extracción de PL en matrices complejas (Folch *et al.*, 1957), en comparación con otros métodos ampliamente utilizados, e incluso con el método oficial Rose-Gottlieb para la extracción de lípidos lácteos (ISO, 2001). Iverson *et al*. (2001) , ensayaron con éxito una modificación del método de Folch, de gran importancia ya que permite el mismo rendimiento para muestras de bajo contenido graso (<3%) reduciendo así la proporción de disolventes a 7,5:1. Cequier-Sánchez *et al*. (2008), evaluaron la sustitución del cloroformo por diclorometano, minimizando de esta forma la toxicidad de las mezclas de disolventes empleadas sin afectar el proceso de extracción. Los métodos que usan mezclas de cloroformo o diclorometano y metanol, resultan altamente eficientes en la extracción de los lípidos polares, sin embargo llevan implícito el procesado de una cantidad pequeña de muestra, largos tiempos de espera y un consumo excesivo de disolventes.

Para solventar tales limitaciones, en la presente Tesis se ha desarrollado un método de extracción con líquidos presurizados (PLE) que utiliza una mezcla de diclorometano y metanol en proporción 2:1.

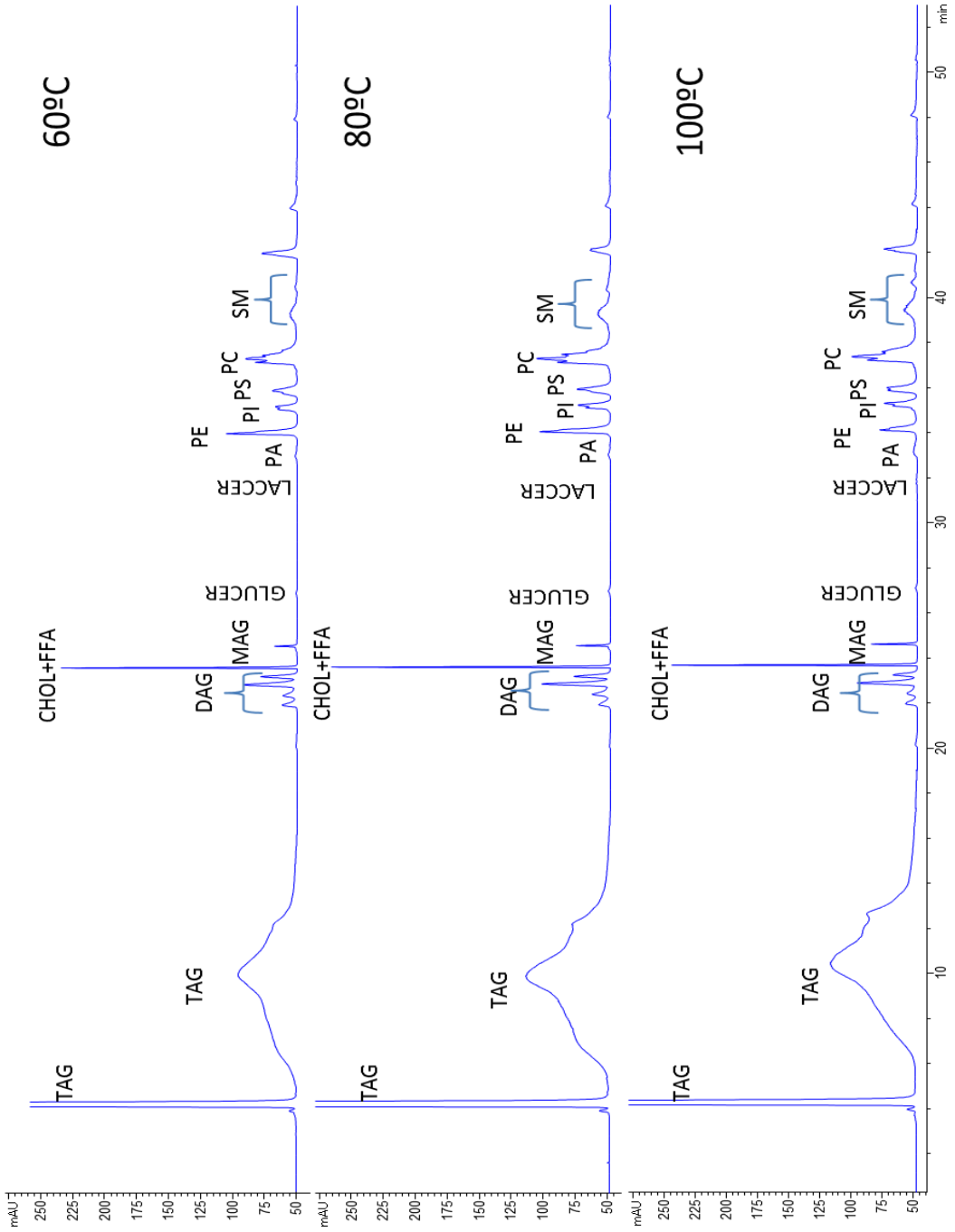
El PLE es una técnica de aislamiento aplicada en muestras sólidas y semisólidas y que consigue incrementar la eficiencia del proceso de extracción, mediante la utilización de disolventes bajo unas condiciones de temperatura y presión elevadas. Por otro lado, este método permite controlar parámetros como el tiempo de duración de cada ciclo o el número de ciclos. La combinación de todos estos factores conduce a una reducción significativa tanto de los tiempos de extracción como del consumo de disolventes. En la Figura 6 se muestra de forma esquemática el funcionamiento del equipo. Una vez cargada la muestra en la celda de extracción, la bomba introduce el/los disolvente/s a la presión que se genera con nitrógeno. Dentro de la celda, la mezcla de disolventes con la muestra es calentada y se mantiene en esas condiciones el tiempo estipulado para la extracción. Una vez finalizado el primer ciclo, el disolvente es desalojado de la celda hacia el vial colector del extracto. Finalmente, el proceso será repetido tantas veces como ciclos se hayan fijado.

Figura 6. Representación esquemática del funcionamiento de PLE.



Para optimizar las condiciones de extracción con PLE (ASE-200, Dionex Corp., Sunnyvale, CA) se empleó una leche en polvo comercial, con un contenido graso máximo del 1%. El rendimiento de extracción se evaluó mediante la adición de una cantidad conocida de grasa anhidra. La presión de trabajo se fijó en 10.3 MPa, basándonos en pruebas previas, ya que no era un factor crítico en el rendimiento de extracción. Se ensayaron distintas condiciones experimentales, aplicando 1, 2 o 3 ciclos de 5 minutos cada uno y variando la temperatura (60º, 80º y 100ºC). Aunque el rendimiento de extracción a las tres temperaturas ensayadas fue cercano al 100%, las condiciones óptimas elegidas usando diclorometano:metanol (2:1) fueron: 60ºC y 2 ciclos de 5 minutos cada uno. La elección de dichas condiciones, se debe al hecho de que, como se observa en la Figura 7, la utilización de temperaturas más elevadas provocaba una reacción de Maillard y un aumento de los niveles de PA como compuesto de degradación (Tabla 2, página 121). Además, el empleo de un mayor número de ciclos o de ciclos de mayor duración no aumentaba el rendimiento del proceso.

Figura 7. Perfil de clases lipídicas de leche desnatada en polvo analizado por HPLC-ELSD. Las condiciones empleadas en la extracción por PLE y fueron: 60º, 80º y 100ºC, 2 ciclos de 5 minutos cada uno.



TAG: triacilglicéridos, DAG :diacilglicéridos, CHOL: cholesterol, MAG : monoacilgliceridos, GLUCER: glocuceramidas, LACCER : lactoceramidas, PL: lípidos polares, PA: ácido fosfatídico, PE: fosfatidiletanolamina, PI: fosfatidilinosiltol, PS: fosfatidilserina, PC: fosfatidilcolina, SM: esfingomielina

Una vez optimizado, el método PLE fue aplicado en muestras de leche de vaca, oveja y cabra. Los resultados fueron comparados con los obtenidos por el método oficial Rose-Gottlieb (ISO, 2001) y por el método Folch (Folch *et al.*, 1957) modificado por Iverson et al.(2001) y que aparece designado como FM a lo largo del texto. La determinación cuantitativa del contenido de grasa de cada una de las leches, se realizó en el Laboratorio Interprofesional Lácteo de Castilla la Mancha (LILCAM) mediante espectroscopía de infrarrojo (IR) utilizando un equipo MilkoScan (Foss Electric Espana S.A., Barcelona,Spain). Esta comparación mostró que el rendimiento de extracción de grasa obtenido con el método PLE propuesto y con el ISO fue similar al detectado por IR. Sin embargo, el método FM extrajo una cantidad significativamente menor de grasa en las tres leches (Figura 1, página 121). Este hecho también fue observado por otros autores al comparar rendimientos de extracción de grasa en muestras alimentarias, empleando varios métodos (Boselli et al., 2001; Mulbry et al., 2009).

Para conocer la composición en clases lipídicas de las leches, se optimizó un método de HPLC-ELSD previamente puesto a punto en nuestro laboratorio (Rodríguez-Alcalá y Fontecha, 2010), que a su vez mejoraba el utilizado hasta ese momento (Rombaut *et al.*, 2005). La combinación de dos columnas de sílica de fase normal en serie, así como ligeras modificaciones en las fases móviles y en los gradientes del método (Tabla 1, página 120), permitieron obtener en un tiempo similar, una mejor separación de los fosfo- y esfingolípidos. Además, también se logró separar los picos de DAG, CHOL y FFA que con la metodología anterior coeluían.

Aplicando este análisis de clases lipídicas a las tres leches, se observó que mientras que los métodos PLE y FM extraían de forma similar los lípidos neutros y los PL, la extracción de fosfo- y esfingolípidos llevada a cabo mediante el método ISO era incompleta (Figura 2, página 123), siendo este resultado similar al encontrado por Avalli y Contarini (2005) en grasa de leche.

Por este motivo el método ISO fue definitivamente descartado y no se utilizó para el estudio de PL

La distribución de fosfo- y esfingolípidos determinada mediante un HPLC (model 1260; Agilent Technologies Inc.) acoplado a un detector ELSD (SEDEX 85 model; Sedere SAS, AlfortvilleCedex,France) puso de manifiesto diferencias

entre el método FM y el método PLE propuesto, ya que este último extrajo una cantidad mayor de PI y PS en todas las muestras (Tabla 3, página 122). Estos resultados, concuerdan con los obtenidos en otros trabajos llevados a cabo en diferentes alimentos y que comparaban métodos similares (Zhou et al., 2010; Cescut et al., 2011).

El análisis de FAME por GC-MS (modelo 6890N; Agilent Technologies Inc.) de los extractos lipídicos obtenidos utilizando los métodos PLE y FM no reveló diferencias significativas para ninguno de los FA (Tabla 4, página 122), lo cual estaría de acuerdo con los datos publicados en otros estudios, (Toschiet al.(2003), Zhouet al.(2010) y Cescutet al.(2011), que comparaban la composición de FA en grasas extraídas de alimentos por métodos similares.

De mismo modo, la determinación mediante GC-FID (Perkin Elmer Ltd, Beaconsfield, UK) de TAG (agrupados por CN) y CHOL, mostró que la distribución y los contenidos obtenidos eran similares independientemente de que muestras fueran extraídas con PLE o con FM (figura 3, página 124), siendo esta la primera vez que se publica esta información.

A la luz de estos resultados se concluyó que el método de extracción lipídica mediante PLE propuesto podría ser empleado como un método de rutina para la extracción de grasa láctea, no sólo porque consigue una extracción total y completa de todos los componentes de la grasa, sino porque también reduce el tiempo y el consumo disolventes. Además, este método permitiría su automatización, lo que representa una ventaja adicional para el análisis cuando el número de muestras es elevado.

1.2. Derivatización lipídica completa y directa de muestras biológicas y de alimentos.

Los resultados discutidos en este apartado forman parte de la publicación **"**A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples", Talanta, 128 (2014) 518-523 (Páginas 129-134).

Una vez desarrollados los métodos que permitían llevar a cabo la extracción completa y la caracterización de todas las clases lipídicas de una muestra grasa, se puso a punto un método de derivatización directa de todos los FA presentes para su posterior análisis por GC-MS. La derivatización directa ofrece la ventaja de poder llevarse a cabo sobre la muestra sin modificar, es decir, que no requiere la extracción previa de la grasa como ocurre en el método oficial (ISO, 2002).

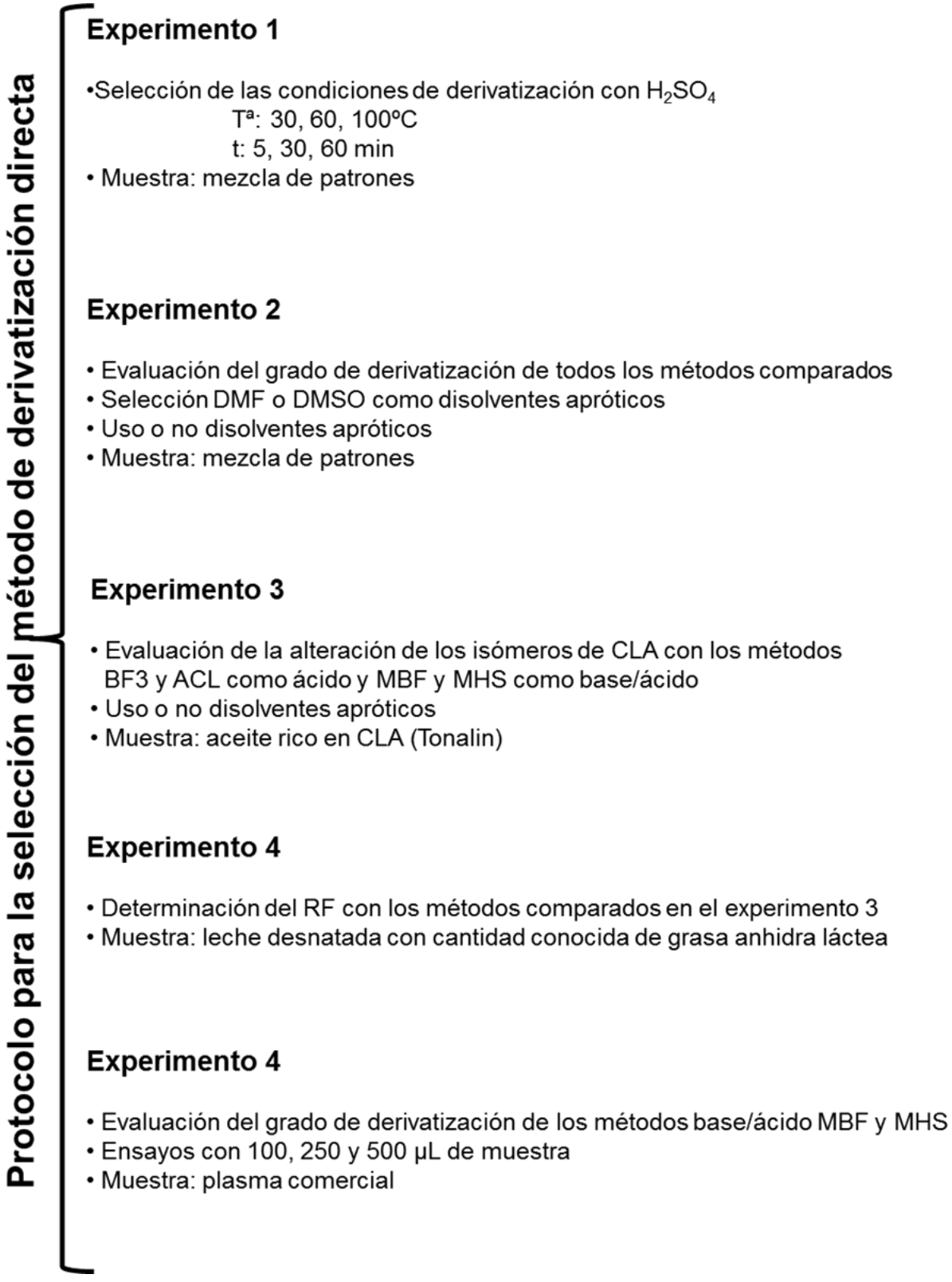
En los alimentos o en las muestras biológicas, los FA se pueden encontrar en forma libre o unidos a diferentes estructuras lipídicas como los glicerolípidos, fosfolípidos o esfingolípidos, los cuales, requieren de una metodología de derivatización específica que los haga más volátiles para así poder ser identificados y cuantificados por GC. Existen métodos de derivatización directa que usan ácidos en metanol como catalizadores (ácido sulfúrico (H_2SO_4), trifluoruro de boro (BF_3) o ácido clorhídrico (HCl)) combinados con calentamiento. Sin embargo, son reactivos inestables que dan lugar a artefactos o alteran la distribución de compuestos específicos, como el CLA, provocando errores en la cuantificación de la composición de la muestra. Para evitar esto, una posible solución es la adición de disolventes apróticos como dimetilformamida (DMF) y dimetilsulfoxido (DMSO) que previenen la interacción del alcohol (metanol) protonado con los dobles enlaces conjugados del CLA (Yamasaki *et al.*, 1999). Además, aunque estos métodos ácidos son idóneos para esterificar los FFA y transmetilar las SM, la transesterification de glicerolípidos y fosfolípidos debe ser catalizada por reactivos básicos como el hidróxido potásico (KOH) y el metóxido sódico (MetNa). Todas estas limitaciones han provocado que en muchos casos se hayan empleado métodos

que no aseguraban la derivatización completa de todos los componentes de la muestra.

Con el objetivo de disponer de un método que permitiera el análisis de todos los FAME, en la presente Tesis se ha desarrollado un método de derivatización total sin extracción previa que, combinando el uso de un catalizador básico y de uno ácido, permite el análisis de los FAME tanto de alimentos como de muestras biológicas.

Para llevar a cabo este estudio se compararon 5 métodos de derivatización extensamente utilizados en la bibliografía (KOH (ISO, 2002), metilato de sodio/BF_3 (MBF) (Bondia-Pons *et al.*, 2004), BF_3 (O'Fallon *et al.*, 2007), cloruro de acetilo en metanol (ACL) (O'Fallon *et al.*, 2007) y H_2SO_4 (Christie, 1990)) y dos métodos propuestos por nuestro laboratorio para GC-MS (MetNa/H_2SO_4 (MHS) e KOH/H_2SO_4 (KHS)). En el caso de los métodos que utilizaban ácido como catalizador, se evaluó la prevención de la alteración por procesos oxidativos o de la formación de artefactos mediante la adición de los disolventes apróticos DMF y DMSO. Para llevar a cabo la comparación se plantearon 5 experimentos utilizando como muestras: una mezcla conocida de glicerolípidos, FFA, CE y CHOL, un producto rico en isómeros de CLA, una leche desnatada con una cantidad conocida de grasa láctea anhidra patrón de referencia y también con una muestra patrón de suero sanguíneo comercial (Figura 8).

Figura 8. Esquema de los experimentos llevados a cabo para la selección del método de derivatización directa para muestras de alimentos y biológicas.



Experimento 1. Con el fin de elegir las condiciones óptimas de temperatura y tiempo de calentamiento cuando se empleaban catalizadores ácidos, la muestra de patrones se derivatizó con H_2SO_4 a las temperaturas de 30ºC, 60ºC y 100ºC, durante 5´, 30´ y 60´. Se concluyó que las mejores condiciones eran la aplicación de 60ºC durante 30 minutos, puesto que eran la temperatura y tiempo mínimos con los que se conseguía una mayor conversión de clase lipídica a FAME (Tabla 1, página 131). Bajo estas condiciones se logró una mayor derivatización de TAG, DAG, MAG, FFA y CE, una mayor formación de los FAME que contenían estas clases lipídicas y una mejor detección de CHOL total (suma del CHOL del CE y CHOL libre), sin que apareciesen diferencias significativas respecto a los ensayos en los que se aplicó mayor tiempo y temperatura.

Experimento 2. Para seleccionar el mejor método directo, se utilizó una muestra de patrones y se comparó el grado de derivatización obtenido con MBF, BF_3 y ACL frente a los métodos propuestos (MHS y KHS), utilizando el disolvente aprótico DMF como protector (el DMSO se desechó por su coelución con los FA en el cromatograma). También se evaluó la idoneidad de la derivatización básica de los dos métodos propuestos (MetNa y KOH). Los resultados mostraron que los métodos de MBF y el MHS eran los que permitían derivatizar la totalidad de la muestra, destacando el MHS, ya que únicamente con él, fue posible cuantificar el CHOL total (Tabla 2, página 132). Los métodos ácidos derivatizaron completamente los FFA, sin embargo, los glicerolípidos, sobre todo los TAG, tuvieron una baja conversión, como también observó Liu (1994) en el análisis de muestras biológicas. El método propuesto de KHS no consiguió la conversión completa de FAME con tanta eficacia como lo hicieron los otros métodos base/ácido, sobre todo con el CE. Como ya describió Christie. (1982), ésto se debe a que las bases hidroxi provocan la hidrólisis de los FA ya derivatizados (FAME). Además, este método tampoco permitió el cálculo del contenido de CHOL total. Estos resultados llevaron a descartar este método para la realización de los experimentos posteriores. Por otro lado, mientras que la catálisis básica con KOH consiguió derivatizar con éxito los glicerolípidos, la conversión incompleta de éstos obtenida con el método MetNa, sugirió una posible formación de gotas emulsionadas que podrían

interferir en la derivatización de la muestra. Sin embargo, la adición de DMF parece disminuir la tensión superficial y el tamaño de la gota emulsionada, evitando así la sobreestimación de algunos FAME que ocurría cuando no se añadía (Tabla 2, página 132).

Experimento 3. Se llevó a cabo la evaluación de la posible alteración de los isómeros de CLA con los métodos ácido/base (MBF y MHS), BF_3 y ACL, así como el uso o no de DMF en un aceite rico en CLA (Tonalin®). Como se observa en la Tabla 3 (página 132), el método de ACL fue el que mostró mayores diferencias en cuanto a los FA respecto al método KOH (usado como método de referencia en este experimento), siendo además algunos de sus valores sobreestimados cuando eran derivatizados en presencia de DMF. La formación de artefactos se relacionó con la aparición de isómeros *trans-trans* del C18:2 derivados de la ausencia de DMF, viéndose afectados los isómeros mayoritarios que se encuentran presentes en la muestra (*cis*9-*trans*11, *trans*10-*cis*12), lo que concuerda con lo descrito en trabajos anteriores (Yamasaki *et al.*, 1999). Los métodos de ACL y de BF_3 fueron los que produjeron más artefactos y un aumento de los isómeros *trans-trans* cuando no se utilizó DMF. Sin embargo los métodos que combinaron base-ácido fueron los que dieron lugar a menores modificaciones. El método propuesto MHS, fue el que provocó menos alteraciones en el perfil de isómeros de CLA, sobre todo cuando se añadía DMF durante la derivatización de aceite de Tonalin®.

Experimento 4. Por otro lado, se evaluó entre los métodos comparados en el experimento anterior, cuál era el mejor factor de respuesta (RF) para una leche desnatada con una cantidad conocida de grasa láctea anhidra patrón de referencia. El RF también es válido para comparar la eficacia con la que un método derivatiza los FA de una muestra ya que valores de RF cercanos a 1 implicarían una eficiente derivatización de cada uno los FA. Los mejores RF, se obtuvieron con el método propuesto MHS, hallándose próximos a 1 para todos los FA, excepto para los SCFA (RF 1.5-3) debido a su elevada volatilidad durante el proceso. El RF obtenido con el método de ACL se hallaba por debajo de 0.5, mientras que los otros dos métodos (BF_3 y MBF) presentaron un RF > 1.

Experimento 5. Por último, se llevó a cabo la evaluación de la derivatización completa de los métodos que no alteraban el perfil de CLA y presentaban mejores RF (MHS y MBF) en diferentes cantidades (100 µL, 200 µL y 500 µL) de una muestra de plasma de referencia comercial. Los resultados mostraron que no existían grandes diferencias entre la derivatización total de ambos métodos cuando se trataba volúmenes de 250 µL y 500 µL. Sin embargo, cuando se derivatizaron 100µL de muestra, el método de MBF no permitió la detección de alguno de los FA que sí se determinaron en cambio al usar MHS (Tabla 4, página 133).

Con estos resultados se concluyó que el método directo propuesto de MHS combinado con DMF es la mejor alternativa para el análisis total de FA, tanto de muestras biológicas como de alimentos, por llevar a cabo una derivatización total, obtener buenos RF, no alterar el perfil de ningún FA (incluidos los isómeros de CLA) y por ser el único que permite la cuantificación del CHOL total.

1.3. Desarrollo de procedimientos cromatográficos para la caracterización exhaustiva de distintas fracciones de lípidos alimentarios

Los resultados discutidos en este apartado forman parte de la publicación **"**Comprehensive study of the lipid classes of krill oil by fractionation and identification of triacylglycerols, diacylglycerols and phospholipid molecular species by using UPLC-QTOF-MS", Food Analitical Methods, 2015, DOI 10.1007/s12161-015-0150-6 (Páginas 137-149).

Se ha abordado el desarrollo de un procedimiento basado en la combinación de distintos métodos y técnicas cromatográficas avanzadas, que nos permita obtener una caracterización exhaustiva de los constituyentes lípídicos presentes en alimentos y grasas o aceites. Se consideró como estrategia inicial la obtención de extractos o fracciones lipídicas diferenciadas por su grado de polaridad (neutras y polares) para su posterior análisis a nivel molecular y

biológico. El fin último del estudio era la posible aplicación de estas fracciones aisladas como potenciales ingredientes bioactivos en alimentos funcionales.

Con el objetivo de desarrollar un método de fraccionamiento de un aceite alimentario, se usó el KO, procedente de un crustáceo rico en PL y que se caracteriza por tener un elevado contenido de los LCPUFA n-3, EPA y DHA, esterificados tanto en los PL como en los TAG. Esta distribución de los LCPUFA se considera de gran relevancia ya que se ha demostrado que éstos son mejor absorbidos cuando se encuentran esterificados al PL (Ulven *et al.*, 2011), a diferencia de lo que ocurre en el aceite de pescado donde se hallan esterificados a TAG. Los potenciales efectos beneficiosos sobre la salud (cáncer, CVD, síndrome metabólico, inflamación, etc.) (Tandy *et al.*, 2009; Berge *et al.*, 2014) atribuidos al KO, han hecho que este aceite, y especialmente su fracción de PL-LCPUFA n-3, hayan sido considerados como ingredientes funcionales.

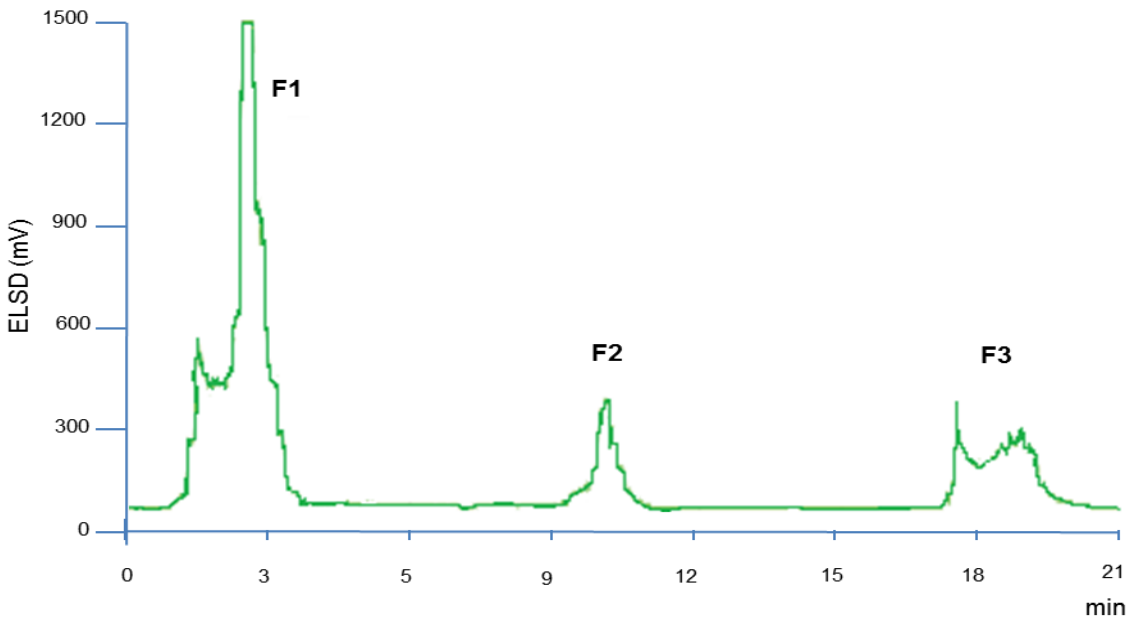
En la presente Tesis se ha abordado un estudio en profundidad para la caracterización del KO, estableciéndose para ello un convenio de colaboración con la empresa AKO3TM (Aker Bio Marine Antarctic AS, Oslo, Norway) y con el Centro para el Desarrollo de la Biotecnología (CDB, CSIC) en Valladolid. Se ha llevado a cabo el aislamiento de fracciones, con especial interés en la fracción polar de PL, para su posterior caracterización mediante la combinación de técnicas cromatográficas avanzadas (LC flash, GC-FID, HPLC-ELSD y UPLC-QToF-MS).

Una caracterización inicial en clases lipídicas del KO, mediante HPLC-ELSD, mostró que estaba mayoritariamente compuesto por TAG (~44%) y por PL (~49%), especialmente PC (Tabla 1, página 141), lo que se corresponde con la información aportada por el fabricante del aceite en su ficha de producto (<50% de TAG y >46% de PL) y con trabajos previamente publicados en los que se determinaron las clases lipídicas en diferentes KO (Phleger et al., 1998; Ju et al., 2009; Tandy et al., 2009; Ali-Nehari y Chun, 2011).

Posteriormente, se desarrolló un método de separación de fracciones lipídicas mediante LC flash-ELSD (Reveleris® Flash SystemChromatography, Grace, Deerfield, IL, USA), utilizando cartuchos de sílica (4 g, Grace Reveleris, Deerfield, USA) y disolventes de polaridad creciente (hexano, dietiléter y

metanol) que dio lugar al aislamiento de 3 fracciones (Figura 9). Los análisis por HPLC-ELSD posteriores determinaron que la F1 (~41% en peso) estaba compuesta principalmente por TAG, la F2 (~14% en peso) por lípidos neutros como DAG, MAG y otros como FFA+CHOL, mientras que última fracción F3 (~45% en peso) contenía únicamente los PL del KO, sobre todo PC (Figura 1 y Tabla 1, página 141). La caracterización de las fracciones aisladas también se llevó a cabo mediante análisis por GC-FID con una columna de TAG según el método descrito por Fontecha *et al*.(2006), detectándose los grupos de TAG en F1 y el CHOL en F2 (Figura 2, página 143).

Figura 9. Perfil cromatográfico obtenido por LC flash para la separación de 3 fracciones en aceite de krill



F: fracción

El análisis de FA del KO y sus fracciones aisladas (Tabla 2, página 142) mostró que más de la mitad de los SFA presentes en el aceite (~43% en KO) se localizaban en los TAG (F1), sobre todo el ácido mirístico (C14:0), resultado similar al descrito por Araujo *et al*. (2014). Este tipo de reparto también se produjo con los MUFA, destacando los ácidos palmitoleico (C16:1) y oleico (C18:1cis9). Sin embargo, los PUFA se repartieron mayoritariamente entre la

fracción de PL (F3) y en menor cantidad en la F2. Destacó el contenido de EPA y DHA presente en los PL, ya que más de la mitad de los LCPUFA detectados en el KO se pudieron identificar en la F3. Ali-Nehari and Chun. (2011), obtuvieron resultados similares tras extraer los PL con SFE.

Los análisis de la F1, realizados mediante UPLC-QToF-MS (ACQUITY UPLC®, Waters, Manchester, UK) mostraban que los TAG se repartieron entre los grupos TAG CN42 al TAG CN60, identificándose 65 especies moleculares siendo los TAG CN48 y TAGCN50 los más abundantes (Tabla 4, página 145 y 146). Estos datos fueron también contrastados mediante los análisis de GC-FID (Tabla 3, página 144). La combinación de los FA, C14:0, C16:0, C16:1 y C18:1 (mayoritarios en F1 de acuerdo con los resultados de FA) da lugar a los TAG (14:0/16:0/18:1), TAG (16:0/16:1/16:1), TAG (16:0/18:1/18:1) y TAG (16:0/16:0/18:1) como las especies moleculares mayoritarias. La ausencia de especies totalmente saturadas debido, en parte, a la presencia de LCPUFA n-3 en casi la mitad de las especies moleculares sugiere que estos FA, aunque especialmente concentrados en la fracción de PL, están extensamente repartidos por todos los TAG. Araujo et al.(2014), en un trabajo llevado a cabo con LC-MS^2, encontraron una distribución distinta de los TAG (entre el TAG CN28 y el TAG CN52), probablemente debido a que la composición del KO no era la misma.

Los análisis mediante UPLC-QToF mostraron que las 22 especies moleculares de DAG se repartieron en grupos entre DAG CN32 y DAG CN44. Al igual que ocurría con los TAG, no se encontró ninguna especie totalmente saturada, debido al elevado contenido de UFA, especialmente de EPA y DHA. Por ello, las especies moleculares con mayor contenido fueron los DAG (16:0/20:5), DAG (18:1/20:5), DAG (20:5/22:6) y sobre todo DAG (20:5/20:5) (Tabla 5, página 146). Es la primera vez que estos datos aparecen descritos en la bibliografía.

Los resultados de los análisis de PL realizados mediante esta metodología se muestran en la Tabla 6 (página 147). Mientras que en este estudio se detectaron 3 Liso-PC (16:0, 18:1 y 18:2), en estudios previos los resultados fueron dependientes tanto del tipo de muestra como de la técnica usada para determinarlos. Así Winther *et al* (2010) detectaron mediante LC-ESI-MS hasta 7 especies moleculares con liso-PC mientras que Le Grandois *et al.*(2009) en

muestras similares no observaron la presencia de ninguna liso-PC uitlizando LC-ESI-MS^2. El número de especies moleculares de PC observadas en este estudio fue de 28, todas ellas insaturadas, lo cual estaría de acuerdo con lo descrito en los dos trabajos antes citados. Sin embargo, en dichos estudios se detectaron 51 y 21 especies de PC, respectivamente. La mayor parte de las especies de PC contenían LCPUFA n-3, siendo PC (16:0/20:5) la especie más abundante alcanzando el 54% del total de PC. Este dato concuerda con lo publicado por Le Grandois*et al.* (2009), quienes identificaron dicha especie (con un 50%) entre las 3 más abundantes (junto a 16:0/22:6 y 16:0/18:1).
Por lo que se refiere a SM, mientras que en nuestro trabajo se identificaron 6 especies moleculares, Zhou *et al.*(2012) utilizando LC-ESI-MS^2 no detectaron la presencia de ninguna en KO.

Se puede concluir que en la presente Tesis se ha logrado desarrollar un método de fraccionamiento lipídico capaz de separar PL, TAG y el grupo constituido por DAG, MAG, FFA y CHOL, en KO, pudiendo ser válida su aplicación para otros aceites alimentarios. Por otro lado, se ha logrado describir exhaustivamente la composición del KO y sus fracciones, observando una localización preferente de los SFA en los TAG, así como un reparto similar de SFA y UFA en el conjunto de DAG, MAG y FFA. Sin embargo, los PL estaban compuestos mayoritariamente por PUFA, e incluian más de la mitad del EPA y del DHA presentes en el KO, los cuales se hallaban repartidos en la mayoría de las especies moleculares. Por todo ello, se puede proponer al KO, y en particular a la fracción de PL, como ingrediente funcional o nutracéutico con potenciales efectos beneficiosos para la salud.

2. Estudio de leche enriquecida en fosfo- y esfingolípidos, procedente de rumiantes con dietas suplementadas con aceites ricos en ácidos grasos poliinsaturados

Los resultados discutidos en este apartado forman parte de la publicación en preparación “Qualitative and quantitative analysis of phospholipids and

sphingolipids in ewes´ milk fat with enhanced nutritional quality through diet supplementation". (Páginas 155-178).

Durante las últimas décadas ha sido una constante de las distintas industrias lácteas, el desarrollo de productos con reducido contenido en grasa o con bajo contenido en SFA para adecuarse a las recomendaciones nutricionales. Aunque son varios los métodos utilizados para mejorar el perfil lipídico de la leche, la suplementación de la dieta de rumiantes con aceites ricos en PUFA ha sido uno de los más extensamente estudiados. Dicha suplementación provoca no sólo una disminución de los SFA, sino también un aumento de la concentración de los PUFA, incluyendo los esenciales como los PUFA n-3 y los funcionales como los isómeros de CLA (Gómez-Cortés *et al.*, 2008; Hervás *et al.*, 2008). Sin embargo, la información disponible en la bibliografía relativa a los posibles efectos que originan en la distribución de las diferentes clases lipídicas, sobre todo en los PL, así como las variaciones en la composición de los FA que los constituyen, es muy escasa. Únicamente en un estudio llevado a cabo en dos grupos de 6 vacas, cuya dieta estaba suplementada con semilla de lino (Lopez *et al.*, 2008), se determinaron los cambios producidos en la distribución de PL. No obstante, dicho estudio no profundiza en los cambios de la composición en FA de los PL. Esto resulta ser de gran importancia no sólo por las ventajas que supondría la obtención de una fracción enriquecida en fosfo- y esfingolípidos, sino porque la mejora del perfil con FA funcionales supondría, como se ha indicado previamente, una mejor biodisponibilidad de dichos FA (Ulven *et al.*, 2011).

Debido a todo lo descrito anteriormente, otro de los objetivos en la presente Tesis fue evaluar los cambios y posibles mejoras producidas en la distribución y concentración de las clases lipídicas de la grasa de la leche, sobre todo de los fosfo- y esfingolípidos, cuando la dieta del rumiante es suplementada con aceites ricos en PUFA.

En este estudio se comparó la composición lipídica de leche de ovejas (dos grupos de n=12) que habían recibido una dieta control y una dieta suplementada con aceite de girasol (Hervás *et al.*, 2008). En primer lugar, se comparó el perfil total de clases lipídicas obtenido mediante HPLC-ELSD y la

distribución de TAG y contenido de CHOL en ambas leches. Tras la separación de cada uno de los PL mediante varias TLC combinadas (Figura 2, página 178), se determinó el contenido en FA por GC-MS y de las especies moleculares mediante MALDI-ToF-MS (BrukerDaltonicGmbH, Bremen, Germany) de cada uno de los fosfo- y esfingolípidos individuales.

Los resultados de este estudio mostraron que el contenido graso de la leche suplementada (SppM) era superior al de la leche control (CtM) (Hervás *et al.*, 2008).

No se observaron diferencias en el contenido total de TAG de las leches estudiadas, determinado mediante HPLC-ELSD (Tabla 1, página 170), aunque sí se hallaron variaciones en cuanto a la distribución porcentual de los grupos de TAG determinados por GC-FID (Tabla 2, página 171). Así, el contenido de TAG de bajo peso molecular (con bajo CN) fue inferior en la leche SppM, debido a una menor presencia de FA de cadena corta y media (del C4:0 al C14:0), aunque esta diferencia fue compensada por un mayor porcentaje de TAG de alto peso molecular (alto CN) debido al aumento del contenido de FA con CN $\geq$ 18 en esta grasa (Hervás *et al.*, 2008). Asimismo, se detectó una disminución significativa en los niveles de DAG y CHOL en la leche SppM cuando ésta se analizó mediante HPLC-ELSD (Tabla 1, página 170). Barbano and Sherbon (1981) también observaron dicha disminución de DAG en leche de vacas cuya dieta había sido suplementada con semillas de girasol y soja, y justificaron este hallazgo por una mayor síntesis endógena de PL a través de los DAG. El menor contenido en CHOL encontrado en la muestra SppM, podría sugerir que la suplementación con aceite de girasol provoca una reducción de la síntesis endógena de CHOL en la leche de estos rumiantes. Aunque este dato podría ser relevante para la elaboración de productos lácteos funcionales, no se ha encontrado información al respecto. En las muestras de SppM, el análisis por HPLC-ELSD también puso de manifiesto aumentos en el contenido de PL, aunque éstos no fueron significativos. Estos datos están de acuerdo con lo descrito por López *et al.* (2008) en leche de vacas suplementadas con una dieta rica en PUFA. Estos autores relacionan este aumento en PL con la presencia de un mayor número de glóbulos grasos aunque de menor tamaño medio en las muestras de leche suplementada, lo que provocaría un aumento de la superficie total de membrana.

En las muestras estudiadas no se observaron diferencias en cuanto al contenido en PC (PL mayoritario), sin embargo la SM aumentó significativamente en la leche SppM llegando a alcanzar un contenido 3 veces superior al encontrado en la CtM. Dicho aumento se considera de gran interés debido a las funciones potencialmente beneficiosas que ejercen la SM y los esfingolípidos en general, sobre la salud humana. Entre ellas destacan sus efectos en el cáncer o en enfermedades de tipo cognitivo (Kuchta *et al.*, 2012; Kullenberg *et al.*, 2012; Contarini y Povolo, 2013). En el trabajo antes citado (Lopez *et al.*, 2008), se detectó igualmente un aumento del 30% en la fracción de SM de leche de vaca suplementada.

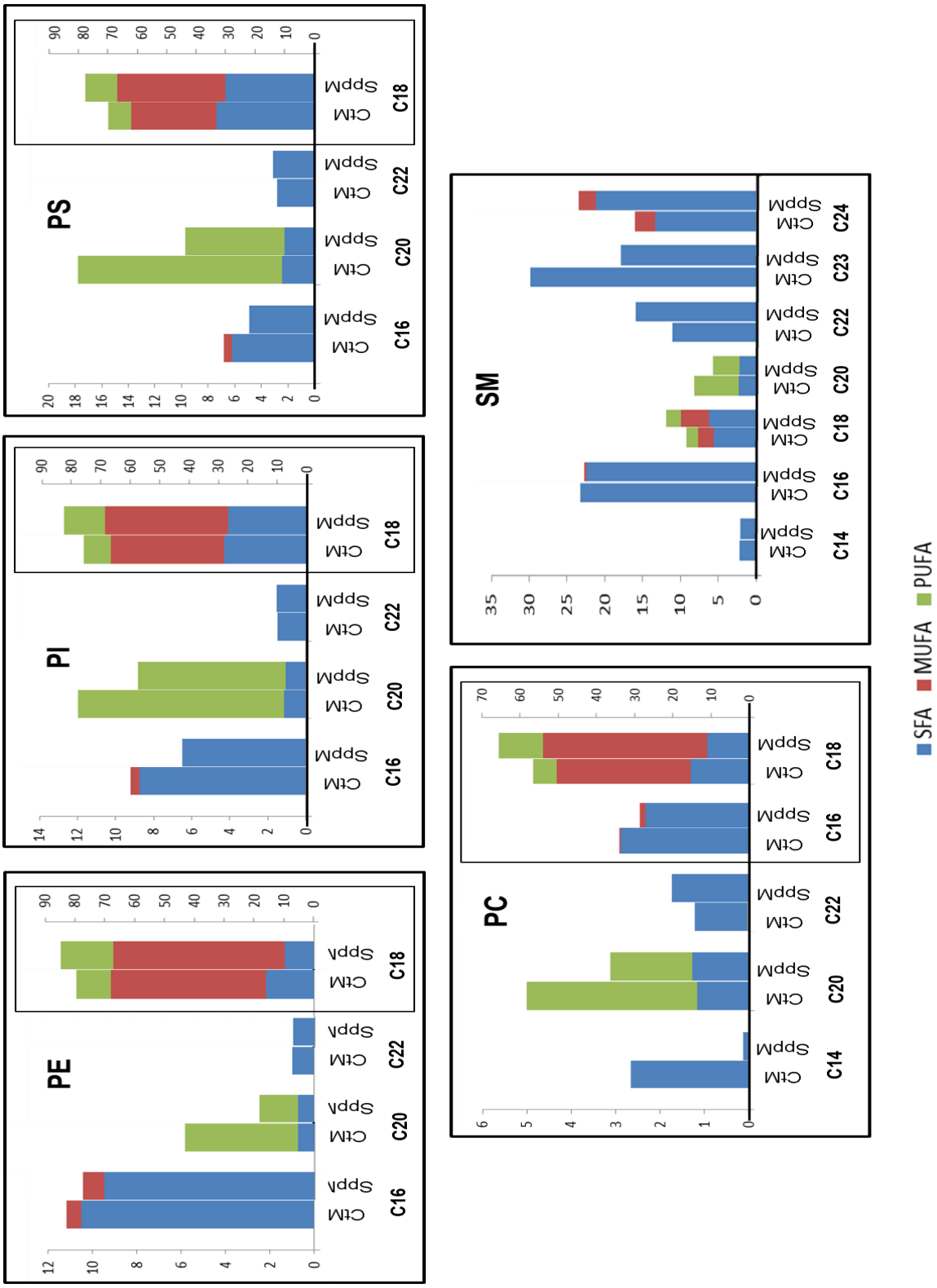
Como se ha indicado anteriormente, se consideró de gran interés el determinar la composición en FA de los PL individuales de las muestras de leche CtM y SppM. Para ello se ensayaron distintos procedimientos combinados de separación de la grasa láctea mediante TLC, que permitieron el aislamiento de los PL individuales en cantidad suficiente para su posterior derivatización y análisis por GC. Los resultados obtenidos para la composición de FAME de cada PL se recogen en la Tabla 3 (página 172). Se observaron variaciones en el contenido de SFA, con disminuciones en el contenido de C14:0, C16:0 y C18:0 presentes en los fosfolípidos PE, PI, PS y PC de la leche SppM que fueron significativas en casi todos los casos. De la misma manera, se detectó un aumento significativo del contenido total de MUFA y en particular de los ácidos C18:1 *trans*10 y VA (precursor del RA) (Oh *et al.*, 2014). En cuanto al contenido en PUFA, todos los fosfolípidos presentes en las muestras SppM, a excepción del PS, experimentaron un aumento en su concentración respecto a la CtM, causado principalmente por la mayor presencia de C18:2 *cis*9, *cis*12 y RA. Es bien conocido desde hace décadas que la suplementación de la dieta del rumiante con PUFA, da lugar en leche a marcados incrementos del contenido del RA, compuesto con actividades potencialmente beneficiosas para la salud (Pariza, 2004). En este estudio, se ha demostrado que el RA en SppM aumenta más de 20 veces en la PE y se incorpora en concentraciones apreciables a los fosfolípidos PI, PS y PC, mientras que en las muestras de leche CtM, no se detectó su presencia en dichos fosfolípidos. Teniendo en cuenta este aspecto, se abriría la posibilidad de utilizar estas fracciones de

MFGM como ingrediente funcional rico en CLA y posiblemente con una mayor biodisponibidad.

Por otra parte, los FA mayoritarios en los fosfolípidos determinados en este estudio (C16:0, C18:0 y el total de C18:1 y C18:2) coinciden con los descritos por Fong *et al.* (2007). Asímismo, algunos estudios previos habían observado la presencia de un elevado contenido en SFA de cadena larga (CN>20) en la SM (Bitman y Wood, 1990). En nuestro estudio, además de describir la composición de SFA de larga cadena presentes en la SM, se ha observado que en las muestras de leche SppM, el contenido de los SFA C22:0 y C24:0 aumenta significativamente tras la suplementación. Sin embargo, en estas mismas muestras se produjo una reducción importante del contenido de C23:0, lo que hizo que se compensara el sumatorio total de SFA entre las muestras CtM y SppM, no apareciendo como significativamente diferentes. En la Figura 10, se muestra la distribución de los principales FA, según el grado de insaturación en cada uno de los PL.

Los cambios observados en los FA de los PL como consecuencia de la suplementación, se pusieron de manifiesto en el estudio de identificación de especies moleculares realizado mediante MALDI-ToF. Tal y como puede observarse, la PE y la PC presentaban un menor número de especies moleculares en SppM que en CtM, sin embargo todas ellas fueron más insaturadas (Tabla 4, página 173 y Tabla 6, página 175). En cuanto al PI y la PS, ambos mostraron un número de especies moleculares similar, aunque las muestras de SppM contenían especies con mayor longitud de cadena y con mayor grado de insaturación que en el caso de la CtM (Tabla 5, página 174). Estos cambios observados en los fosfolípidos se correlacionan con una mayor presencia de C18:1 y C18:2 tras la suplementación. No existen trabajos previos comparando muestras de estas características, sin embargo el número de especies de estos PL en CtM fue similar a los encontrados por Fong *et al.* (2007).

Figura 10. Distribución de los principales FA (%), según el grado de insaturación, de cada PL en leche control (CtM) y suplementada (SppM)



PE: fosfatidiletanolamina, PI: fosfatidilinositol, PS: fosfatidilserina, PC: fosfatidilcolina, SM: esfingomielina. En esta gráfica se representan los FA ≥1%de la tabla 3 (página 172)

En la Tabla 7 de la página 176, se puede observar que el número de especies de SM identificadas en ambas leches fue similar, y al igual que describen Jensen (2002) y López *et al.* (2008), más del 50% de la especies de SM fueron saturadas. No obstante, en este estudio se ha comprobado que las muestras SppM poseen una cantidad considerable de especies moleculares de SM con C16:1 que no fueron detectadas en la leche CtM y que por lo tanto se originarían como consecuencia de la suplementación.

En conclusión, el suplemento de la dieta de las ovejas con aceite de girasol, causa, como era de esperar una mejora del perfil de FA de PL con un contenido significativamente mayor de PUFA, induciendo además una reducción de la producción endógena de CHOL y DAG. En cuanto a la distribución de fosfo- y esfingolípidos, la suplementación conduce, como se ha comentado, a un incremento de la presencia de PUFA en su composición además de causar un significativo aumento del contenido de SM, aspectos ambos que pueden estar relacionados con los potenciales efectos beneficiosos para la salud que se han descrito para estos compuestos.

3. Estudio de la actividad biológica y bioaccesibilidad de fracciones y extractos lipídicos ricos en fosfo- y esfingolípidos obtenidos de mazada mediante ensayos *in vitro* y estudios en humanos.

La capacidad de evaluar el papel que juegan los distintos componentes presentes en los alimentos en la prevención de enfermedades y en la promoción de la salud, se basa en lograr la disponibilidad de biomarcadores precisos y fiables que reflejen cambios por exposición a nutrientes y a sus efectos biológicos en el metabolismo. En este apartado, hemos considerado la posibilidad que nos ofrecen los procedimientos y las metodologías desarrolladas anteriormente para emplear los fosfo- y los esfingolípidos como biomarcadores y así ser capaces de determinar tanto su biodisponibilidad como su actividad biológica.

Para ello se han planteado dos estudios encaminados a determinar la actividad antiproliferativa en cultivos celulares de los fosfo- y esfingolípidos presentes en

las muestras de mazada y en los extractos de PL aislados, así como su biodisponibilidad tras la ingesta en un estudio nutricional en humanos.

3.1. Estudio de la actividad antiproliferativa de mazada y de sus fracciones lipídicas aisladas en líneas celulares humanas de cáncer.

Los resultados discutidos en este apartado forman parte de la publicación "Antiproliferative activity of buttermilk lipid fractions isolated using food grade and non-food grade solvents on human cancer cell lines", enviado a Journal of Functional Foods (Páginas 183-206).

Como se ha indicado anteriormente, son numerosos los estudios realizados durante los últimos años en los que se describen los beneficiosos efectos que ejercen los fosfo- y esfingolípidos ingeridos con la dieta sobre la salud humana. Así, la mayoría de ellos han dirigido sus esfuerzos hacia aquellos alimentos ricos o enriquecidos en estos PL, con el fin de determinar su actividad frente a algunas enfermedades como el cáncer, infecciones, enfermedad cognitiva, enfermedades hepáticas, etc. (Contarini y Povolo, 2013). Quizá, la determinación de la actividad antitumoral de los PL, haya sido el área de investigación en la que más estudios se han realizado. En la mayor parte de los trabajos en los que se han observado efectos beneficiosos (Kullenberg*et al.*(2012) se han utilizando PL de origen vegetal como la soja, o de origen marino como el KO o las algas. Sin embargo, no son muchos los trabajos que han examinado el posible papel de los PL lácteos como compuestos anticancerígenos. Aunque algunos grupos han evaluado la bioactividad de la MFGM completa, no han determinado con exactitud si la funcionalidad era atribuible a las proteínas asociadas a la MFGM o a los PL, o bien se trataba una actividad sinérgica entre ambos componentes (Duan, 2005; Spitsberg, 2005; Fong *et al.*, 2007).

Tal y como se sugirió en un trabajo previo (Castro-Gómez *et al.*, 2013), para la evaluación de la actividad y la determinación de la dosis necesaria para que dichos compuestos causen el efecto deseado, tanto en estudios *in vitro* como *in vivo*, se considera crítico el uso de un método de aislamiento adecuado que

permita el mantenimiento de la funcionalidad de los compuestos bioactivos. En el mencionado estudio se puso de manifiesto la importancia de evaluar la posible actividad antiproliferativa de la mazada y de sus fracciones lipídicas aisladas utilizando disolventes de grado alimentario (FG).

Para ello se llevó a cabo un estudio en colaboración con el laboratorio de Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) de la Universidad de Campinas, Brasil, con el objetivo de determinar la posible bioactividad de los diferentes aislados de PL obtenidos tanto con disolventes orgánicos no aptos para uso alimentario (NFG) y como con disolventes FG, en cultivos de 9 líneas tumorales humanas (dos de piel y ovario, pecho, riñón, pulmón, colon y médula) (Tabla 1, página 200). Se evaluó la actividad antiproliferativa de muestras de mazada completa, de su extracto lipídico, y de sus fracciones polar y no polar, a distintas concentraciones. Los extractos lipídicos fueron obtenidos mediante el método validado por Castro-Gómez *et al.* (2014), empleando bien una mezcla de diclorometano:metanol (2:1 v/v) como disolventes NFG o bien etanol como disolvente FG. Por otra parte se ensayaron dos métodos de fraccionamiento con LC flash, uno de los cuales empleaba un cartucho de sílica y hexano, dietileter y metanol como disolventes NFG (Castro-Gómez *et al.*, 2015) y el otro utilizaba un cartucho de sílica en fase reversa (C18) y metanol y etanol como disolventes FG.

No se encontraron diferencias significativas en cuanto al rendimiento de extracción entre los métodos FG y NFG, mostrando además valores coincidentes con los publicados por otros grupos en mazada extraída por diferentes métodos (Fauquant *et al.*, 1985; Rombaut *et al.*, 2006; Sodini *et al.*, 2006). Los perfiles lipídicos obtenidos mediante HPLC-ELSD (Figura 1, página 203 y Figura 2, página 204) fueron similares, excepto en el método de extracción FG, donde la concentración de DAG fue casi tres veces superior. Por otro lado, aunque no existen diferencias significativas entre los métodos en cuanto al contenido total de PL extraídos, sí se aprecian variaciones en la distribución de cada uno de los fosfo- y esfingolípidos. En ambos extractos, los PL mayoritarios fueron PE, PC y SM, sin embargo el extracto FG presentó una cantidad significativamente mayor de PE y menor de PC que el procedente del NFG. De la grasa extraída con el método NFG se obtuvieron dos fracciones por LC flash, la F1 que estaba mayoritariamente constituida por TAG y la F2 con un

contenido de PL (>94%). La distribución de PL, en esta fracción era diferente a la encontrada en la grasa de origen, siendo la PE, el PI y la PS los fosfolípidos mayoritarios, mientras que la PC y la SM no alcanzaron el 1% del total de PL. Del extracto lipídico obtenido utilizando disolventes FG también se obtuvieron dos fracciones en orden inverso de elución. Mientras que la F1 contenía mayoritariamente PL, encontrándose todas las especies de PL en proporciones similares a las de la grasa original, la F2 contenía mayoritariamente TAG (90%), detéctandose solo una ligera presencia de PL (PC y SM con 4% y 6 %, respectivamente). Los contenidos de las clases lipídicas de ambas extracciones y fraccionamientos se muestran en la Tabla 2 (página 201) y la Tabla 3 (página 202).

Existen trabajos previos en los que se describen fraccionamientos lipídicos realizados mediante métodos como TLC o SPE, pero en los que no se tiene en cuenta la toxicidad de los disolventes orgánicos empleados (Caboni *et al.*, 1996; Fong *et al.*, 2007). Dicha toxicidad se considera un factor clave para la utilización de los extractos en alimentos así como para el mantenimiento de su funcionalidad. Por otro lado, la cantidad de fracción lipídica obtenida mediante estas técnicas es del orden de miligramos, lo que dificulta la realización de estudios posteriores sobre su actividad biológica *in vitro* o *in vivo*. Algunas técnicas como la MF y UF o los SFE han adquirido gran relevancia durante los últimos años puesto que no utilizan disolventes orgánicos en el proceso de extracción y permiten la obtención de volúmenes importantes de muestra lipídica o de extractos. Sin embargo, algunos de estos procedimientos presentan el inconveniente de que los aislados que se obtienen poseen un grado de pureza limitado, además de requerir un equipamiento costoso para llevarlos a cabo (Costa *et al.*, 2010; Konrad *et al.*, 2013). Hasta el momento no se han encontrado en la bibliografia estudios previos en los que se estudien fracciones lipídicas de MFGM obtenidas mediante LC flash.

Los resultados del estudio de la actividad antiproliferativa de la mazada en polvo y de sus extractos lipídicos sobre cultivos de células tumorales humanas se muestran en la Figura 3 (página 205) y en la Figura 4 (página 206). A pesar de que en algunos estudios previos la bioactividad de la MFGM ha sido atribuida tanto a las proteínas como a los lípidos que la componen (Spitsberg,

2005; Dewettinck *et al.*, 2008; Zanabria *et al.*, 2013), en este estudio, la mazada, en diferentes dosis, no presentó actividad antiproliferativa, probablemente debido a que en la muestra en polvo la concentración de los compuestos bioactivos de la MFGM eran insuficientes. Sin embargo, el extracto total de grasa obtenido de la mazada, mediante el método FG, mostró una inhibición parcial del crecimiento celular (TG_{50}) en la línea K361 (cáncer de médula/leucemia humano) a la máxima concentración ensayada. Las fracciones aisladas con disolventes NFG no presentaron ningún tipo de actividad antiproliferativa. Estos resultados sugirieron que aunque las muestras obtenidas presentaban un perfil lipídico similar, únicamente aquellas fracciones grasas obtenidas con disolventes FG mantenían la bioactividad de sus componentes. De la misma manera, la fracción lipídica F1, extraída con disolventes FG y altamente enriquecida en PL, fue la que presentó una mayor actividad antiproliferativa inhibiendo el crecimiento de las líneas tumorales ADR/RES (ovario con resistencia a múltiples fármacos) a una concentración de 96.7 µg/mL y el de las líneas tumorales HT29 (cáncer de colon) a <250 µg/mL. En el caso del cáncer de ovario, los resultados concuerdan con los descritos por van Vlerken *et al.* (2007) para la ceramida, presente en la SM, la cual en combinación con un agente quimioterapéutico reactivaba la señal apoptótica en células SKOV3 de ese tipo de cáncer. Los datos de actividad antiproliferativa en la F1 (rica en PL) en células de cáncer de colon, también coinciden con los obtenidos tanto en experimentos *in vitro* con células SW-480 y Caco-2 (Dial *et al.*, 2006; Fukunaga *et al.*, 2008) como *in vivo* en ratas con tumores de colon inducidos (Fukunaga *et al.*, 2008), dónde se observa un efecto inhibitorio tras el tratamiento con PL tanto de origen vegetal como marino.

Aunque mucho menos numerosa, existe bibliografía en relación con la actividad antitumoral de los PL lácteos, así Zhang *et al.*(2008) detectaron inhibición del cáncer de colon en ratones ICR tras administrarles SM obtenida de leche de vaca. La actividad anticancerígena de SM láctea se ha relacionado con su función como mensajero esencial en el control del crecimiento, en la diferenciación y en la apoptosis celular. Por otro lado, Snow *et al.* (2010) describieron la reducción del cáncer de colon en ratas Fischer 344 tras el consumo de MFGM.

La fracción F1 obtenida en condiciones FG también mostró actividad TG_{50} frente a células 786-0 (cáncer de riñon) y frente a K562 (cáncer de medula/leucemia) a concentraciones de entre 25 µg/mL y 250, respectivamente. Estos resultados no habían sido publicados con anterioridad. Por otro lado, la F2 obtenida con disolventes FG y con un perfil mayoritariamente neutro, también mostro actividad TG_{50} frente a células ADR/RES (ovario resistente a múltiples fármacos) probablemente debido a la presencia de PC y SM en la muestra (9% del total de la fracción).

A partir de estos resultados se concluyó que el empleo de disolventes FG para el aislamiento lipídico de la MFGM y sus fracciones es un factor crítico para el mantenimiento la actividad antitumoral de los extractos. Además, la presencia de fosfo- y esfingolípidos lácteos parece resultar crucial para la presencia de actividad antiproliferativa frente a cultivos celulares de cáncer humanos HT-29 (colon) y NCI-ADR/RES (ovario), lo que sugiere que además de las proteínas, también los PL asociados a la MFGM parecen tener una elevada actividad antiproliferativa.

3.2. Estudio de biodisponibilidad en humanos de los compuestos lipídicos presentes en un producto lácteo enriquecido con mazada

Los resultados discutidos en este apartado forman parte de la publicación en preparación “Postprandial variations on plasma lipid classes and fatty acids content after consumption of skimmed milk enriched with buttermilk in healthy subjects” (Páginas 209-231).

Los estudios encaminados a determinar la biodisponibilidad de un producto, un suplemento o un ingrediente, se aplican fundamentalmente para productos farmacéuticos administrados oralmente, y se centran en determinar el proceso mediante el cual se libera un principio activo y es trasladado hasta su lugar de acción. De igual manera en alimentación funcional se considera fundamental el desarrollo de estudios que permitan determinar las variaciones que tiene lugar durante las intervenciones alimentarias en modelos animales y en humanos

tanto a nivel fisiológico como en fluidos y tejidos biológicos (absorción, metabolismo, distribución en tejidos y eliminación) al consumir un alimento o ingrediente bioactivo.

El objetivo de este trabajó fue la monitorización de la concentración de todas las clases lipídicas, en especial la de PL, y de la composición en FA del plasma humano en un estudio seriado a lo largo de 5 horas tras el consumo de una bebida enriquecida en mazada (como fuente de PL) con el fin de evaluar la biodisponibilidad de estos compuestos. Este ensayo fue realizado en colaboración con la empresa CAPSA (Central Lechera Asturiana) y el Departamento de Ciencias de la Salud de la Universidad Católica San Antonio de Murcia.

Se llevó a cabo una intervención alimentaria con 12 voluntarios sanos que consumieron 400 mL de una bebida láctea compuesta de leche desnatada enriquecida con mazada en polvo (12.5% p/v). La mazada incorporada a la bebida había sido seleccionada mediante el análisis de su composición global con especial atención a la fracción lipídica. El contenido en grasa de la bebida era del 1% y la concentración de PL de un 22% sobre el total de la grasa. La composición en clases lipídicas y FA de la bebida se muestran en la Tabla 2 (página 227) y en la Tabla 3 (página 228).

Una vez ingerido el producto lácteo, se tomaron muestras de plasma sanguíneo de los voluntarios a intervalos de una hora desde el momento de la ingesta hasta transcurridas 5 horas. Se determinaron los perfiles lipídicos de cada una de las muestras estudiando las variaciones que se producen en las clases lipídicas y en los FAME mediante HPLC-ELSD y GC-MS, respectivamente.

Los resultados pusieron de manifiesto que a pesar del bajo contenido graso de la bebida ingerida, la concentración de los compuestos lipídicos determinados en plasma, experimentó variaciones detectables a lo largo del tiempo de estudio. Aunque la concentración total de grasa en plasma, determinada gravimétricamente, no sufrió modificaciones significativas durante la primera hora de ensayo, a partir de ese momento se observó una tendencia al aumento del contenido de grasa hasta la tercera hora, momento en el que se alcanzó el valor máximo en la mayoría de los voluntarios. Posteriormente se produjo un

descenso continuado hasta alcanzar las concentraciones basales, transcurridas 4-5 h desde el momento de la ingesta (Figura 1, página 230). Esta misma tendencia fue observada por Tholstrup*et al.*(1998) en adultos jóvenes que habían consumido grasa de leche en ayunas. A pesar de lo observado para el contenido total de grasa en plasma, los análisis con HPLC-ELSD mostraban que cada clase lipídica se comporta de manera diferente. La monitorización de la concentración de cada una de las clases lipídicas aparece representada en la Figura 2 (página 231). La evolución de los lípidos neutros TAG y DAG fue muy similar, experimentando un aumento de concentración continuo y prolongado desde el momento de la ingesta hasta después de transcurridas 2 horas. A partir de ese momento, los niveles fueron disminuyendo hasta alcanzar los niveles basales a las 5 horas. Estos resultados están de acuerdo con lo observado en voluntarios jóvenes tras el consumo de mantequilla, en los cuales se alcanzó un nivel máximo de TAG en plasma a las dos horas (Goltz *et al.*, 2012). Este tipo de evolución es bien conocida y se explica como una consecuencia del metabolismo de los TAG que son incorporados al núcleo del QM en el enterocito y transportados por el plasma, lo que causa un aumento de su concentración durante las primeras horas tras la ingesta (Michalski, (2009). La acción continua de las lipasas sobre los TAG provoca su hidrólisis y consecuentemente la liberación de los DAG, MAG y FA, cuyas concentraciones se incrementan en plasma y que a continuación prosiguen su metabolismo hasta su absorción total por las diferentes células del organismo. Por esta razón se observa una evolución ascendente en el contenido de compuestos intermedios del metabolismo lipídico en plasma, como los MAG, desde la ingesta hasta las 2-3 horas. El contenido en CE no experimentó variaciones en plasma durante todo el estudio, probablemente debido a que su concentración en la bebida suministrada era prácticamente inapreciable. Datos similares fueron descritos por Sutherland *et al.* (2007), tras administrar un batido de leche con 70% de contenido graso a voluntarios sanos y registrarlo durante 6 horas. El CHOL libre presentó la misma evolución en el tiempo, lo que concuerda con el hecho de que dicho compuesto no es absorbido de manera eficiente, tal y como se ha descrito en la bibliografía (Castro y Fielding, 1985).

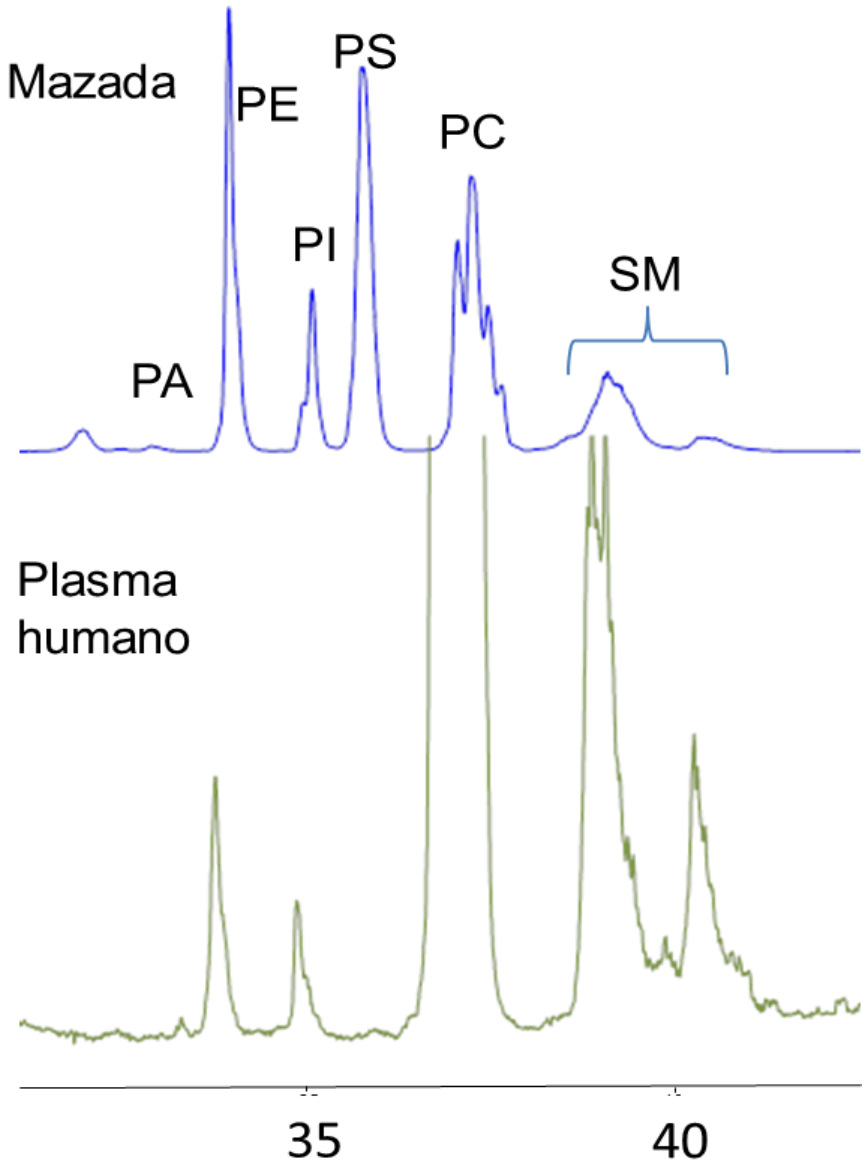
En el contenido total de PL se observa una tendencia de aumento prolongado desde el momento de la ingesta hasta las 5 horas después, alcanzando un

incremento del 6%. Esta misma tendencia fue observada por Simonsson *et al.*(1982), Castro and Fielding (1985) y Cohn *et al.*(1988) en plasma humano tras el consumo de una dieta rica en grasa. Respecto a los PL considerados individualmente, todos siguieron una tendencia similar, aunque el mayor aumento de concentración se observó en la SM y en el PI. En humanos, también se ha descrito la evolución de la PC en plasma tras el consumo de yema de huevo (Simonsson *et al.*, 1982). Del mismo modo, Agren *et al.*(2006), determinaron la concentración de los PL durante las 5 primeras horas tras la ingesta, y sugieren que todos los cambios observados son atribuibles a la dieta, ya que los PL que se sintetizan en el hígado y son transportados por las VLDL se mantienen en valores constantes. Estos autores también describen la ausencia de PS en plasma humano, al igual que ocurre en los resultados de nuestro estudio, donde no fue posible detectar la PS (Figura 11). No se han encontrado estudios en la bibliografía sobre la evolución de las concentraciones de PE y PI presentes en plasma tras el consumo de un alimento rico en PL.

Las concentraciones de FAME obtenidos del plasma de los voluntarios se muestran en la Tabla 4 (página 229). Tal y como se esperaba, se produce un aumento del contenido total de FA en plasma tras la ingesta del producto hasta las 3 horas, momento en el que alcanza su máximo para después descender hasta su valor inicial. Este comportamiento mostraba similitud con el de los TAG, pero no así con el de los PL, como consecuencia de su elevado contenido en plasma (≥ 75% del total de lípidos). En plasma, los FA mayoritarios fueron C16:0, C18:0, C18:1 *cis*9, C18:2 *cis*9, *cis*12 y C20:4 lo que se corresponde con lo descrito previamente por Vors*et al.* (2013) tras la ingesta de leche por voluntarios sanos. Malpuech-Brugere*et al.*(2010), observaron un pico máximo de los FA (C16:0 y C18:1), 5 horas después del consumo de aceite de girasol. Esta diferencia en el tiempo de absorción probablemente se deba al hecho de que el estudio se realizó con voluntarios que eran obesos. A pesar de la presencia en la bebida láctea de FA de cadena corta y media, en plasma sólo fue posible detectar FA con CN ≥12, debido a su rápida absorción en el intestino, lo cual hace que aparezcan como FFA fácilmente incorporables por las células (Cook y Sellin, 1998). La suma de SFA, MUFA y PUFA también

alcanzaron su concentración máxima a las 3 horas, lo que concuerda con lo descrito por otros estudios en los que se suministraban alimentos ricos en grasa a voluntarios en ayunas (Koutsari *et al.*, 2004; Jans *et al.*, 2012).

Figura 11. Comparación de la distribución de los fosfo- y esfingolípidos en la grasa de mazada y en plasma humano, obtenida mediante HPLC-ELSD



PE: fosfatidiletanolamina, PI: fosfatidilinosiltol, PS: fosfatidilserina, PC: fosfatidilcolina, SM: esfingomielina.

Este estudio concluye que la administración de un producto lácteo enriquecido en mazada (como fuente de PL), en la dieta de los voluntarios produce un incremento en la concentración de los TAG de plasma así como de los FA

mayoritarios, hasta las 2-3 horas, para posteriormente disminuir su concentración hasta niveles basales. Sin embargo, los fosfo- y esfingolípidos, aunque fueron igualmente absorbidos y pueden ser determinados en plasma, a excepción de la PS, mantienen una tendencia de aumento en su concentración hasta pasadas 5 horas tras la ingesta.

Estos resultados sugieren que estos compuestos presentes en una bebida láctea mostraban una elevada biodisponibilidad permaneciendo en plasma hasta su absorción total.

5. RESUMEN

En la leche, la grasa se presenta en forma glóbulos rodeados por una membrana celular denominada MFGM. Dicha membrana es una tricapa lipídica que envuelve a los lípidos neutros, mayoritariamente TAG, lo que les permite permanecer en emulsión y protegidos de procesos lipolíticos. La composición de la MFGM consiste en una mezcla compleja de glicoproteínas, fosfolípidos, esfingolípidos, glicolípidos (cerebrosidos y gangliosidos), colesterol, enzimas y otros componentes minoritarios.

A los fosfo- y esfingolípidos consumidos en los alimentos se les atribuye una gran variedad de beneficios para la salud humana entre los que destacan sus efectos positivos sobre diferentes tipos de cáncer y sobre el deterioro cognitivo. En la dieta, el principal aporte de PL lo constituyen alimentos de origen vegetal como la soja o animal como la yema de huevo o el krill, sin embargo estos presentan un perfil incompleto de PL o su consumo está desaconsejado por el riesgo de trasmisión de priones, como ocurre con el consumo de cerebros animales.
La mencionada MFGM no sólo es una fuente segura de PL sino que además, muestra un perfil completo de fosfo- y esfingolípidos en su composición. A pesar de esto, los estudios de bioactividad de los PL lácteos son escasos y los trabajos publicados utilizando MFGM, atribuyen la actividad biológica indistintamente tanto a los PL como a las proteínas que forman parte de ella.

El contenido de PL en leche es reducido (<1% respecto al total de grasa), sin embargo hay subproductos lácteos, como la mazada, en los que aumenta significativamente la concentración de estos sobre el total de grasa. El suero de mantequilla o mazada, se define como un producto en polvo amarillo con 11,4% de grasa y un contenido en fosfolípidos del >20% sobre la fracción grasa, mientras que la composición de la fracción no grasa es similar a la de la leche. Como se ha indicado, la mazada es el subproducto (de muy bajo coste en el mercado) del proceso de fabricación de mantequilla, y consiste en la fase acuosa de la nata, que se libera durante la agitación cuando los granos de mantequilla se están formando. En los últimos años, la mazada está despertando un interés creciente más allá de sus propiedades tecnológicas, por su contenido en PL de elevada actividad presentes en la MFGM (aprox. 30-45

% p/p). Estos PL se encuentran altamente relacionados con el metabolismo celular y se consideran mensajeros secundarios, implicados en la transducción de señales transmembrana y en la regulación del crecimiento, la proliferación, la diferenciación y la apoptosis celular. También juegan un papel clave en la señalización neuronal y están vinculados a las enfermedades relacionadas con la edad, la coagulación de la sangre, la inmunidad y las respuestas inflamatorias. Estos resultados permiten considerar la MFGM como un potencial nutracéutico o ingrediente bioactivo.

Los fosfo- y esfingolípidos lácteos forman parte de de la compleja matriz que constituye la grasa láctea y cuyo análisis exige la utilización de una amplia gama de metodologías que permitan el estudio exhaustivo de su composición. Por esta razón, en la presente Tesis Doctoral se han desarrollado una serie de metodologías que permiten mejorar la extracción lipídica, el aislamiento de los PL y de los lípidos neutros. Asimismo, se han puesto a punto diferentes técnicas cromatográficas que permiten la caracterización exhaustiva de la grasa y sus fracciones.

Por un lado, se ha optimizado un método de HPLC-ELSD para la detección y cuantificación de todas las clases lipídicas presentes en una muestra grasa, permitiendo una mejor separación de los lípidos neutros y PL que los métodos más utilizados para este tipo de análisis.

Por otro lado, se ha desarrollado un método de extracción lipidíca completa mediante PLE. Esta técnica se caracteriza por obtener un alto rendimiento de extracción en un menor tiempo y utilizando una menor cantidad de disolventes que los métodos tradicionalmente empleados, lo cual, se consigue mediante una adecuada combinación de factores como temperatura, tiempo y número de ciclos de extracción. Para la optimización de las condiciones de extracción con PLE, se empleó leche desnatada en polvo que contenía una cantidad conocida de grasa láctea anhidra.

En dichas condiciones optimizadas, se obtuvieron los extractos lipídicos de leches de vaca, oveja y cabra para su comparación con aquellos extractos obtenidos mediante otros métodos de extracción usados tradicionalmente, utilizándose IR como método cuantitativo control de concentración grasa. La

comparación del rendimiento de extracción, del perfil de clases lipídicas, de los FAME y de la distribución de los grupos de TAG, permitieron concluir que el método de extracción lipídica mediante PLE propuesto, podría ser empleado como un método de rutina para la extracción de grasa láctea. Esta afirmación viene sustentada no sólo por el hecho de que el método propuesto permite una extracción total y completa de todos los componentes de la grasa, sino por la notable reducción tanto del tiempo de extracción como de la cantidad de disolventes utilizados, a lo que se añade la posibilidad de automatización del proceso para el análisis de un número elevado de muestras.

En lo que se refiere a la determinación de los FA, es sabido que en grasa, éstos pueden encontrarse tanto esterificados a estructuras lipídicas, como glicero-, fosfo- o esfingolípidos, o bien en forma libre (FFA). Para llevar a cabo su identificación y cuantificación mediante GC, es necesario el uso de un método de derivatización que asegure la volatilización de todos ellos. Existen métodos que permiten la derivatización de los glicerolípidos y los fosfolípidos mediante un proceso de catálisis básica y que normalmente llevan implícita la necesidad de una extracción lipídica previa. Sin embargo, cuando se pretende determinar los FAME de los FFA o los FA que se encuentran formando parte de los esfingolípidos se requiere del uso de catalizadores ácidos, los cuales suelen crear artefactos además de modificar el perfil de algunos FA, pudiendo conducir a una errónea caracterización de los FA de la muestra.
Por ese motivo en esta Tesis se ha desarrollado un método de derivatización que, combinando una base (metóxido sódico) con un ácido en metanol (ácido sulfúrico), junto con la adición de un disolvente aprótico (DMF) para evitar la formación de artefactos y modificaciones del perfil de FA, permite la derivatización total y directa de las muestras.
Se llevó a cabo una comparación, del método propuesto con cinco de los métodos de derivatización básicos, ácidos y básico/ácido más utilizados en la bibliografía. Los resultados mostraron que el método propuesto, metóxido sódico/ácido sulfúrico, era el único método que derivatizaba todos los FA y permitía detectar el CHOL total tanto en mezclas de patrones, como en muestras lácteas y biológicas. Además se observó que dicho método, en presencia de DMF como disolvente aprótico, no modificaba el perfil de FA en

una muestra rica en CLA. En este estudio se concluyó que el método directo propuesto, utilizando metóxido sódico/ácido sulfúrico combinado con DMF, constituye la mejor alternativa para el análisis total de FA, tanto de muestras biológicas como de alimentos, puesto que permite derivatización total, sin alterar el perfil de ningún FA (incluidos los isómeros de CLA) siendo además el único que permite la cuantificación del CHOL total.

Una vez abordado el procedimiento basado en la combinación de métodos y técnicas cromatográficas que permitieran caracterizar exhaustivamente los constituyentes lípídicos presentes en alimentos, se propuso como objetivo la obtención de extractos o fracciones lipídicas diferenciadas por su grado de polaridad (neutras y polares) para su posterior análisis, así como para su aplicación como ingredientes bioactivos en alimentos funcionales.
Para llevar a cabo esta tarea, se empleó KO, un aceite rico en EPA y DHA, caracterizado por tener estos LCPUFA n-3 esterificados a los PL y no a los TAG como ocurre en el aceite de pescado. Esta peculiaridad resulta muy relevante dado que los FA en PL presentan una mayor absorción que aquellos esterificados en TAG. El uso combinado de los disolventes hexano, dietileter y metanol y de cartuchos de sílica, permitieron alcanzar la separación de tres fracciones mediante LC flash. Mientras que en el KO los contenidos de TAG y PL fueron distribuidos en concentraciones similares, la F1 estuvo mayoritariamente constituida por TAG, la F2 por lípidos neutros diferentes a TAG y la F3 por PL, fundamentalmente PC. Además, se llevó a cabo el análisis de los FAME y de las especies moleculares de cada uno de ellas. Los resultados de dichos análisis permitieron concluir que el método de fraccionamiento lipídico desarrollado era capaz de separar PL de lípidos neutros en KO, pudiendo ser válida su aplicación a otros aceites alimenticios. Por otro lado, permitió la descripción exhaustiva de la composición del KO y sus fracciones, observándose que los SFA se distribuyen de manera preferente en los TAG. Sin embargo, los PL presentaban mayoritariamente PUFA en su composición, incorporando más de la mitad del EPA y del DHA presentes en el KO, los cuales se encontraban repartidos en la mayoría de las especies moleculares. Por todo ello se puede sugerir la utilización del KO, y en particular de la fracción de PL, como un posible ingrediente funcional o nutracéutico.

En los últimos años, la mejora del perfil graso de la leche, ha sido el objetivo de muchos de los trabajos llevados a cabo tanto en el campo de la investigación como en la industria. Una de las estrategias más utilizadas ha sido la suplementación de la dieta del ganado con aceites ricos en PUFA. De esta forma se logra una mejora en el perfil de FA de la leche, disminuyendo el contenido de los SFA y aumentando el de los UFA, en particular de aquellos con reconocida actividad biológica. Sin embargo, se dispone de escasa información en relación a los cambios ocurridos en la fracción polar de la leche, así como en el resto de clases lipídicas. Esto es de gran importancia puesto que la mejora del perfil mediante la incorporación de FA funcionales en la fracción polar supondría, como se ha indicado previamente, una mejor biodisponibilidad de dichos FA. Con el fin de obtener dicha información, se extrajo mediante PLE la grasa de dos leches de oveja, una procedente de animales cuya dieta había sido suplementada con aceite de girasol y otra de animales que consumieron dieta control. Se analizó de perfil total de clases lipídicas de ambas leches y se separó cada uno de los fosfo- y esfingolípidos mediante diferentes metodologías de TLC. Tras el aislamiento individual de cada PL, fueron determinados sus FA y especies moleculares. Con los resultados obtenidos con los citados análisis se pudo concluir que la suplementación de la dieta de las ovejas con aceite de girasol, no sólo mejora el perfil total de FA, sino que además reduce la producción endógena de CHOL y DAG y provoca diferencias en la distribución de los fosfolípidos cuya composición se ve mejorada por el aumento de UFA, así como un aumento de SM, con conocidos efectos beneficiosos para la salud.

Por otro lado, es importante evaluar el papel que juegan los distintos componentes presentes en los alimentos en la prevención de enfermedades y en la promoción de la salud. Por ello, en otro estudio, se consideró la posibilidad de determinar la actividad antiproliferativa en cultivos celulares de fosfo- y esfingolípidos presentes en las muestras de mazada y en extractos de PL aislados. Además, se evaluó la importancia del uso de disolventes FG en el aislamiento de las fracciones lipídicas y su papel crítico en el mantenimiento de la posible bioactividad.

Para ello, se extrajo la grasa de mazada mediante PLE, aislándose posteriormente los PL y los lípidos neutros mediante LC flash. Se emplearon etanol y metanol como disolventes FG y diclorometano, metanol, hexano y dietileter como disolventes NFG. La actividad antiproliferativa de la mazada, los extractos lipídicos y las fracciones obtenidas por ambos métodos, fue evaluada en nueve líneas tumorales humanas. Los resultados de estos experimentos revelaron que, el empleo de disolventes FG para el aislamiento lipídico de la MFGM y sus fracciones constituye un factor crítico para el mantenimiento de la actividad antitumoral de los extractos. Además, la fracción enriquecida en fosfo- y esfingolípidos lácteos mostró actividad antiproliferativa en las células de cáncer humanas HT-29 (colon) y NCI-ADR/RES (ovario), lo que sugiere que además de las proteínas asociadas a la MFGM, los PL también poseen actividad anticancerígena.

Finalmente se diseñó un ensayo encaminado a determinar la biodisponibilidad de los compuestos lipídicos, principalmente de PL, en humanos. El estudio seriado de 5 horas, se inició tras el consumo de 400mL de una bebida láctea compuesta por leche desnatada y mazada (como fuente de PL), extrayendo muestras de plasma a los voluntarios a intervalos de una hora. En dichas muestras se monitorizó la concentración de todas las clases lipídicas, con especial interés en la de fosfo- y esfingolípidos, así como la composición en FA. Los resultados obtenidos pusieron de manifiesto que a pesar del bajo contenido graso de la bebida ingerida (1%), la concentración de los compuestos lipídicos determinados en plasma, sufrió variaciones detectables a lo largo del tiempo de estudio. Así, la administración a humanos de un producto lácteo enriquecido con mazada condujo a un incremento en la concentración total de grasa, de los TAG y de los FA mayoritarios de plasma durante las 2-3 horas iniciales, para posteriormente disminuir sus concentraciones hasta los niveles basales. Sin embargo, en el caso de los fosfo- y esfingolípidos aunque también fueron absorbidos, se observó una tendencia al aumento de su presencia en plasma (a excepción de la PS), hasta transcurridas 5 horas tras la ingesta. Estos resultados permitieron concluir que los compuestos presentes en la bebida láctea mostraban una elevada biodisponibilidad, manteniéndose en plasma hasta su absorción total.

En la presente Tesis Doctoral se han desarrollado métodos de extracción lipídica, aislamiento de fracciones y técnicas cromatográficas que han permitido la caracterización exhaustiva de la composición de diferentes grasas, con especial interés en la fracción polar. Con ellas se han podido complementar la escasa información al respecto de la mejora que se produce en la composición y distribución de los fosfo- y esfingolípidos de la leche de ovejas cuando la dieta es suplementada con aceites ricos en PUFA. Además, se ha observado que la fracción de fosfo- y esfingolípidos aislada de grasa láctea tiene una potencial actividad antiproliferativa en células tumorales humanas en ensayos *in vitro*. Esto es de gran importancia debido a que estos compuestos polares presentan una gran biodisponibilidad en humanos cuando son consumidos, pudiendo llegar a ejercer beneficiosos efectos sobre la salud.

6. CONCLUSIONES

Los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral han permitido extraer las siguientes conclusiones:

1. Se han desarrollado procedimientos de extracción lipídica mediante líquidos presurizados, optimizando las variables presión, temperatura y tiempo, que permiten obtener la fracción grasa de alimentos con un elevado rendimiento, tanto si se emplea la mezcla de disolventes diclorometano:metanol (2:1), como disolventes de grado alimentario (etanol) en condiciones que harían posible su aplicación de forma rutinaria.

2. El empleo combinado de técnicas cromatográficas LC Flash, HPLC-ELSD y GC-MS han permitido aislar fracciones enriquecidas de distintas clases lipídicas según su grado de polaridad (lípidos neutros y lípidos polares) para su posterior caracterización y/o empleo en estudios de bioactividad de muestras alimenticias o de aceites de distinto origen.

3. Se ha desarrollado un método de derivatización directa y completa de muestras alimenticias o biológicas, líquidas u homogeneizadas combinando la catálisis básica con metóxido sódico y la metilación con ácido sulfúrico en metanol, que asegura la transesterificacion de los glicerolípidos y fosfolípidos, la esterificación de los ácidos grasos libres y la transmetilación de las esfingomielinas. Además, la incorporación de un disolvente aprótico (dimetilformamida) evitó la formación de artefactos e isómeros *trans*.

4. Se ha caracterizado en profundidad el perfil lipídico de aceite de krill y sus fracciones permitiendo observar una localización preferente de los ácidos grasos saturados en los tracilglicéridos, así como de los ácidos grasos poliinsaturados, incluyendo los ácidos grasos eicosapentanoico y docosahexanoico, en los lípidos polares, repartidos en todas las especies moleculares de fosfatidilcolina. Esto permitiría considerar al aceite de krill, y

sobre todo a la fracción de lípidos polares, como un ingrediente funcional o nutracéutico con potenciales efectos beneficiosos para la salud.

5. La suplementación de la dieta de rumiantes con aceites o semillas ricos en ácidos grasos poliinsaturados, además de mejorar significativamente el perfil nutricional de ácidos grasos de la leche, reduce de manera significativa el contenido del colesterol y de los diacilglicéridos, y conduce a una mejora en la composición y distribución de los lípidos polares lácteos, destacando el aumento de las esfingomielinas con reconocidos efectos beneficiosos.

6. La fracción de lípidos polares obtenidos de mazada láctea enriquecida en fosfo- y esfingolípidos, presenta actividad antiproliferativa frente a líneas celulares de tumores humanos de colon y ovario, así como actividad TG_{50} en células de leucemia y de riñón. Además, se ha comprobado que el uso de disolventes de grado alimentario en la extracción y posterior aislamiento de estas fracciones de lípidos polares, es un factor crítico a tener en cuenta para el mantenimiento de dicha actividad antiproliferativa.

7. Las fracciones lipídicas, tanto neutra como polar (fosfo- y esfingolípidos), presentes en un producto lácteo desnatado al que se ha incorporado mazada láctea, presentan diferentes niveles de biodisponibilidad. Mientras que los lípidos neutros muestran su máximo de absorción a las 2-3 horas tras el consumo, para posteriormente disminuir su concentración hasta niveles basales, los fosfo- y esfingolípidos presentan un aumento más lento aunque prolongado en el tiempo. Esto implicaría un mayor tiempo de permanencia en sangre (plasma), manteniéndose hasta su absorción total.

7. BIBLIOGRAFÍA

Abd El Baky, H. H., El Baz, F. K., El Baroty, G. S., Asker, M. S., Ibrahim, E. A. 2014. Phospholipids of some marine macroalgae: Identification, antivirus, anticancer and antimicrobial bioactivities. Der Pharma Chemica 6(5):370-382.

Agren, J. J., Hallikainen, M., Vidgren, H., Miettinen, T. A., Gylling, H. 2006. Postprandial lipemic response and lipoprotein composition in subjects with low or high cholesterol absorption efficiency. Clinica chimica acta; international journal of clinical chemistry 366(1-2):309-315.

Ali-Nehari, A., Chun, B.S. 2011. Characterization of purified phospholipids from krill (*Euphausia superba*) residues deoiled by supercritical carbon dioxide. Korean Journal of Chemical Engineering 29(7):918-924.

Alonso, L., Fontecha, J., Lozada, L., Fraga, M. J., Juarez, M. 1999. Fatty acid composition of caprine milk: major, branched-chain, and *trans* fatty acids. Journal of Dairy Science 82(5):878-884.

Anadón, A., Martínez-Larrañaga, M. R., Martínez, M. A., Ares, I., Ramos, E., Gómez-Cortés, P., Juárez, M., De la Fuente, M. A. 2010. Acute oral safety study of dairy fat rich in trans-10 C18:1 versus vaccenic plus conjugated linoleic acid in rats. Food and Chemical Toxicology 48(2):591-598.

Araujo, P., Zhu, H., Breivik, J., Hjelle, J., Zeng, Y. 2014. Determination and structural elucidation of triacylglycerols in krill oil by chromatographic techniques. Lipids 49(2):163-172.

Argov, N., Lemay, D. G., German, J. B. 2008. Milk fat globule structure and function: nanoscience comes to milk production. Trends in Food Science and Technology 19(12):617-623.

Arouri, A., Mouritsen, O. G. 2013. Membrane-perturbing effect of fatty acids and lysolipids. Progress in Lipid Research 52(1):130-140.

Aureli, M., Loberto, N., Chigorno, V., Prinetti, A., Sonnino, S. 2011. Remodeling of Sphingolipids by Plasma Membrane Associated Enzymes. Neurochemical Research 36(9):1636-1644.

Avalli, A., Contarini, G. 2005. Determination of phospholipids in dairy products by SPE/HPLC/ELSD. Journal of Chromatography A 1071(1-2):185-190.

Baenke, F., Peck, B., Miess, H., Schulze, A. 2013. Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. Disease Models & Mechanisms 6(6):1353-1363.

Barbano, D. M., Sherbon, J. W. 1981. Polyunsaturated Protected Lipid: Effect on Milk Phospholipids. Journal of Dairy Science 64(11):2170-2174.

Bendsen, N. T., Christensen, R., Bartels, E. M., Astrup, A. 2011. Consumption of industrial and ruminant *trans* fatty acids and risk of coronary heart disease: a systematic review and meta-analysis of cohort studies. European Journal of Clinical Nutrition 65(7):773-783.

Benton, D., Donohoe, R. T., Sillance, B., Nabb, S. 2001. The influence of phosphatidylserine supplementation on mood and heart rate when faced with an acute stressor. Nutritional Neuroscience 4(3):169-178.

Berge, K., Musa-Veloso, K., Harwood, M., Hoem, N., Burri, L. 2014. Krill oil supplementation lowers serum triglycerides without increasing low-density lipoprotein cholesterol in adults with borderline high or high triglyceride levels. Nutrition Research 34(2):126-133.

Berra, B., Colombo, I., Sottocornola, E., Giacosa, A. 2002. Dietary sphingolipids in colorectal cancer prevention. European Journal of Cancer Prevention 11(2):193-197.

Bitman, J., Wood, D. L. 1990. Changes in milk fat phospholipids during lactation. Journal of Dairy Science 73(5):1208-1216.

Bondia-Pons, I., Castellote, A. I., López-Sabater, M. C. 2004. Comparison of conventional and fast gas chromatography in human plasma fatty acid determination. Journal of Chromatography B 809(2):339-344.

Boselli, E., Velazco, V., Caboni, M. F., Lercker, G. 2001. Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food. Journal of Chromatography A 917(1-2):239-244.

Braverman, N. E., Moser, A. B. 2012. Functions of plasmalogen lipids in health and disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1822(9):1442-1452.

Brouwer, I. A., Wanders, A. J., Katan, M. B. 2010. Effect of animal and industrial *trans* fatty acids on HDL and LDL cholesterol levels in humans – A quantitative review. PLoS One 5(3):e9434.

Brownbill, R. A., Petrosian, M., Ilich, J. Z. 2005. Association between dietary conjugated linoleic acid and bone mineral density in postmenopausal women. Journal of the American College Nutrition 24(3):177-181.

Buang, Y., Wang, Y.-M., Cha, J.-Y., Nagao, K., Yanagita, T. 2005. Dietary phosphatidylcholine alleviates fatty liver induced by orotic acid. Nutrition 21(7–8):867-873.

Bunea, R., El Farrah, K., Deutsch, L. 2004. Evaluation of the effects of Neptune Krill Oil on the clinical course of hyperlipidemia. Alternative Medicine Review : A Journal of Clinical Therapeutic 9(4):420-428.

Caboni, M., Menotta, S., Lercker, G. 1996. Separation and analysis of phospholipids in different foods with a light-scattering detector. Journal of the American Oil Chemists' Society 73(11):1561-1566.

Calvano, C. D., De Ceglie, C., Aresta, A., Facchini, L. A., Zambonin, C. G. 2013. MALDI-TOF mass spectrometric determination of intact phospholipids as markers of illegal bovine milk adulteration of high-quality milk. Analytical and Bioanalytical Chemistry 405(5):1641-1649.

Calvo, M. V., Castro-Gómez, P., Fontecha, F. J., García-Serrano, A. M., Juárez, M., Rodríguez Alcalá, L. M. 2014. Grasa láctea: una fuente natural de compuestos bioactivos. Alimentación, Nutrición y Salud 21(3):57-63

Carrapiso, A., García, C. 2000. Development in lipid analysis: Some new extraction techniques and *in situ* transesterification. Lipids 35(11):1167-1177.

Castro-Gómez, M., Holgado, F., Rodríguez-Alcalá, L., Montero, O., Fontecha, J. 2015. Comprehensive study of the lipid classes of krill oil by fractionation and identification of triacylglycerols, diacylglycerols, and phospholipid molecular species by using UPLC/QToF-MS. Food Analytical Methods DOI: 10.1007/s12161-015-0150-6

Castro-Gomez, M. P., Rodriguez-Alcala, L. M., Calvo, M. V., Romero, J., Mendiola, J. A., Ibanez, E., Fontecha, J. 2014. Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using pressurized liquid system and chromatographic techniques. Journal of Dairy Science 97(11):6719-6728

Castro-Gómez, P., Fontecha, J., Rodríguez-Alcalá, L. M. 2014. A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples. Talanta 128(0):518-523.

Castro-Gómez, P., Rodríguez Alcalá, L. M., Calvo, M. V., Holgado, F., Carvalho, J. E., Fontecha, F. J. 2013. Antiproliferative activity of buttermilk, milk fat globule membrane and isolated lipid fractions on ten human cancer cell lines. Annals of Nutrition and Metabolism 63(S1):1623.

Castro, G. R., Fielding, C. J. 1985. Effects of postprandial lipemia on plasma cholesterol metabolism. The Journal of Clinical Investigation 75(3):874-882.

Cequier-Sánchez, E., Rodríguez, C., Ravelo, A. n. G., Zárate, R. 2008. Dichloromethane as a Solvent for Lipid Extraction and Assessment of Lipid Classes and Fatty Acids from Samples of Different Natures. Journal of Agricultural Food Chemistry 56(12):4297-4303.

Cescut, J., Severac, E., Molina-Jouve, C., Uribelarrea, J. L. 2011. Optimizing pressurized liquid extraction of microbial lipids using the response surface method. Journal of Chromatography A 1218(3):373-379.

Chardigny, J. M., Destaillats, F., Malpuech-Brugére, C., Moulin, J., Bauman, D. E., Lock, A. L., Barbano, D. M., Mensink, R. P., Bezelgues, J. B., Chaumont, P., Combe, N., Cristiani, I., Joffre, F., German, J. B., Dionisi, F., Boirie, Y., Sébédio, J. L. 2008. Do *trans* fatty acids from industrially produced sources and from natural sources have the same effect on cardiovascular disease risk factors in healthy subjects? Results of the *trans* Fatty Acids Collaboration study. American Journal of Clinical Nutrition 87(3):558-566.

Chatterton, D. E., Nguyen, D. N., Bering, S. B., Sangild, P. T. 2013. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. The International Journal of Biochemistry & Cell Biology 45(8):1730-1747.

Chirkov, S. N. 2002. The Antiviral Activity of Chitosan (Review). Applied Biochemistry and Microbiology 38(1):1-8.

Christie, W. W. 1982. A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. Journal of Lipid Research 23(7):1072-1075.

Christie, W. W. 1990. Gas chromatography and lipids, a practical guide. The oily Press, Ayr, Scotland.

Cohn, J., Kamili, A., Wat, E., Chung, R. W., Tandy, S. 2010. Dietary Phospholipids and Intestinal Cholesterol Absorption. Nutrients 2(2):116-127.

Cohn, J. S., McNamara, J. R., Cohn, S. D., Ordovas, J. M., Schaefer, E. J. 1988. Postprandial plasma lipoprotein changes in human subjects of different ages. Journal of Lipid Research 29(4):469-479.

Cohn, J. S., Wat, E., Kamili, A., Tandy, S. 2008. Dietary phospholipids, hepatic lipid metabolism and cardiovascular disease. Current Opinion in Lipidology 19(3):257-262.

Collomb, M., Schmid, A., Sieber, R., Wechsler, D., Ryhanen, E.-L. 2006. Conjugated linoleic acids in milk fat: Variation and physiological effects. International Dairy Journal 16(11):1347-1361.

Contarini, G., Povolo, M. 2013. Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies. International Journal of Molecular Sciences 14(2):2808-2831.

Conway, V., Couture, P., Gauthier, S., Pouliot, Y., Lamarche, B. 2014. Effect of buttermilk consumption on blood pressure in moderately hypercholesterolemic men and women. Nutrition 30(1):116-119.

Conway, V., Couture, P., Richard, C., Gauthier, S. F., Pouliot, Y., Lamarche, B. 2013. Impact of buttermilk consumption on plasma lipids and surrogate markers of cholesterol homeostasis in men and women. Nutrition, Metabolism, and Cardiovascular Diseases: NMCD 23(12):1255-1262.

Cook, S. I., Sellin, J. H. 1998. Review article: short chain fatty acids in health and disease. Alimentary Pharmacology & Therapeutics 12(6):499-507.

Corredig, M., Roesch, R. R., Dalgleish, D. G. 2003. Production of a Novel Ingredient from Buttermilk. Journal of Dairy Science 86(9):2744-2750.

Costa, M. R., Elias-Argote, X. E., Jiménez-Flores, R., Gigante, M. L. 2010. Use of ultrafiltration and supercritical fluid extraction to obtain a whey buttermilk powder enriched in milk fat globule membrane phospholipids. International Dairy Journal 20(9):598-602.

Cowart, L. A. 2009. Sphingolipids: players in the pathology of metabolic disease. Trends in Endocrinology and Metabolism: TEM 20(1):34-42.

Crichton, G., C Howe, P., Buckley, J., Coates, A., Murphy, K. 2012. Dairy consumption and cardiometabolic health: outcomes of a 12-month crossover trial. Nutrition & Metabolism 9(1):1-11.

Crook, T. H., Tinklenberg, J., Yesavage, J., Petrie, W., Nunzi, M. G., Massari, D. C. 1991. Effects of phosphatidylserine in age-associated memory impairment. Neurology 41(5):644-649.

D'Souza, K., Epand, R. M. 2014. Enrichment of phosphatidylinositols with specific acyl chains. Biochimica et Biophysica Acta (BBA) - Biomembranes 1838(6):1501-1508.

Danthine, S., Blecker, C., Paquot, M., Innocente, N., Deroanne, C. 2000. Progress in milk fat globule membrane research: A review. Évolution des connaissances sur la membrane du globule gras du lait: Synthèse bibliographique 80(2):209-222.

Demirbilek, S., Karaman, A., Gurunluoglu, K., Tas, E., Akin, M., Aksoy, R. T., Turkmen, E., Edali, M. N., Baykarabulut, A. 2006. Polyenylphosphatidylcholine pretreatment protects rat liver from ischemia/reperfusion injury. Hepatology research : the official journal of the Japan Society of Hepatology 34(2):84-91.

Deutsch, L. 2007. Evaluation of the Effect of Neptune Krill Oil on Chronic Inflammation and Arthritic Symptoms. Journal of the American College of Nutrition 26(1):39-48.

Dewettinck, K., Rombaut, R., Thienpont, N., Le, T. T., Messens, K., Van Camp, J. 2008. Nutritional and technological aspects of milk fat globule membrane material. International Dairy Journal 18(5):436-457.

Di Marzo, V., Griinari, M., Carta, G., Murru, E., Ligresti, A., Cordeddu, L., Giordano, E., Bisogno, T., Collu, M., Batetta, B., Uda, S., Berge, K., Banni, S. 2010. Dietary krill oil increases docosahexaenoic acid and reduces 2-arachidonoylglycerol but not N-acylethanolamine levels in the brain of obese Zucker rats. International Dairy Journal 20(4):231-235.

Dial, E. J., Doyen, J. R., Lichtenberger, L. M. 2006. Phosphatidylcholine-associated nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit DNA synthesis and the growth of colon cancer cells *in vitro*. Cancer Chemotherapy and Pharmacology 57(3):295-300.

Dillehay, D. L., Webb, S. K., Schmelz, E. M., Merrill, A. H., Jr. 1994. Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice. Journal of Nutrition 124(5):615-620.

Doria, M. L., Cotrim, C. Z., Simoes, C., Macedo, B., Domingues, P., Domingues, M. R., Helguero, L. A. 2013. Lipidomic analysis of phospholipids from human mammary epithelial and breast cancer cell lines. Journal of Cellular Physiology 228(2):457-468.

Duan, R. D. 2005. Anticancer compounds and sphingolipid metabolism in the colon. In vivo 19(1):293-300.

Duan, R. D., Nilsson, A. 2009. Metabolism of sphingolipids in the gut and its relation to inflammation and cancer development. Progress in Lipid Research 48(1):62-72.

Ehehalt, R., Braun, A., Karner, M., Fullekrug, J., Stremmel, W. 2010. Phosphatidylcholine as a constituent in the colonic mucosal barrier--physiological and clinical relevance. Biochimica et Biophysica Acta 1801(9):983-993.

El-Loly, M. M. 2011. Composition, properties and nutritional aspects of milk fat globule membrane - a Review. Polish Journal of Food and Nutrition Science 61(1):7-32.

El Alwani, M., Wu, B. X., Obeid, L. M., Hannun, Y. A. 2006. Bioactive sphingolipids in the modulation of the inflammatory response. Pharmacology Theraphy 112(1):171-183.

Elwood, P. C., Pickering, J. E., Givens, D. I., Gallacher, J. E. 2010. The Consumption of Milk and Dairy Foods and the Incidence of Vascular Disease and Diabetes: An Overview of the Evidence. Lipids 45(10):925-939.

Erős, G., Ibrahim, S., Siebert, N., Boros, M., Vollmar, B. 2009a. Oral phosphatidylcholine pretreatment alleviates the signs of experimental rheumatoid arthritis. Arthritis Research & Therapy 11(2):R43.

Erős, G., Varga, G., Varadi, R., Czobel, M., Kaszaki, J., Ghyczy, M., Boros, M. 2009b. Anti-inflammatory action of a phosphatidylcholine, phosphatidylethanolamine and N-acylphosphatidylethanolamine-enriched diet in carrageenan-induced pleurisy. European Surgical Research. 42(1):40-48.

Eshigina, S., Gapparov, M. M., Mal'tsev, G., Kulakov, S. N. 2007. [Influence of dietary therapy containing sunflower oil fortified with phospholipids on the lipid metabolism in patients with hypertension and obesity. Voprosy Pitaniya 76(1):58-62.

Evers, J. M., Haverkamp, R. G., Holroyd, S. E., Jameson, G. B., Mackenzie, D. D. S., McCarthy, O. J. 2008. Heterogeneity of milk fat globule membrane structure and composition as observed using fluorescence microscopy techniques. International Dairy Journal 18(12):1081-1089.

Fagan, P., Wijesundera, C. 2004. Liquid chromatographic analysis of milk phospholipids with on-line pre-concentration. Journal of Chromatography A 1054(1-2):241-249.

Fauquant, C., Briard-Bion, V., Leconte, N., Guichardant, M., Michalski, M.-C. 2007. Membrane phospholipids and sterols in microfiltered milk fat globules. European Journal of Lipid Science and Technology 109(12):1167-1173.

Fauquant, J., Vieco, E., Brule, G., Maubois, J.-L. 1985. Clarification des lactosérums doux par agrégation thermocalcique de la matière grasse résiduelle. Lait 65(647-648):1-20.

Field, C. J., Blewett, H. H., Proctor, S., Vine, D. 2009. Human health benefits of vaccenic acid. Applied Physiology, Nutrition, and Metabolism 34(5):979-991.

Folch, J., Lees, M., Stanley, G. H. S. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry. 226(1):497-509.

Fong, B. Y., Norris, C. S., MacGibbon, A. K. H. 2007. Protein and lipid composition of bovine milk-fat-globule membrane. International Dairy Journal 17(4):275-288.

Fontecha, J. 2010. Lípidos funcionales de la dieta. Alimentos saludables y de diseño específico. Alimentos funcionales. Instituto Tomás Pascuál Sanz para la Nutrición y la Salud, ed. IM&C

Fontecha, J., M. Rodriguez-Alcala, L., V. Calvo, M., Juarez, M. 2011. Bioactive Milk Lipids. Current Nutrition & Food Science 7(3):155-159.

Fontecha, J., Mayo, I., Toledano, G., Juarez, M. 2006. Triacylglycerol composition of protected designation of origin cheeses during ripening. Authenticity of milk fat. Journal of Dairy Science 89(3):882-887.

Frasch, S. C., Bratton, D. L. 2012. Emerging roles for lysophosphatidylserine in resolution of inflammation. Progress in Lipid Research 51(3):199-207.

Frede, E., Precht, D., Timmen, H. 1990. Lipide: Fettsäuren, Fette und Fettbegleitstoffe einschlieblich fettlöslicher Vitamine. Kompendium Zur Milchwirtschaftlichen Chemie:57-78.

Fukunaga, K., Hossain, Z., Takahashi, K. 2008. Marine phosphatidylcholine suppresses 1,2-dimethylhydrazine-induced colon carcinogenesis in rats by inducing apoptosis. Nutrition Research 28(9):635-640.

Gallier, S., Gragson, D., Cabral, C., Jiménez-Flores, R., Everett, D. W. 2010. Composition and Fatty Acid Distribution of Bovine Milk Phospholipids from Processed Milk Products. Journal of Agricultural Food Chemistry 58(19):10503-10511.

Garcia, C., Lutz, N. W., Confort-Gouny, S., Cozzone, P. J., Armand, M., Bernard, M. 2012. Phospholipid fingerprints of milk from different mammalians determined by 31P NMR: towards specific interest in human health. Food Chemistry 135(3):1777-1783.

Gardocki, M. E., Jani, N., Lopes, J. M. 2005. Phosphatidylinositol biosynthesis: Biochemistry and regulation. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1735(2):89-100.

Gaullier, J.-M., Halse, J., Hoye, K., Kristiansen, K., Fagertun, H., Vik, H., Gudmundsen, O. 2004. Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. American Journal of Clinical Nutrition 79(6):1118-1125.

Gayet-Boyer, C., Tenenhaus-Aziza, F., Prunet, C., Lamarche, B., Chardigny, J. 2011. Ruminant *trans* fatty acids intake and cardiovascular risk factors: A quantitative review of intervention studies. Parma: IDF World Dairy Summit.

Gebauer, S. K., Destaillats, F., Mouloungui, Z., Candy, L., Bezelgues, J. B., Dionisi, F., Baer, D. J. 2011. Effect of *trans* fatty acid isomers from ruminant sources on risk factors of cardiovascular disease: study design and rationale. Contemporary Clinical Trials 32(4):569-576.

German, J. B. 1999. Butyric acid: a role in cancer prevention. Nutrition Bulletin 24(4):203-209.

German, J. B., Dillard, C. J. 2006. Composition, structure and absorption of milk lipids: A source of energy, fat-soluble nutrients and bioactive molecules. Critical Reviews in Food Science and Nutrition 46(1):57-92.

Goltz, S. R., Campbell, W. W., Chitchumroonchokchai, C., Failla, M. L., Ferruzzi, M. G. 2012. Meal triacylglycerol profile modulates postprandial absorption of carotenoids in humans. Molecular Nutrition & Food Research 56(6):866-877.

Gómez-Cortés, P., Bach, A., Luna, P., Juárez, M., de la Fuente, M. A. 2009. Effects of extruded linseed supplementation on n-3 fatty acids and conjugated linoleic acid in milk and cheese from ewes. Journal of Dairy Science 92(9):4122-4134.

Gómez-Cortés, P., Frutos, P., Mantecón, A. R., Juárez, M., De La Fuente, M. A., Hervás, G. 2008. Milk production, conjugated linoleic acid content, and in vitro ruminal fermentation in response to high levels of soybean oil in dairy ewe diet. Journal of Dairy Science 91(4):1560-1569.

Goudjil, H., Fontecha, J., Luna, P., De La Fuente, M. A., Alonso, L., Jua?rez, M. 2004. Quantitative characterization of unsaturated and *trans* fatty acids in ewe's milk fat. Lait 84(5):473-482.

Gundermann, K. J., Kuenker, A., Kuntz, E., Drozdzik, M. 2011. Activity of essential phospholipids (EPL) from soybean in liver diseases. Pharmacological Reports: PR 63(3):643-659.

Guo, Z., Vikbjerg, A. F., Xu, X. 2005. Enzymatic modification of phospholipids for functional applications and human nutrition. Biotechnology Advances 23(3):203-259.

Hartmann, P., Szabó, A., Erős, G., Gurabi, D., Horváth, G., Németh, I., Ghyczy, M., Boros, M. 2009. Anti-inflammatory effects of phosphatidylcholine in neutrophil leukocyte-dependent acute arthritis in rats. European Journal of Pharmacology 622(1):58-64.

Hassig, C. A., Tong, J. K., Schreiber, S. L. 1997. Fiber-derived butyrate and the prevention of colon cancer. Chemistry & Biology 4(11):783-789.

Hauff, S., Vetter, W. 2009. Quantification of fatty acids as methyl esters and phospholipids in cheese samples after separation of triacylglycerides and phospholipids. Analytica Chimica Acta 636(2):229-235.

Haug, A., Hostmark, A. T., Harstad, O. M. 2007. Bovine milk in human nutrition–a review. Lipids in Health and Disease 6(25):1-16.

Hellhammer, J., Waladkhani, A. R., Hero, T., Buss, C. 2010. Effects of milk phospholipid on memory and psychological stress response. British Food Journal 112(10):1124-1137.

Hervás, G., Luna, P., Mantecón, A. R., Castañares, N., De La Fuente, M. A., Juárez, M., Frutos, P. 2008a. Effect of diet supplementation with sunflower oil on milk production, fatty acid profile and ruminal fermentation in lactating dairy ewes. Journal of Dairy Research 75(4):399-405.

Hilmarsson, H., Larusson, L. V., Thormar, H. 2006. Virucidal effect of lipids on visna virus, a lentivirus related to HIV. Archives of Virology 151(6):1217-1224.

Hintze, K. J., Snow, D., Burtenshaw, I., Ward, R. E. 2011. Nutraceutical properties of milk fat globular membrane. Biotechnology and Biopolymers:321-342.

Hori, G., Wang, M.-F., Chan, Y.-C., Komtatsu, T., Wong, Y., Chen, T.-H., Yamamoto, K., Nagaoka, S., Yamamoto, S. 2001. Soy Protein Hydrolyzate with Bound Phospholipids Reduces Serum Cholesterol Levels in Hypercholesterolemic Adult Male Volunteers. Bioscience, Biotechnology, and Biochemistry 65(1):72-78.

Hossain, Z., Hosokawa, M., Takahashi, K. 2009. Growth inhibition and induction of apoptosis of colon cancer cell lines by applying marine phospholipid. Nutrition and Cancer 61(1):123-130.

Hur, S. J., Park, G. B., Joo, S. T. 2007. Biological activities of conjugated linoleic acid (CLA) and effects of CLA on animal products. Livestock Science 110(3):221-229.

Ierna, M., Kerr, A., Scales, H., Berge, K., Griinari, M. 2010. Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. BMC Musculoskeletal Disorders 11(1):1-11.

Innis, S. M. 2007. Dietary (n-3) Fatty Acids and Brain Development. Journal of Nutrition 137(4):855-859.

Iqbal, J., Hussain, M. M. 2009. Intestinal lipid absorption. American Journal of Physiology. Endocrinology and Metabolism 296(6):E1183-1194.

ISO, I. S. 2001. Milk and milk products-Extraction methods for lipids and liposoluble compounds. ISO 14156-IDF 172:2001.

ISO, I. S. 2002. Milk fat-Preparation of fatty acid methyl esters. ISO 15884-IDF:182:2002.

Iverson, S. J., Lang, S. L. C., Cooper, M. H. 2001. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36(11):1283-1287.

Jäger, R., Purpura, M., Kingsley, M. 2007. Phospholipids and sports performance. Journal of the International Society of Sports Nutrition 4:5-5.

Jannace, P. W., Lerman, R. H., Santos, J. I., Vitale, J. J. 1992. Effects of oral soy phosphatidylcholine on phagocytosis, arachidonate concentrations, and killing by human polymorphonuclear leukocytes. American Journal of Clinical Nutrition 56(3):599-603.

Jans, A., Konings, E., Goossens, G. H., Bouwman, F. G., Moors, C. C., Boekschoten, M. V., Afman, L. A., Müller, M., Mariman, E. C., Blaak, E. E. 2012. PUFAs acutely affect triacylglycerol-derived skeletal muscle fatty acid uptake and increase postprandial insulin sensitivity. American Journal of Clinical Nutrition 95(4):825-836.

Jansen, M., Treutner, K.-H., Schmitz, B., Otto, J., Jansen, P., Neuss, S., Schumpelick, V. 2004. Phospholipids reduce gastric cancer cell adhesion to extracellular matrix *in vitro*. BMC Gastroenterology 4(1):33.

Jantscheff, P., Schlesinger, M., Fritzsche, J., Taylor, L. A., Graeser, R., Kirfel, G., Furst, D. O., Massing, U., Bendas, G. 2011. Lysophosphatidylcholine pretreatment reduces VLA-4 and P-Selectin-mediated b16.f10 melanoma cell adhesion *in vitro* and inhibits metastasis-like lung invasion in vivo. Molecular Cancer Therapeutics 10(1):186-197.

Jayaraman, T., Kannappan, S., Ravichandran, M. K., Anuradha, C. V. 2008. Impact of Essentiale L on ethanol-induced changes in rat brain and erythrocytes. Singapore Medical Journal 49(4):320-327.

Jensen, R. G. 2002. The composition of bovine milk lipids: January 1995 to December 2000. Journal of Dairy Science 85(2):295-350.

Jiang, Y., Noh, S. K., Koo, S. I. 2001. Egg phosphatidylcholine decreases the lymphatic absorption of cholesterol in rats. The Journal of Nutrition 131(9):2358-2363.

Jicha, G. A., Markesbery, W. R. 2010. Omega-3 fatty acids: potential role in the management of early Alzheimer's disease. Clinical Interventions in Aging 5:45-61.

Ju, S.J., Kang, H.-K., Kim, W., Harvey, H. R. 2009. Comparative lipid dynamics of euphausiids from the Antarctic and Northeast Pacific Oceans. Marine Biology 156(7):1459-1473.

Juárez Iglesias, M. 2015. Los nutrientes de la leche en la salud cardiovascular. Nutrición hospitalaria: órgano oficial de la Sociedad Española de Nutrición Parenteral y Enteral 31(2):26-32.

Juárez, M., Fontecha, J. 2009. Componentes Bioactivos de la Grasa Láctea. in Funcionalidad de Componentes Lácteos. J. Fontecha, M. Recio, and M. A. Pilosof, ed. Universidad Miguel Hernández. Elche Alicante.

Jutzeler van Wijlen, R. P., Colombani, P. C. 2010. Grass-based ruminant production methods and human bioconversion of vaccenic acid with estimations of maximal dietary intake of conjugated linoleic acids. International Dairy Journal 20(7):433-448.

Karner, M., Kocjan, A., Stein, J., Schreiber, S., von Boyen, G., Uebel, P., Schmidt, C., Kupcinskas, L., Dina, I., Zuelch, F., Keilhauer, G., Stremmel, W. 2014. First Multicenter Study of Modified Release Phosphatidylcholine in Ulcerative Colitis: A Randomized, Placebo-Controlled Trial in Mesalazine-Refractory Courses. American Journal of Gastroenterology 109(7):1041-1051.

Kay, J. K., Mackle, T. R., Auldist, M. J., Thomson, N. A., Bauman, D. E. 2004. Endogenous Synthesis of *cis*-9, *trans*-11 Conjugated Linoleic Acid in Dairy Cows Fed Fresh Pasture. Journal of Dairy Science 87(2):369-378.

Keenan, T. W., Patton, S. 1995. The structure of milk: implications for sampling and storage. A. The milk lipid globule membrane. Pages 5-50 in Handbook of Milk Composition. R. G. Jensen, ed. Academic Press, San Diego, CA.

Khanal, R. C. 2004. Factors affecting conjugated linoleic acid (CLA) content in milk, meat, and egg: a review. Pakistan Journal of Nutrition 3(2):82.

Kingsley, M. I., Miller, M., Kilduff, L. P., McEneny, J., Benton, D. 2006. Effects of phosphatidylserine on exercise capacity during cycling in active males. Medicine and Science in Sports and Exercise 38(1):64.

Kingsley, M. I., Wadsworth, D., Kilduff, L. P., McEneny, J., Benton, D. 2005. Effects of phosphatidylserine on oxidative stress following intermittent running. Medicine and Science in Sports and Exercise 37(8):1300.

Kirsten, R., Heintz, B., Nelson, K., Hesse, K., Schneider, E., Oremek, G., Nemeth, N. 1994. Polyenylphosphatidylcholine improves the lipoprotein profile in diabetic patients. International Journal of Clinical Pharmacology abd Therapeutics 32(2):53-56.

Kivinen, A., Tarpila, S., Salminen, S., Vapaatalo, H. 1992. Gastroprotection with milk phospholipids: a first human study. Milchwissenschaft 47:694-696.

Konagai, C., Yanagimoto, K., Hayamizu, K., Han, L., Tsuji, T., Koga, Y. 2013. Effects of krill oil containing n-3 polyunsaturated fatty acids in phospholipid form on human brain function: a randomized controlled trial in healthy elderly volunteers. Clinical Interventions in Aging 8:1247-1257.

Konrad, G., Kleinschmidt, T., Lorenz, C. 2013. Ultrafiltration of whey buttermilk to obtain a phospholipid concentrate. International Dairy Journal 30(1):39-44.

Koutsari, C., Zagana, A., Tzoras, I., Sidossis, L. S., Matalas, A. L. 2004. Gender influence on plasma triacylglycerol response to meals with different monounsaturated and saturated fatty acid content. European Journal of Clinical Nutrition 58(3):495-502.

Kuchta, A. M., Kelly, P. M., Stanton, C., Devery, R. A. 2012. Milk fat globule membrane – a source of polar lipids for colon health? A review. International Journal of Dairy Technology 65(3):315-333.

Kullenberg, D., Taylor, L. A., Schneider, M., Massing, U. 2012. Health effects of dietary phospholipids. Lipids in Health and Disease 11:3.

Ladd, S. L., Sommer, S. A., LaBerge, S., Toscano, W. 1993. Effect of phosphatidylcholine on explicit memory. Clinical Neuropharmacology 16(6):540-549.

Lagarde, M., Bernoud, N., Brossard, N., Lemaitre-Delaunay, D., Thies, F., Croset, M., Lecerf, J. 2001. Lysophosphatidylcholine as a preferred carrier form of docosahexaenoic acid to the brain. Journal of Molecular Neuroscience : MN 16(2-3):201-204.

Le Grandois, J., Marchioni, E., Zhao, M., Giuffrida, F., Ennahar, S. d., Bindler, F. o. 2009. Investigation of Natural Phosphatidylcholine Sources: Separation and Identification by Liquid Chromatography−Electrospray Ionization−Tandem Mass Spectrometry (LC−ESI−MS2) of Molecular Species. Journal of Agricultural Food Chemistry 57(14):6014-6020.

Le, T. T., Miocinovic, J., Nguyen, T. M., Rombaut, R., van Camp, J., Dewettinck, K. 2011. Improved solvent extraction procedure and high-performance liquid chromatography-evaporative light-scattering detector method for analysis of polar lipids from dairy materials. Journal of Agricultural Food Chemistry 59(19):10407-10413.

Legrand, P. 2008. Nutritional interest of different fatty acids from milk fat. Sciences des Aliments 28(1-2):34-43.

Lemonnier, L. A., Dillehay, D. L., Vespremi, M. J., Abrams, J., Brody, E., Schmelz, E. M. 2003. Sphingomyelin in the suppression of colon tumors: prevention versus intervention. Archives of Biochemistry and Biophysics 419(2):129-138.

Leventis, P. A., Grinstein, S. 2010. The distribution and function of phosphatidylserine in cellular membranes. Annual Review of Biophysics 39(1):407-427.

Leyck, S., Dereu, N., Etschenberg, E., Ghyczy, M., Graf, E., Winkelmann, J., Parnham, M. J. 1985. Improvement of the gastric tolerance of non-steroidal anti-inflammatory drugs by polyene phosphatidylcholine (Phospholipon 100). European Journal of Pharmacology 117(1):35-42.

Li, Y. C., Park, M. J., Ye, S.-K., Kim, C.-W., Kim, Y.-N. 2006. Elevated Levels of Cholesterol-Rich Lipid Rafts in Cancer Cells Are Correlated with Apoptosis Sensitivity Induced by Cholesterol-Depleting Agents. The American Journal of Pathology 168(4):1107-1118.

Lichtenberger, L., Romero, J. J., Dial, E. J. 2009. Gastrointestinal safety and therapeutic efficacy of parenterally administered phosphatidylcholine-associated indomethacin in rodent model systems. British Journal of Pharmacology 157(2):252-257.

Lieber CS, A. B., Bibi EJ. 2005. Polyenylphosphatidylcholine (PPC) is beneficial for the treatment of hepatitis C. Hepatology 42(S1):695A.

Lieber, C. S., Decarli, L. M., Mak, K. M., Kim, C. I., Leo, M. A. 1990. Attenuation of alcohol-induced hepatic fibrosis by polyunsaturated lecithin. Hepatology 12(6):1390-1398.

Liu, H. X., Adachi, I., Horikoshi, I., Ueno, M. 1995. Mechanism of promotion of lymphatic drug absorption by milk fat globule membrane. International Journal of Pharmaceutics 118(1):55-64.

Liu, K. S. 1994. Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. Journal of the American Oil Chemists´ Society 71(11):1179-1187.

Lopez, C., Briard-Bion, V., Menard, O., Rousseau, F., Pradel, P., Besle, J.-M. 2008. Phospholipid, Sphingolipid, and Fatty Acid Compositions of the Milk Fat Globule Membrane are Modified by Diet. Journal of Agricultural Food Chemistry 56(13):5226-5236.

Lopez, C., Madec, M.-N., Jimenez-Flores, R. 2011. Lipid rafts in the bovine milk fat globule membrane revealed by the lateral segregation of phospholipids and heterogeneous distribution of glycoproteins. Food Chemistry 120(1):22-33.

Luna, P., Bach, A., Juarez, M., de la Fuente, M. A. 2008. Effect of a Diet Enriched in Whole Linseed and Sunflower Oil on Goat Milk Fatty Acid Composition and Conjugated Linoleic Acid Isomer Profile. Journal of Dairy Science 91(1):20-28.

MacKenzie, A., Vyssotski, M., Nekrasov, E. 2009. Quantitative Analysis of Dairy Phospholipids by 31P NMR. Journal of the American Oil Chemists´ Society 86(8):757-763.

Maczek, C., Bock, G., Jurgens, G., Schonitzer, D., Dietrich, H., Wick, G. 1998. Environmental influence on age-related changes of human lymphocyte membrane viscosity using severe combined immunodeficiency mice as an *in vivo* model. Experimental Gerontology 33(5):485-498.

Maines, L., Fitzpatrick, L., French, K., Zhuang, Y., Xia, Z., Keller, S., Upson, J., Smith, C. 2008. Suppression of Ulcerative Colitis in Mice by Orally Available Inhibitors of Sphingosine Kinase. Digestive Diseases and Sciences 53(4):997-1012.

Maines, L., Fitzpatrick, L., Green, C., Zhuang, Y., Smith, C. 2010. Efficacy of a novel sphingosine kinase inhibitor in experimental Crohn's disease. Inflammopharmacology 18(2):73-85.

Mak, K. M., Wen, K., Ren, C., Lieber, C. S. 2003. Dilinoleoylphosphatidylcholine reproduces the antiapoptotic actions of polyenylphosphatidylcholine against ethanol-induced hepatocyte apoptosis. Alcoholism, Clinical and Experimental Research 27(6):997-1005.

Malpuech-Brugere, C., Mouriot, J., Boue-Vaysse, C., Combe, N., Peyraud, J. L., LeRuyet, P., Chesneau, G., Morio, B., Chardigny, J. M. 2010. Differential impact of milk fatty acid profiles on cardiovascular risk biomarkers in healthy men and women. European Journal of Clinical Nutrition 64(7):752-759.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., Rosen, H. 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 296(5566):346-349.

MAPA. 2009. La alimentación en España. Dirección general de alimentación, Madrid.

Mather, I., Keenan, T. 1998. Origin and Secretion of Milk Lipids. Journal of Mammary Glandule Biology Neoplasia 3(3):259-273.

McDaniel, M. A., Maier, S. F., Einstein, G. O. 2003. "Brain-specific" nutrients: a memory cure? Nutrition 19(11-12):957-975.

Meck, W. H., Williams, C. L. 1999. Choline supplementation during prenatal development reduces proactive interference in spatial memory. Developmental Brain Research 118(1):51-59.

Merrill, A. H. 2011. Sphingolipid and Glycosphingolipid Metabolic Pathways in the Era of Sphingolipidomics. Chemical Reviews 111(10):6387-6422.

Mi, W., van Wijk, N., Cansev, M., Sijben, J. W., Kamphuis, P. J. 2013. Nutritional approaches in the risk reduction and management of Alzheimer's disease. Nutrition 29(9):1080-1089

Michalski, M.C. 2009. Specific molecular and colloidal structures of milk fat affecting lipolysis, absorption and postprandial lipemia. European Journal of Lipid Science and Technology 111(5):413-431.

Miranda, D. T., Batista, V. G., Grando, F. C., Paula, F. M., Felicio, C. A., Rubbo, G. F., Fernandes, L. C., Curi, R., Nishiyama, A. 2008. Soy lecithin supplementation alters macrophage phagocytosis and lymphocyte response to concanavalin A: a study in alloxan-induced diabetic rats. Cell Biochemistry and Function 26(8):859-865.

Mitchell, P. L., McLeod, R. S. 2008. Conjugated linoleic acid and atherosclerosis: studies in animal models. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire 86(4):293-301.

Moate, P. J., Chalupa, W., Boston, R. C., Lean, I. J. 2007. Milk Fatty Acids. I. Variation in the Concentration of Individual Fatty Acids in Bovine Milk. Journal of Dairy Science 90(10):4730-4739.

Modrak, D. E., Cardillo, T. M., Newsome, G. A., Goldenberg, D. M., Gold, D. V. 2004. Synergistic Interaction between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer. Cancer Research 64(22):8405-8410.

Modrak, D. E., Gold, D. V., Goldenberg, D. M. 2006. Sphingolipid targets in cancer therapy. Molecular Cancer Therapeutics 5(2):200-208.

Modrak, D. E., Lew, W., Goldenberg, D. M., Blumenthal, R. 2000. Sphingomyelin potentiates chemotherapy of human cancer xenografts. Biochemical and Biophysical Research Communications 268(2):603-606.

Modrak, D. E., Rodriguez, M. D., Goldenberg, D. M., Lew, W., Blumenthal, R. D. 2002. Sphingomyelin enhances chemotherapy efficacy and increases apoptosis in human colonic tumor xenografts. International Journal of Oncology 20(2):379-384.

Monteleone, P., Beinat, L., Tanzillo, C., Maj, M., Kemali, D. 1990. Effects of phosphatidylserine on the neuroendocrine response to physical stress in humans. Neuroendocrinology 52(3):243-248.

Morin, P., Jimenez-Flores, R., Pouliot, Y. 2007. Effect of processing on the composition and microstructure of buttermilk and its milk fat globule membranes. International Dairy Journal 17(10):1179-1187.

Morin, P., Jiménez-Flores, R., Pouliot, Y. 2004. Effect of Temperature and Pore Size on the Fractionation of Fresh and Reconstituted Buttermilk by Microfiltration. Journal of Dairy Science 87(2):267-273.

Mozaffarian, D., Aro, A., Willett, W. C. 2009. Health effects of *trans*-fatty acids: experimental and observational evidence. European Journal of Clinical Nutrition 63 Suppl 2:S5-21.

Mozzi, R., Buratta, S., Goracci, G. 2003. Metabolism and Functions of Phosphatidylserine in Mammalian Brain. Neurochemistry Research 28(2):195-214.

Mulbry, W., Kondrad, S., Buyer, J., Luthria, D. 2009. Optimization of an Oil Extraction Process for Algae from the Treatment of Manure Effluent. Journal of the American Oil Chemists´ Society 86(9):909-915.

Murgia, S., Mele, S., Monduzzi, M. 2003. Quantitative characterization of phospholipids in milk fat via 31P NMR using a monophasic solvent mixture. Lipids 38(5):585-591.

Nagata, T., Yaguchi, T., Nishizaki, T. 2011. DL- and PO-phosphatidylcholines as a promising learning and memory enhancer. Lipids in Health and Disease 10(1):25.

Navder, K. P., Baraona, E., Lieber, C. S. 1997. Polyenylphosphatidylcholine attenuates alcohol-induced fatty liver and hyperlipemia in rats. The Journal of Nutrition 127(9):1800-1806.

Niederau, C., Strohmeyer, G., Heintges, T., Peter, K., Gopfert, E. 1998. Polyunsaturated phosphatidyl-choline and interferon alpha for treatment of chronic hepatitis B and C: a multi-center, randomized, double-blind, placebo-controlled trial. Leich Study Group. Hepato-gastroenterology 45(21):797-804.

Nilsson, A., Duan, R. D. 2006. Absorption and lipoprotein transport of sphingomyelin. Journal of Lipid Research 47(1):154-171.

Noh, S. K., Koo, S. I. 2004. Milk sphingomyelin is more effective than egg sphingomyelin in inhibiting intestinal absorption of cholesterol and fat in rats. The Journal of Nutrition 134(10):2611-2616.

O'Fallon, J. V., Busboom, J. R., Nelson, M. L., Gaskins, C. T. 2007. A direct method for fatty acid methyl ester synthesis: Application to wet meat tissues, oils, and feedstuffs. Journal of Animal Science 85(6):1511-1521.

O'Keeffe, M., St-Onge, M. P. 2013. Saturated Fat and Cardiovascular Disease: A Review of Current Evidence. Current Cardiovascular Risk Reports 7(2):154-162.

Oh, J.-J., Lee, J.-S., Lim, J.-N., Wang, T., Kim, S.-H., Lee, H.-G. 2014. *Trans* vaccenic acid (*trans*-11 18:1), a precursor of *cis*-9, *trans*-11-conjugated linoleic acid, exerts a direct anti-carcinogenic function in T47D breast carcinoma cells. Food Science and Biotechnology 23(2):641-646.

Ohlsson, L., Burling, H., Nilsson, A. 2009. Long term effects on human plasma lipoproteins of a formulation enriched in butter milk polar lipid. Lipids in Health Disease 8:44.

Oosthuizen, W., Vorster, H., Vermaak, W., Smuts, C., Jerling, J., Veldman, F., Burger, H. 1998. Lecithin has no effect on serum lipoprotein, plasma fibrinogen and macro molecular protein complex levels in hyperlipidaemic men in a double-blind controlled study. European Journal of Clinical Nutrition 52(6):419.

Oosting, A., Kegler, D., Wopereis, H. J., Teller, I. C., van de Heijning, B. J., Verkade, H. J., van der Beek, E. M. 2012. Size and phospholipid coating of lipid droplets in the diet of young mice modify body fat accumulation in adulthood. Pediatric Research 72(4):362-369.

Pandey, N. R., Sparks, D. L. 2008. Phospholipids as cardiovascular therapeutics. Current Opinion in Investigational Drugs 9(3):281-285.

Pariza, M. W. 2004. Perspective on the safety and effectivenes of conjugated linoleic acid. American Journal of Clinical Nutrion 79(6, Suppl.):1132S-1136S.

Pariza, M. W., Park, Y., Cook, M. E. 2001. The biologically active isomers of conjugated linoleic acid. Proggress in Lipid Research 40(4):283-298.

Park, K. M., Fulgoni, V. L., 3rd. 2013. The association between dairy product consumption and cognitive function in the National Health and Nutrition Examination Survey. The British Journal of Nutrition 109(6):1135-1142.

Parodi, P. W. 2004. Milk fat in human nutrition. Australian Journal of Dairy Technology 59(1):3-59

Parodi, P. W. 2009. Has the association between saturated fatty acids, serum cholesterol and coronary heart disease been over emphasized? International Dairy Journal 19(6-7):345-361.

Patel, N., Vogel, R., Chandra-Kuntal, K., Glasgow, W., Kelavkar, U. 2014. A novel three serum phospholipid panel differentiates normal individuals from those with prostate cancer. PLoS One 9(3):e88841.

Patton, S., Keenan, T. W. 1975. The Milk fat globule membrane. Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes 415(3):273-309.

Pepeu, G. 2003. Is There Evidence That Phospholipid Administration Is Beneficial for Your Brain? in Nutrition and Biochemistry of Phospholipids. AOCS Publishing.

Pepeu, G., Pepeu, I. M., Amaducci, L. 1996. A review of phosphatidylserine pharmacological and clinical effects. Is phosphatidylserine a drug for the ageing brain?. Pharmacological Research 33(2):73-80.

Pettus, B. J., Bielawska, A., Subramanian, P., Wijesinghe, D. S., Maceyka, M., Leslie, C. C., Evans, J. H., Freiberg, J., Roddy, P., Hannun, Y. A., Chalfant, C. E. 2004. Ceramide 1-Phosphate Is a Direct Activator of Cytosolic Phospholipase A2. Journal of Biological Chemistry 279(12):11320-11326.

Phleger, C. F., Nichols, P. D., Virtue, P. 1998. Lipids and trophodynamics of Antarctic zooplankton. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 120(2):311-323.

Ravnskov, U. 2014. Lack of evidence that saturated fat causes cardiovascular disease. British Medical Journal 348:g3205.

Richmond, B. L., Boileau, A. C., Zheng, S., Huggins, K. W., Granholm, N. A., Tso, P., Hui, D. Y. 2001. Compensatory phospholipid digestion is required for cholesterol absorption in pancreatic phospholipase A deficient mice. Gastroenterology 120(5):1193-1202.

Ristić Medić, D., Ristić, V., Arsić, A., Poštić, M., Ristić, G., Blaženčić Mladenović, V., Tepšić, J. 2006. Effects of soybean D-LeciVita product on serum lipids and fatty acid composition in type 2 diabetic patients with hyperlipidemia. Nutrition, Metabolism and Cardiovascular Diseases 16(6):395-404.

Robertson, B., Burri, L., Berge, K. 2014. Genotoxicity test and subchronic toxicity study with Superba™ krill oil in rats. Toxicology Reports 1(0):764-776.

Rodríguez-Alcalá, L. M., Calvo, M. V., Villar-Tajadura, M. A., Castro-Gómez, M. d. P., Holgado, F., Juárez, M., Fontecha., J. 2010. Lípidos bioactivos en productos lácteos. en Avances en la investigación de la alimentación funcional. J. Fontecha, ed. Programa CYTED.

Rodríguez-Alcalá, L. M., Fontecha, J. 2010. Major lipid classes separation of buttermilk, and cows, goats and ewes milk by high performance liquid chromatography with an evaporative light scattering detector focused on the phospholipid fraction. Journal of Chromatography A 1217(18):3063-3066.

Rombaut, Camp, J. V., Dewettinck, K. 2006. Phospho- and sphingolipid distribution during processing of milk, butter and whey. Journal of Food Technology 41(4):435-443.

Rombaut, R., Camp, J. V., Dewettinck, K. 2005. Analysis of Phospho- and Sphingolipids in Dairy Products by a New HPLC Method. Journal of Dairy Science 88(2):482-488.

Rombaut, R., Dewettinck, K. 2006. Properties, analysis and purification of milk polar lipids. International Dairy Journal 16(11):1362-1373.

Rombaut, R., Dewettinck, K. 2007. Thermocalcic Aggregation of Milk Fat Globule Membrane Fragments from Acid Buttermilk Cheese Whey. Journal of Dairy Science 90(6):2665-2674.

Rombaut, R., Dewettinck, K., Van Camp, J. 2007. Phospho- and sphingolipid content of selected dairy products as determined by HPLC coupled to an evaporative light scattering detector (HPLC-ELSD). Journal of Food Composition and Analysis 20(3-4):308-312.

Ryder, J. W., Portocarrero, C. P., Song, X. M., Cui, L., Yu, M., Combatsiaris, T., Galuska, D., Bauman, D. E., Barbano, D. M., Charron, M. J., Zierath, J. R., Houseknecht, K. L. 2001. Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. Diabetes 50(5):1149-1157.

Saito, H., Ishihara, K. 1997. Antioxidant activity and active sites of phospholipids as antioxidants. Journal American Oil Chemistry Society 74:1531-1536.

Sakakima, Y., Hayakawa, A., Nagasaka, T., Nakao, A. 2007. Prevention of hepatocarcinogenesis with phosphatidylcholine and menaquinone-4: *In vitro* and *in vivo* experiments. Journal of Hepatology 47(1):83-92.

Sakakima, Y., Hayakawa, A., Nakao, A. 2009. Phosphatidylcholine induces growth inhibition of hepatic cancer by apoptosis via death ligands. Hepato-Gastroenterology 56(90):481-484.

Sales-Campos, H., Souza, P. R., Peghini, B. C., da Silva, J. S., Cardoso, C. R. 2013. An overview of the modulatory effects of oleic acid in health and disease. Mini Reviews in Medicinal Chemistry 13(2):201-210.

Sampalis, F., Bunea, R., Pelland, M. F., Kowalski, O., Duguet, N., Dupuis, S. 2003. Evaluation of the effects of Neptune Krill Oil on the management of premenstrual syndrome and dysmenorrhea. Alternative Medicine Review : a Journal of Clinical Therapeutic 8(2):171-179.

Sánchez-Juanes, F., Alonso, J. M., Zancada, L., Hueso, P. 2009a. Distribution and fatty acid content of phospholipids from bovine milk and bovine milk fat globule membranes. International Dairy Journal 19(5):273-278.

Sánchez-Juanes, F., Alonso, J. M., Zancada, L., Hueso, P. 2009b. Glycosphingolipids from bovine milk and milk fat globule membranes: A comparative study. Adhesion to enterotoxigenic *Escherichia coli* strains. Biological Chemistry 390(1):31-40.

Schmelz, E. M. 2004. Sphingolipids in the chemoprevention of colon cancer. Frontiers in Bioscience: a Journal and Virtual Library 9:2632.

Schmelz, E. M., Dillehay, D. L., Webb, S. K., Reiter, A., Adams, J., Merrill Jr, A. H. 1996. Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1, 2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. Cancer Research 56(21):4936-4941.

Schneider, H., Braun, A., Füllekrug, J., Stremmel, W., Ehehalt, R. 2010. Lipid Based Therapy for Ulcerative Colitis—Modulation of Intestinal Mucus Membrane Phospholipids as a Tool to Influence Inflammation. International Journal of Molecular Sciences 11(10):4149-4164.

Sevastou, I., Kaffe, E., Mouratis, M.-A., Aidinis, V. 2013. Lysoglycerophospholipids in chronic inflammatory disorders: The PLA2/LPC and ATX/LPA axes. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1831(1):42-60.

Simonsson, P., Nilsson, A., Akesson, B. 1982. Postprandial effects of dietary phosphatidylcholine on plasma lipoproteins in man. American Journal of Clinical Nutrition 35(1):36-41.

Singal, A. K., Jampana, S. C., Weinman, S. A. 2011. Antioxidants as therapeutic agents for liver disease. Liver International 31(10):1432-1448.

Singh, H. 2006. The milk fat globule membrane—A biophysical system for food applications. Current Opinion in Colloid & Interface Science 11(2–3):154-163.

Singh, R., de Meester, F., Mechirova, V., Pella, D., Otsuka, K. 2008. Fatty Acids in the Causation and Therapy of Metabolic Syndrome. Pages 263-284 in Wild-Type Food in Health Promotion and Disease Prevention. F. De Meester and R. Watson, ed. Humana Press.

Sinibaldi, L., Goldoni, P., Pietropaolo, V., Cattani, L., Peluso, C., Di Taranto, C. 1992. Role of phospholipids in BK virus infection and haemagglutination. Microbiologica 15(4):337-344.

Snow, D. R., Jimenez-Flores, R., Ward, R. E., Cambell, J., Young, M. J., Nemere, I., Hintze, K. J. 2010. Dietary Milk Fat Globule Membrane Reduces the Incidence of Aberrant Crypt Foci in Fischer-344 Rats. Journal of Agricultural Food Chemistry 58(4):2157-2163.

Sodini, I., Morin, P., Olabi, A., Jimenez-Flores, R. 2006. Compositional and Functional Properties of Buttermilk: A Comparison Between Sweet, Sour, and Whey Buttermilk. Journal of Dairy Science 89(2):525-536.

Soel, S. M., Choi, O. S., Bang, M. H., Yoon Park, J. H., Kim, W. K. 2007. Influence of conjugated linoleic acid isomers on the metastasis of colon cancer cells *in vitro* and *in vivo*. The Journal of Nutritional Biochemistry 18(10):650-657.

Song, H. J., Grant, I., Rotondo, D., Mohede, I., Sattar, N., Heys, S. D., Wahle, K. W. J. 2005. Effect of CLA supplementation on immune function in young healthy volunteers. European Journal of Clinical Nutrition 59(4):508-517.

Speelmans, G., Vriesema, A. J. M., Hofman, Z., Kivit, I., Hageman, R. J. J. 2004. Enteral composition for the prevention and/or treatment of sepsis. Google Patents. WO 2004068969 A1.

Spence, A. J., Jimenez-Flores, R., Qian, M., Goddik, L. 2009. Phospholipid enrichment in sweet and whey cream buttermilk powders using supercritical fluid extraction. Journal of Dairy Science 92(6):2373-2381.

Spitsberg, V. L. 2005. Invited review: Bovine milk fat globule membrane as a potential nutraceutical. Journal of Dairy Science 88(7):2289-2294.

Sprong, C., Hulstein, M., Van der Meer, R. 1998. Phospholipid-rich butter milk decreases the gastro-intestinal survival and translocation of listeria in rats. Gastroenterology 114:A1090.

Sprong, R. C., Hulstein, M. F. E., Van der Meer, R. 2001. Bactericidal Activities of Milk Lipids. Antimicrobial Agents and Chemotherapy 45(4):1298-1301.

Starks, M. A., Starks, S. L., Kingsley, M., Purpura, M., Jäger, R. 2008. The effects of phosphatidylserine on endocrine response to moderate intensity exercise. Journal of the International Society of Sports Nutrition 5:11-11.

Steijns, J. M. 2008. Dairy products and health: Focus on their constituents or on the matrix? International Dairy Journal 18(5):425-435.

Stremmel, W., Hanemann, A., Ehehalt, R., Karner, M., Braun, A. 2010. Phosphatidylcholine (lecithin) and the mucus layer: Evidence of therapeutic efficacy in ulcerative colitis?. Digestive Diseases 28(3):490-496.

Sutherland, W. H., de Jong, S. A., Walker, R. J. 2007. Effect of dietary cholesterol and fat on cell cholesterol transfer to postprandial plasma in hyperlipidemic men. Lipids 42(10):901-911.

Tall, A. R., Blum, C. B., Grundy, S. M. 1983. Incorporation of radioactive phospholipid into subclasses of high-density lipoproteins. The American Journal of Physiology 244(5):E513-516.

Tanaka, K., Hosozawa, M., Kudo, N., Yoshikawa, N., Hisata, K., Shoji, H., Shinohara, K., Shimizu, T. 2013. The pilot study: sphingomyelin-fortified milk has a positive association with the neurobehavioural development of very low birth weight infants during infancy, randomized control trial. Brain & development 35(1):45-52.

Tandy, S., Chung, R. W. S., Wat, E., Kamili, A., Berge, K., Griinari, M., Cohn, J. S. 2009. Dietary Krill Oil Supplementation Reduces Hepatic Steatosis, Glycemia, and Hypercholesterolemia in High-Fat-Fed Mice. Journal of Agricultural Food Chemistry 57(19):9339-9345.

Taylor, L., Pletschen, L., Arends, J., Unger, C., Massing, U. 2010. Marine phospholipids—a promising new dietary approach to tumor-associated weight loss. Support Care Cancer 18(2):159-170.

Tholstrup, T., Sandström, B., Hermansen, J., Hølmer, G. 1998. Effect of modified dairy fat on postprandial and fasting plasma lipids and lipoproteins in healthy young men. Lipids 33(1):11-21.

Timby, N., Domellöf, E., Hernell, O., Lönnerdal, B., Domellöf, M. 2014. Neurodevelopment, nutrition, and growth until 12 mo of age in infants fed a low-energy, low-protein formula supplemented with bovine milk fat globule membranes: a randomized controlled trial. American Journal of Clinical Nutrition.

Toschi, T. G., Bendini, A., Ricci, A., Lercker, G. 2003. Pressurized solvent extraction of total lipids in poultry meat. Food Chemistry. 83(4):551-555.

Tou, J. C., Jaczynski, J., Chen, Y.-C. 2007. Krill for Human Consumption: Nutritional Value and Potential Health Benefits. Nutrition Reviews 65(2):63-77.

Touitou, E. 2012. Sanitizing compositions. Google Patents. CA 2775929 A1.

Treede, I., Braun, A., Sparla, R., Kuhnel, M., Giese, T., Turner, J. R., Anes, E., Kulaksiz, H., Fullekrug, J., Stremmel, W., Griffiths, G., Ehehalt, R. 2007. Anti-inflammatory effects of phosphatidylcholine. The Journal of Biological Chemistry 282(37):27155-27164.

Tweedy, J. R., Garcia, C. A. 1982. Lecithin treatment of cognitively impaired Parkinson's patients. European journal of clinical investigation 12(1):87-90.
Uauy, R., Dangour, A. D. 2006. Nutrition in brain development and aging: role of essential fatty acids. Nutrition Reviews 64(5 Pt 2):S24-33.

Ulven, S., Kirkhus, B., Lamglait, A., Basu, S., Elind, E., Haider, T., Berge, K., Vik, H., Pedersen, J. 2011. Metabolic Effects of Krill Oil are Essentially Similar to Those of Fish Oil but at Lower Dose of EPA and DHA, in Healthy Volunteers. Lipids 46(1):37-46.

Van Hooijdonk, A. C., Kussendrager, K. D., Steijns, J. M. 2000. *In vivo* antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. British Journal of Nutrition 84:S127-S134.
van Meer, G., Voelker, D. R., Feigenson, G. W. 2008. Membrane lipids: where they are and how they behave. Nature reviews. Molecular Cell Biology 9(2):112-124.

van Vlerken, L. E., Duan, Z., Seiden, M. V., Amiji, M. M. 2007. Modulation of intracellular ceramide using polymeric nanoparticles to overcome multidrug resistance in cancer. Cancer Research 67(10):4843-4850.

Vance, D. E., Vance, J. E. 2008. Chapter 8 - Phospholipid biosynthesis in eukaryotes. Pages 213-VI in Biochemistry of Lipids, Lipoproteins and Membranes (Fifth Edition). D. E. V. E. Vance, ed. Elsevier, San Diego.

Vanderghem, C., Bodson, P., Danthine, S., Paquot, M., Deroanne, C., Blecker, C. 2010. Milk fat globule membrane and buttermilks: From composition to valorization. La membrane du globule gras du lait et les babeurres: De leur composition à leur valorisation 14(3):485-500.

Veereman-Wauters, G., Staelens, S., Rombaut, R., Dewettinck, K., Deboutte, D., Brummer, R. J., Boone, M., Le Ruyet, P. 2012. Milk fat globule membrane (INPULSE) enriched formula milk decreases febrile episodes and may improve behavioral regulation in young children. Nutrition 28(7-8):749-752.

Vigerust, N., Bjørndal, B., Bohov, P., Brattelid, T., Svardal, A., Berge, R. 2013. Krill oil versus fish oil in modulation of inflammation and lipid metabolism in mice transgenic for TNF-α. European Journal of Nutrition 52(4):1315-1325.

Visioli, F., Strata, A. 2014. Milk, Dairy Products, and Their Functional Effects in Humans: A Narrative Review of Recent Evidence. Advances in Nutrition: An International Review Journal 5(2):131-143.

Vors, C., Pineau, G., Gabert, L., Drai, J., Louche-Pelissier, C., Defoort, C., Lairon, D., Desage, M., Danthine, S., Lambert-Porcheron, S., Vidal, H., Laville, M., Michalski, M. C. 2013. Modulating absorption and postprandial handling of dietary fatty acids by structuring fat in the meal: a randomized crossover clinical trial. American Journal of Clinical Nutrition 97(1):23-36.

Waldemar, T. 2002. Egg Lipids. in Chemical and Functional Properties of Food Lipids CRC Press.

Wang, T., Hammond, E., Fehr, W. 1997. Phospholipid fatty acid composition and stereospecific distribution of soybeans with a wide range of fatty acid composition. Journal of the American Oil Chemist´ Society 74(12):1587-1594.

Wang, X. D., Andersson, R., Soltesz, V., Wang, W. Q., Ar'rajab, A., Bengmark, S. 1994. Phospholipids prevent enteric bacterial translocation in the early stage of experimental acute liver failure in the rat. Scandinavian Journal of Gastroenterology 29(12):1117-1121.

Wang, Y., Jacome-Sosa, M. M., Proctor, S. D. 2012. The role of ruminant *trans* fat as a potential nutraceutical in the prevention of cardiovascular disease. Food Research International 46(2):460-468.

Wat, E., Tandy, S., Kapera, E., Kamili, A., Chung, R. W., Brown, A., Rowney, M., Cohn, J. S. 2009. Dietary phospholipid-rich dairy milk extract reduces hepatomegaly, hepatic steatosis and hyperlipidemia in mice fed a high-fat diet. Atherosclerosis 205(1):144-150.

Weihrauch, J., Son, Y.-S. 1983. Phospholipid content of foods. Journal of the American Oil Chemists' Society 60(12):1971-1978.

Wijendran, V., Huang, M. C., Diau, G. Y., Boehm, G., Nathanielsz, P. W., Brenna, J. T. 2002. Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. Pediatric Research 51(3):265-272.

Winther, B., Hoem, N., Berge, K., Reubsaet, L. 2010. Elucidation of phosphatidylcholine composition in krill oil extracted from *Euphausia superba*. Lipids 46(1):25-36.

Wójcicki, J., Pawlik, A., Samochowiec, L., Kaldoǹska, M., Myśliwiec, Z. 1995. Clinical evaluation of lecithin as a lipid-lowering agent. Phytotherapy Research 9(8):597-599.

Xu, Y., Leo, M. A., Lieber, C. S. 2005. DLPC attenuates alcohol-induced cytotoxicity in HepG2 cells expressing CYP2E1. Alcohol and Alcoholism 40(3):172-175.

Yamasaki, M., Kishihara, K., Ikeda, I., Sugano, M., Yamada, K. 1999. A recommended esterification method for gas chromatographic measurement of conjugated linoleic acid. Journal of the American Oil Chemists' Society 76(8):933-938.

Yamashita, A., Oka, S., Tanikawa, T., Hayashi, Y., Nemoto-Sasaki, Y., Sugiura, T. 2013. The actions and metabolism of lysophosphatidylinositol, an endogenous agonist for GPR55. Prostaglandins & Other Lipid Mediators 107(0):103-116.

Yan, X., Li, H., Xu, J., Zhou, C. 2010. Analysis of phospholipids in microalga Nitzschia closterium by UPLC-Q-TOF-MS. Chinese Journal of Oceanology and Limnology 28(1):106-112.

Zanabria, R., Tellez, A., Griffiths, M., Corredig, M. 2014. The antiproliferative properties of the milk fat globule membrane are affected by extensive heating. Dairy Science of Technology 94(5):439-453.

Zanabria, R., Tellez, A. M., Griffiths, M., Corredig, M. 2013. Milk fat globule membrane isolate induces apoptosis in HT-29 human colon cancer cells. Food & Function 4(2):222-230.

Zeisel, S. H. 2004. Nutritional importance of choline for brain development. Journal of the American College of Nutrition 23(6 SUPPL.):621S-626S.

Zhang, P., Li, B., Gao, S., Duan, R.-D. 2008. Dietary sphingomyelin inhibits colonic tumorigenesis with an up-regulation of alkaline sphingomyelinase expression in ICR mice. Anticancer Research 28(6A):3631-3635.

Zhao, W. S., Zhai, J. J., Wang, Y. H., Xie, P. S., Yin, X. J., Li, L. X., Cheng, K. L. 2009. Conjugated linoleic acid supplementation enhances antihypertensive effect of ramipril in Chinese patients with obesity-related hypertension. American Journal of Hypertension 22(6):680-686.

Zhou, L., Le Grandois, J., Marchioni, E., Zhao, M., Ennahar, S., Bindler, F. 2010. Improvement of total lipid and glycerophospholipid recoveries from various food matrices using pressurized liquid extraction. Journal of Agricultural Food Chemistry 58(18):9912-9917.

Zhou, L., Zhao, M., Ennahar, S., Bindler, F., Marchioni, E. 2012. Liquid Chromatography–Tandem Mass Spectrometry for the Determination of Sphingomyelin Species from Calf Brain, Ox Liver, Egg Yolk, and Krill Oil. Journal of Agricultural Food Chemistry 60(1):293-298.

Zhou, Q., Gao, B., Zhang, X., Xu, Y., Shi, H., Yu, L. L. 2014. Chemical profiling of triacylglycerols and diacylglycerols in cow milk fat by ultra-performance convergence chromatography combined with a quadrupole time-of-flight mass spectrometry. Food Chemistry 143:199-204.

Zierenberg, O., Grundy, S. 1982. Intestinal absorption of polyenephosphatidylcholine in man. Journal of Lipid Research 23(8):1136-1142.