UNIVERSIDAD DE CÓRDOBA

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

DESARROLLO DE PLATAFORMAS DE ANÁLISIS ORIENTADO Y GLOBAL EN METABOLÓMICA VEGETAL Y CLÍNICA Y EN NUTRIMETABOLÓMICA

Mara Isabel Orozco Solano

Córdoba, diciembre de 2012

TITULO: *DESARROLLO DE PLATAFORMAS DE ANÁLISIS ORIENTADO Y GLOBAL EN METABOLÓMICA VEGETAL Y CLÍNICA Y EN NUTRIMETABOLÓMICA*

AUTOR: *MARA ISABEL OROZCO SOLANO*

© Edita: Servicio de Publicaciones de la Universidad de Córdoba.
Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

TÍTULO DE LA TESIS: DESARROLLO DE PLATAFORMAS DE ANÁLISIS ORIENTADO Y GLOBAL EN METABOLÓMICA VEGETAL Y CLÍNICA Y EN NUTRIMETABOLÓMICA

DOCTORANDA: MARA ISABEL OROZCO SOLANO

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El trabajo realizado por la doctoranda Mara Isabel Orozco Solano en el desarrollo de su Tesis Doctoral ha sido muy bueno, no sólo por el volumen de trabajo realizado, sino también por su alta calidad. Prueba de ello son las 13 contribuciones científicas que han derivado en publicaciones en revistas internacionales de prestigio. Los trabajos desarrollados en el último período se encuentran actualmente en período de revisión y otros se han remitido para su publicación.

Cabe destacar que la tesis doctoral no sólo ha derivado en una abundante publicación científica, sino que, además, forma parte de la nueva línea de investigación en el grupo donde se integra la doctoranda y también en la UCO, en la que no se había investigado en Metabolómica hasta la fecha.

Sin duda, puede afirmarse que la doctoranda ha conseguido alcanzar el nivel deseado para un doctor con un prometedor futuro como investigador.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de noviembre de 2012

Firma de los directores

Prof. Dr. M.D. Luque de Castro

Dr. F. Priego Capote

Fdo.: María Dolores Luque de Castro

Fdo.: Feliciano Priego Capote

DESARROLLO DE PLATAFORMAS DE ANÁLISIS ORIENTADO Y GLOBAL EN METABOLÓMICA VEGETAL Y CLÍNICA Y EN NUTRIMETABOLÓMICA

Los directores,

Fdo.: María Dolores Luque de Castro
Catedrática del Departamento
de Química Analítica
Universidad de Córdoba

Fdo.: Feliciano Priego Capote
Doctor Contratado Ramón
y Cajal del Departamento
de Química Analítica
Universidad de Córdoba

Trabajo presentado para optar al grado de
Doctora en Ciencias, Sección Químicas

Fdo.: Mara Isabel Orozco Solano
Licenciada en Química

María Dolores Luque de Castro y **Feliciano Priego Capote**, en calidad de directores de la Tesis Doctoral presentada por la licenciada en Química, Mara Isabel Orozco Solano, con el título **"Desarrollo de plataformas de análisis orientado y global en metabolómica vegetal y clínica y en nutrimetabolómica"**

CERTIFICAN:

Que la citada Tesis Doctoral se ha realizado en los laboratorios del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba y que, a su juicio, reúne los requisitos necesarios exigidos en este tipo de trabajos.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, 2012

Fdo.: María Dolores Luque de Castro Fdo.: Feliciano Priego Capote

AGRADECIMIENTOS

Un trabajo de investigación no es un trabajo individual, existió mucha gente ayudándome de diferentes formas de las cuales estaré inmensamente agradecida. Desearía poder citar a todas esas personas que de alguna forma contribuyeron a que el desarrollo de esta tesis se diera, sin embargo resaltaré en primer lugar, agradecer a mis directores de tesis María Dolores Luque y Feliciano Priego Capote por su profesionalismo y su continuo esfuerzo. Ellos han sido un excelente modelo a seguir. Debo agradecer la confianza depositada al aceptarme como su estudiante de doctorado, dándome la oportunidad de trabajar en su grupo de investigación y de adquirir la destreza y profesionalismo que sobresale en cada uno de los trabajos realizados con ellos. Los dos conforman un grupo que aporta a cada uno de nosotros como doctorandos las destrezas y capacidades propias de una dotada experiencia y rigor científico. A la Dra María Dolores Luque agradecerle todo el apoyo constante y la motivación en los momentos más díficiles de mi estancia lejos de casa; su gran capacidad investigadora, su tenacidad en el trabajo y su invalorable paciencia hicieron posible la realización de esta Memoria. A el Dr. Feliciano Priego, un pilar a seguir en nuestro grupo, su gran dedicación, su paciencia al orientarme en un mejorar continuo y sus valiosos aportes permitieron que el desarrollo de esta investigación fuera posible.

Quiero agradecer a todo el grupo de investigación FQM-227 por su enorme apoyo, por ser mi familia durante esta larga estancia muy lejos de casa; a los nuevos compañeros que integran el grupo, Antonio y María, quienes desde ya se han ganado por su carisma y gran disponibilidad, se han ganado ya un lugar especial en la historia del grupo.

En especial a Chema y a Jose Ruíz, quienes fueron mis primeros maestros en el desmontaje de un equipo, me enseñaron a no tener miedo y a ser muy sistemática en el trabajo. Por los ratos de amistad y por ese apoyo continuo durante la finalización de esta tesis. Siempre estuvieron ahí cuando más lo necesite, infinitas gracias.

ÍNDICE

OBJETIVOS

El incuestionable crecimiento exponencial que ha experimentado la metabolómica en los últimos años es consecuencia tanto de la importancia de las contribuciones de esta "ómica de las moléculas pequeñas" como de su significativa aportación en áreas que habían sido dominio exclusivo de ómicas desarrolladas con anterioridad; lo que pone de manifiesto que se trata de una disciplina aceptada por la comunidad científica. Las diferentes subdisciplinas de la metabolómica han adquirido muy distinto grado de desarrollo. La importancia de algunas de ellas —como la lipidómica y la nutrimetabolómica o metabolómica nutricional— es tal que muchos autores las consideran disciplinas como tales y del mismo rango que la metabolómica.

Aunque las estrategias típicas de la metabolómica han sido tradicionalmente el análisis orientado o "targeted analysis", el perfil metabolómico global o "global metabolomics profiling" y la huella metabólica dactilar o "metabolomics fingerprinting", que en el caso de estudios en cultivos celulares se amplía a la huella pedicular o "metabolomics footprinting", actualmente se tiende a la simplificación estableciendo sólo distinción entre análisis orientado (targeted analysis) y análisis global (untargeted analysis). Estas dos estrategias difieren en el muestreo, etapas de preparación de la muestra, instrumentación requerida y tipo de información que proporcionan.

El *objetivo genérico* de la investigación que se recoge en esta Memoria fue desarrollar plataformas, tanto para análisis metabolómico orientado como global, que tuvieran una manifiesta utilidad en nutrimetabolómica y en metabolómica clínica, de forma que supusieran una contribución a la eliminación de las lagunas existentes en estas áreas de la

metabolómica, incrementando así la posibilidad de resolución de problemas por ella misma o integrada con otras ómicas (biología de sistemas).

Para el desarrollo de las plataformas la doctoranda ha tenido el apoyo de: (i) El equipamiento analítico del grupo de investigación en el que se integra, que consta de sistemas miniaturizados de preparación de muestra (laboratorio en una válvula, lab-on-valve o LOV), estaciones automáticas de extracción en fase sólida (SPE) acopladas en línea con cromatografía líquida (LC) o microcromatografía líquida (μ-LC) con detectores de fluorescencia inducida por láser (LIF), de absorción molecular (DAD), de espectrometría de masas con detector de tiempo de vuelo (TOF/MS), o en tándem con triple cuadrupolo (QqQ); cromatografía de gases con detector de masas de impacto electrónico (GC–MS/MS). (ii) La búsqueda y estudio de la información bibliográfica sobre metabolómica. (iii) La experiencia del grupo en el diseño de métodologías analíticas en las que materializar la aplicación de las plataformas instrumentales (con especial énfasis en la preparación de la muestra, tradicional línea de investigación del grupo). Con este soporte se planificaron los siguientes *objetivos concretos*, que se fueron materializando y se recogen en los diferentes capítulos que componen esta Memoria:

1. Revisar de forma exhaustiva y crítica la investigación existente sobre la fracción insaponificable del olivo como una etapa previa al diseño de plataformas para una adecuada contribución al estudio del metaboloma del olivo. La experiencia del grupo en estos estudios bibliográficos ha dado como resultado la publicación que se recoge como Capítulo 1.

Los objetivos concretos en investigación de laboratorio se dividieron en función de la estrategia utilizada, análisis orientado o global, y dentro del primero en función de la disciplina, nutrimetabolómica o metabolómica clínica. Con estos criterios se agrupó la investigación en bloques o partes en los que los objetivos fueron:

2. Desarrollar una plataforma basada en GC–MS/MS para la determinación de la fracción insaponificable (constituida en este caso por los esteroles y los alcoholes alifáticos) en hojas y frutos del olivo. Especial énfasis se hizo en la etapa de preparación de la muestra, en la que la lixiviación y la derivatización se aceleraron de forma drástica mediante ultrasonidos, tal como se muestra en el Capítulo 2. La aplicación de la plataforma a la caracterización de las dos fracciones en diferentes variedades de olivo constituye el Capítulo 3.

3. Realizar aportaciones de interés en el campo de la nutrimetabolómica con dos orientaciones diferentes: (i) Poner de manifiesto la influencia que ejerce el enriquecimiento de los aceites vegetales con extractos de inhibidores de la oxidación del olivo frente a los que se enriquecen con inhibidores sintéticos cuando se someten al proceso de fritura. (ii) Comprobar el efecto de la ingestión por individuos obesos de los aceites de fritura en función del tipo de aceite y de las características de los inhibidores de la oxidación utilizados para el enriquecimiento. La influencia del enriquecimiento de los aceites se estudió considerando la degradación experimentada por la fracción insaponificable (Capítulo 4) y por el contenido en inhibidores (Capítulo 5); mientras que los estudios de la influencia de estos aceites en pacientes obesos se realizó en muestras de suero en las que se determinaron alcoholes alifáticos, esteroles y triterpenos (Capítulo 6) y ácidos grasos (Capítulo 7) mediante GC–MS/MS en ambos casos y utilizando ultrasonidos para acelerar la etapa de derivatización. Un último estudio en nutrimetabolómica se realizó con una plataforma totalmente automatizada (SPE–LC–QqQ) para el estudio de fenoles presentes en aceite de oliva virgen y sus metabolitos en muestras de plasma de individuos sanos tras una ingesta abundante de aceite de oliva.

4. Diseñar plataformas para análisis cuantitativo en metabolómica clínica, en las que la automatización y miniaturización fuesen la clave para su aplicación al análisis sistemático con consumo mínimo de muestra. Con estas premisas junto con la no menos importante

de poner de manifiesto la aplicabilidad de la metabolómica en una variedad de fluidos biológicos, se diseñó una plataforma basada en el uso de un sistema LOV para miniaturizar la etapa SPE y la derivatización de los analitos (ácidos siálicos) para una mejor separación mediante μ-LC y una máxima sensibilidad en la detección de la fluorescencia de los productos inducida por láser. El método, aplicado a la determinación de los analitos en muestras de suero, orina, saliva y leche materna, se recoge en el Capítulo 9. Un método totalmente automatizado para estos analitos se desarrolló mediante el acoplamiento en línea de una estación automatizada de SPE, un LC y un detector de masas de triple cuadrupolo. Se consigue de esta forma reducir el volumen de muestra necesario para el análisis puesto que la totalidad de los metabolitos retenidos en el cartucho de SPE se eluye mediante la fase móvil cromatográfica. Se consigue de esta forma rebajar los límites de detección y cuantificación en un orden de magnitud, tal como se muestra en el Capítulo 10.

5. Diseñar y aplicar plataformas globales con las que obtener el perfil metabolómico del plasma obtenido de individuos sanos sometidos alimentación con contenido lipídico diferente en cantidad y en calidad. Un excelente diseño del estudio y del muestreo establecieron las bases para que la aplicación de plataformas basadas en LC–TOF/MS a las fracciones polar y no polar de las muestras de plasma proporcionaran, tras el tratamiento adecuado de los datos, los resultados que se recogen en los Capítulos 11 y 12.

La formación de la futura doctora, que constituye el *objetivo último* de toda tesis doctoral, ha incluido el máster en "Química Fina y Nanotecnología", en el que la doctoranda ha cursado el número de créditos correspondientes. Paralelamente a que la investigación recogida en la parte principal de la Memoria se ha publicado (o se encuentra en vías de publicación) en revistas analíticas o clínicas, dependiendo de su aspecto

predominante, esta investigación también se ha divulgado en congresos y reuniones científicas, con un total de 3 presentaciones, tal como se recoge en el Anexo.

INTRODUCCIÓN

En esta introducción se pretende dar una visión general de los aspectos que constituyen la columna vertebral de la investigación que se presenta en esta Memoria de Tesis Doctoral: La metabolómica en el contexto de las ómicas, sus características genéricas y analíticas y sus subdisciplinas, entre ellas la que entronca directamente con gran parte de la investigación realizada: La nutrimetabolómica. Las diferentes etapas de una plataforma analítica para estudios metabolómicos, además de otras herramientas utilizadas en la disciplina, como el análisis estadístico multivariante, también serán objeto de esta introducción.

La metabolómica

La más reciente de las grandes ómicas, como se considera a la metabolómica, puede definirse como una disciplina genérica dedicada al estudio cualitativo y cuantitativo de todas las especies de bajo peso molecular (típicamente <1500 Da) presentes en un sistema biológico dado. Recientemente, Ryan et al. [1] parecen decantarse por una definición que abarque la caracterización y la cuantificación de todos los metabolitos en una muestra, pero con la puntualización de que la metabolómica considera la resolución espacial (debida a la compartimentalización celular del metabolismo) y los cambios temporales del metaboloma en respuesta a los estímulos ambientales, extensibles a cualquier tipo de xenoestímulo. Por su parte el metaboloma se define como el complemento cuantitativo de los metabolitos, compuestos de bajo peso molecular presentes en un fluido biológico, célula u organismo en unas condiciones fisiológicas dadas [2]. Estas condiciones abarcan diferentes perturbaciones, tales como variaciones genéticas, estados patológicos o respuestas a estímulos externos.

1. Generalidades sobre la metabolómica

La metabolómica, al ser la última de las grandes ómicas, ha soportado su desarrollo en otras disciplinas ómicas. Este soporte justifica su evolución, similar a la que han experimentado la genómica, transcriptómica y proteómica, aunque en la actualidad presenta un menor desarrollo. Sin embargo, el creciente uso de la metabolómica y el indudable apoyo que supone para otras ómicas hacen previsible su consolidación en un futuro próximo. Una muestra del crecimiento de la nueva disciplina frente a la más consolidada proteómica se muestra en la Fig. 1, en la que se recoge la evolución de la proteómica y la metabolómica desde el comienzo de la primera (1997).

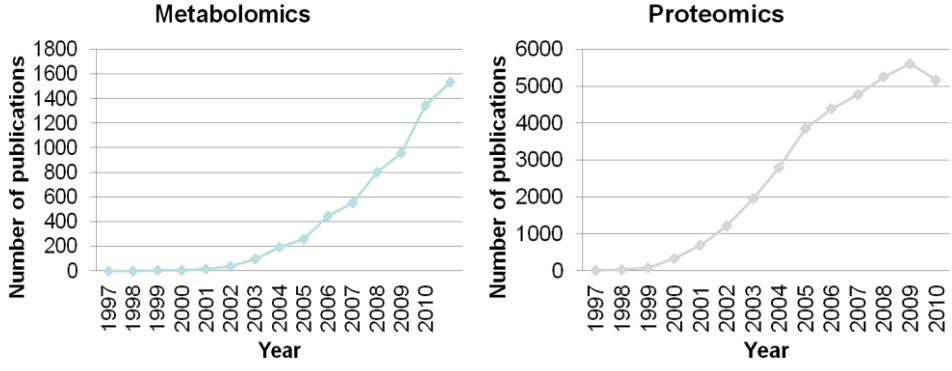


Figura 1. *Diagrama de la evolución del número de publicaciones sobre metabolómica y proteómica en el período 1997–2010.*

Aspectos en los que la metabolómica se diferencia claramente de las restantes ómicas son los siguientes: a) El número de metabolitos es menor que el número de genes, transcritos o proteínas, lo que reduce la complejidad de su estudio (e.g. publicaciones recientes sugieren que el número de metabolitos en el ser humano es de aproximadamente 7800

comparado con el gran el número de genes reportados en bases de datos para humanos (≈ 20000 [3]). (b) La obtención de un perfil metabolómico es más barato y rápido que los análisis en proteómica o en transcriptómica. c) La tecnología implicada en metabolómica es más genérica, ya que un metabolito dado es el mismo en cualquier organismo que lo contenga.

La metabolómica ha demostrado recientemente su potencial en muchas áreas, tales como la respuesta al estrés ambiental [4], la toxicología [5], la nutrición [6–10], los efectos de la manipulación genética [11], el diagnóstico de enfermedades [12], la medicina tradicional [13], la comparación de diferentes etapas del crecimiento [14] y el descubrimiento de nuevos productos naturales [15], entre otras. Como nueva estrategia con capacidad para la búsqueda de marcadores, la metabolómica ha focalizado también su atención en la identificación de biomarcadores con potencial prognóstico y/o diagnóstico. Con este propósito se han desarrollado plataformas analíticas que se han aplicado con éxito al análisis de biofluidos y/o tejidos en áreas tales como la fisiología, el diagnóstico, la genómica funcional, la farmacología, la toxicología y la nutrición, entre las más importantes.

Hay que puntualizar que el objetivo de la metabolómica es integrar los datos obtenidos mediante las plataformas analíticas en bases de datos que puedan utilizarse como herramientas eficaces para el diagnóstico de modelos de predicción. Para ello se requiere el manejo de un número grande de muestras para obtener resultados transferibles con los que se puedan establecer comparaciones entre organismos. Ello implica minimizar las fuentes de variabilidad entre muestras, que pueden ser debidas a factores intra- o inter-individuales principalmente causados por el estado fisiológico, la dieta, el ambiente o las condiciones genéticas o patológicas.

2. Estrategias analíticas en metabolómica

El papel que juegan las plataformas analíticas (y los métodos propuestos a través de ellas) en metabolómica es la de puente que une las muestras biológicas originales con los biomarcadores relacionados con una determinada enfermedad, un estado fisiológico o con rutas relacionadas con diferentes patologías. En otras palabras, la función de la metabolómica depende de la plataforma analítica diseñada y su capacidad para conseguir un perfil global de los metabolitos, de forma que estará más próxima a un comportamiento ideal cuanto más se ajuste a las siguientes características: (i) Proporcionar una información amplia sobre la muestra (e.g. tipos de metabolitos, rangos de concentración); (ii) ofrecer una frecuencia de muestreo grande (e.g. requerir poca o nula preparación de la muestra, análisis rápido); (iii) ser robusta (experimentar cambios pequeños o nulos en la respuesta instrumental para variaciones experimentales en un rango amplio); (iv) alterar la composición de la muestra mínimamente; (v) combinar la capacidad de identificación y cuantificación; (vi) ser asequible a laboratorios comunes (e.g. tener un precio bajo y ser fáciles de implantar).

Dependiendo de la situación en estudio el análisis en metabolómica abarca diferentes estrategias que se clasifican de la siguiente forma:

a) Análisis orientado o "targeted analysis", mediante el que se pretende el estudio cualitativo y cuantitativo de uno o, más comúnmente, un pequeño grupo de metabolitos de características químicas similares o implicados en una o varias rutas bioquímicas.

b) Análisis global "global metabolomics profiling" o "untargeted analysis", con el que se intenta cubrir un amplio rango de metabolitos utilizando una única plataforma analítica o varias que actúan de forma complementaria (e.g. GC–MS, LC–MS, CE–MS). Requerimientos de este tipo de análisis

son: (i) Extraer el mayor número posible de los metabolitos de la muestra con la eficacia adecuada; (ii) evitar la exclusión de metabolitos por sus propiedades químicas; (iii) ser al menos semicuantitativo a través de su relación con una muestra control; (iv) ser preferentemente cuantitativo, lo que es imprescindible para la interpretación de las rutas metabólicas, el cálculo de balances atómicos o el desarrollo de modelos de predicción; (v) proporcionar una alta eficiencia ("high throughput").

En estudios celulares, además, se considera, según se trabaje con fluidos intra y/o extracelulares (es decir, el endometaboloma o el exometaboloma o secretoma), la huella dactilar metabolómica y/o la huella pedicular, respectivamente. Mediante ambas huellas se pretende conseguir información para la clasificación de grupos de muestras o el cribado, principalmente.

La complejidad de la plataforma analítica depende sobre todo del objetivo del estudio metabolómico y del estado físico de la muestra biológica. Si el objetivo es un método orientado, la preparación de la muestra se focalizará en los metabolitos de interés y se utilizarán etapas de limpieza, aislamiento y preconcentración de los analitos; por el contrario, los métodos para análisis global son no selectivos y con ellos se pretende abarcar el mayor número posible de metabolitos. El estado físico de la muestra también determina el número de etapas requeridas para la preparación de la muestra, que es más laboriosa en general para muestras sólidas.

3. La relación de la metabolómica con otras ómicas: La biología de sistemas

Aunque el conocimiento de los organismos vivos a nivel molecular está todavía en su infancia, es evidente que las extensas investigaciones sobre la "cascada ómica", que engloba las cuatro grandes ómicas, son los soportes sobre los que se edificará la nueva ciencia basada en estas disciplinas (la biología de sistemas), que supera ampliamente el concepto clásico de la biología molecular. El análisis integrado de la respuesta de un organismo a una perturbación a nivel de genoma, transcriptoma, proteoma y/o metaboloma llevará a un mejor conocimiento de los mecanismos bioquímicos y biológicos implicados en los sistemas complejos (Fig. 2). La biología de sistemas introduce un nuevo modelo con el que integrar la respuesta de los sistemas y sus interacciones utilizando las técnicas más adecuadas para obtener datos cuantitativos para la construcción y validación de modelos. Es en este ámbito en el que el subfijo ómica (que proviene de la palabra latina "ome", que significa masa en el sentido de masivo o mucho) cobra su más amplio sentido dado el enorme número de datos que se requiere manejar, que ya es grande para cada una de las disciplinas, pero que se multiplica en el caso de la biología de sistemas. Actualmente existen unas 200 ómicas [16], muchas de las cuales son subdisciplinas de las grandes ómicas y están o pueden estar también relacionadas con la biología de sistemas.

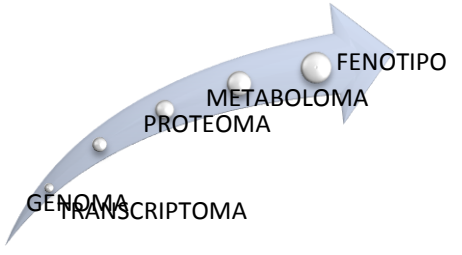


Figura 2. *El análisis integrado de los fenómenos a los diferentes niveles orgánicos describe la respuesta del organismo a las enfermedades, la genética y las perturbaciones ambientales.*

El hecho de que la metabolómica sea la más reciente de las grandes ómicas justifica que, mientras el desarrollo tecnológico en genómica, transcriptómica y proteómica ha sido muy significativo, las herramientas para un estudio extenso del metaboloma estén menos desarrolladas, si bien su relación con el dogma fundamental de la biología molecular haga que muchos de los logros de esta última sean utilizables en metabolómica [17]. Esta disciplina es el "punto final" de la cascada ómica, la más próxima al fenotipo y por tanto con una gran carga de información respecto a él, pero no existe aún (y es poco probable que se pueda conseguir) una única plataforma instrumental que permita analizar todos los metabolitos, de la misma forma que ocurre con las restantes grandes ómicas.

4. Las subdisciplinas de la metabolómica: Generalidades

La amplia variedad temática en metabolómica ha dado lugar a una serie de subdisciplinas que, en función de su extensión, pueden clasificarse en primarias y secundarias. Entre las primarias se encuentran la lipidómica, la nutrimetabolómica o metabolómica nutricional y la xenometabolómica, que se consideran a continuación.

Lipidómica: Esta subdisciplina es tan amplia, variada y su importancia tan grande que algunos autores la consideran como una ómica independiente y de la misma importancia que la propia metabolómica. Un criterio simple para definirla es como "la caracterización completa de todos los lípidos en un tipo de célula particular, tejido o biofluido". Un criterio más amplio y más complejo la define como "el conocimiento integral de todos los lípidos de un sistema biológico con respecto a señalizadores celulares, estructura de la membrana, modulación transcripcional y translacional, interacciones célula–célula y célula–proteína y la respuesta temporal causada por

cambios ambientales". Subdisciplinas de la lipidómica son la acilolipidómica, esfingolipidómica, esterolipidómica, glicerolipidómica, glicerofosfolipidómica, policetolipidómica, prenolipidómica, sacarolipidómica, etc., abarcando, además, las subclases y familias de lípidos [18].

Nutrimetabolómica o metabolómica nutricional: Se refiere al "estudio de la dosis y cambios temporales en compuestos celulares de bajo peso molecular en respuesta a tratamientos dietéticos" y constituye, junto con la nutrigenómica, la base en la que se pretende soportar la pretendida alimentación personalizada, que puede proporcionar enormes beneficios a la población [18,19]. Es quizás la subdisciplina en la que actualmente más se investiga y a la que se dedica, además de más soporte económico, más contribución de la población tanto de individuos sanos dispuestos a someterse a dietas experimentales, como de pacientes con interés en conocer cómo una determinada dieta puede mejorar su estado.

Xenometabolómica: Consiste en "el estudio de los compuestos no endógenos (drogas, fármacos y sus metabolitos, contaminantes, componentes dietéticos, hierbas medicinales, etc.) en un tejido o biofluido", y abarca desde la metabolómica clínica a la ambiental, pasando por la farmacológica. Algunos autores consideran esta parte de la metabolómica como disciplina independiente a la que dan el nombre de metabonómica, considerando como metabolómica propiamente dicha sólo la vegetal.

Subdisciplinas secundarias de la metabolómica son la fármacometabolómica, la metabolómica ambiental, la cardiometabolómica, la secretómica, la eritrómica, la ratiómica, la señalómica, la cosmetobolómica y tantas otras que han ido surgiendo y seguirán

haciéndolo a tenor de la importancia que adquieran las aplicaciones de la metabolómica en parcelas concretas.

La subdisciplina a la que se ha dedicado la investigación realizada en esta Tesis es de forma mayoritaria la nutrimetabolómica, pero también la lipidómica, por lo que se ha considerado de interés dedicar las dos siguientes secciones a estas subdisciplinas.

5. Las subdisciplinas de la metabolómica: La lipidómica

Los lípidos pueden definirse de forma genérica como "sustancias biológicas, generalmente de naturaleza hidrofóbica y en muchos casos solubles en disolventes orgánicos". Una definición más compleja, pero mucho más explicativa, los considera como "moléculas pequeñas hidrofóbicas o anfipáticas (ambifílicas) que se originan enteramente o en parte por condensaciones de tioésteres mediante carbaniones (ácidos grasos, policétidos, etc.) o por condensación de unidades isopreno, también de tipo carbanión (prenoles, esteroles, etc.) [20]. Estas propiedades químicas cubren un amplio rango de moléculas, tales como ácidos grasos, fosfolípidos, esteroles, esfingolípidos y terpenos, entre otros [21]. La extensa investigación que se ha desarrollado sobre lípidos ha dado como resultado un creciente interés en la estructura de la típica doble capa de los fosfolípidos en la membrana y en su interacción con las proteínas de la membrana [22], su importancia como moléculas mensajeras secundarias [23] y la aceptación actual de que muchas enfermedades (e.g. arteroesclerosis, obesidad, hipertensión, diabetes) están asociadas a cambios en la composición lipídica de células y tejidos [18]. El papel crucial de los lípidos en la membrana celular puede resumirse en los siguientes aspectos: (i) Sirven de barrera para establecer los gradientes químicos y eléctricos apropiados para los orgánulos celulares; (ii) actúan

como matriz para facilitar conformaciones y dinámicas específicas para que las interacciones proteína–proteína y lípido–proteína sean efectivas; (iii) constituyen la reserva/depósito de mensajeros secundarios lipídicos para la propagación de la señalización en el crecimiento celular, la diferenciación, la muerte programada y la respuesta a estímulos; (iv) son el depósito de energía celular para las diferentes, múltiples funciones celulares. Por tanto, las funciones de los lípidos están relacionadas con la respuesta inmune, la acción antiinflamatoria, la proliferación celular, la apoptosis, la diabetes, el cáncer, las enfermedades cardiovasculares, los desórdenes neurodegenerativos y las enzimas que metabolizan los lípidos (blanco de los fármacos). Existe una serie de aspectos característicos de los lipidomas celulares que complican estos estudios y restringen la universalidad de los resultados. Son los siguientes:

1. Los lípidos celulares son muy diferentes entre las distintas especies, tipos de células, orgánulos celulares, membranas y microdominios de membrana.
2. El lipidoma de cada tipo de célula comprende diferentes porcentajes molares de clases y subclases de lípidos específicos y de especies moleculares que tienen diferentes longitudes, grados de insaturación, distinta localización de los dobles enlaces y distintas ramificaciones de las cadenas alifáticas.
3. El lipidoma celular es dinámico, depende de las condiciones nutricionales, influencias hormonales, estado de salud, niveles de ejercicio y muchos otros factores.
4. Los lípidos, en un número de decenas de miles, están presentes en el lipidoma celular a concentraciones de atomoles a nanomoles de lípidos por miligramo de proteína.

Actualmente, la lipidómica considerada como la determinación simultánea de todas las especies lipídicas existentes en una muestra constituye un campo de investigación emergente que ha sido revisado de forma extensa por Wenk [24].

Los objetivos a largo plazo de la lipidómica son los siguientes: (i) Revelar los mecanismos biológicos de las enfermedades con las que están relacionados los lípidos; (ii) descubrir nuevos biomarcadores para su diagnosis temprana; (iii) auxiliar en el descubrimiento de nuevos fármacos; (iv) evaluar la eficacia de estos fármacos.

Obviamente, la lipidómica requiere la existencia de métodos analíticos sensibles y seguros, cuyo desarrollo se ha visto dificultado por la ausencia de homogeneidad estructural de los lípidos, las diferencias en la composición y concentración de los ácidos grasos, los múltiples tipos de enlaces entre cadenas de ácidos grasos, cadenas glicerol y las diferencias en la estructura del grupo principal. Éstas son probablemente las razones por las que todavía no existen para los lípidos protocolos generalmente aceptados, tal como ocurre en el caso de las proteínas.

5.1. Los metabolitos lipídicos objeto de estudio en esta Tesis: Los ácidos grasos

Un ácido graso (FA) es una molécula orgánica de naturaleza lipídica formada por una larga cadena hidrocarbonada lineal en cuyo extremo hay un grupo carboxilo y donde cada átomo de carbono se une al siguiente y al precedente por medio de un enlace covalente sencillo o doble. Los FAs son componentes clave de las membranas biológicas y del almacenamiento de grasas en los animales, las plantas y los microorganismos, por lo que son compuestos de gran interés en medicina y en la industria. Existen tres

grupos de ácidos grasos dependiendo del grado de insaturación: Saturados, monoinsaturados y poliinsaturados (PUFAs). La posición de un doble enlace desde el extremo metilo de la molécula permite clasificar a la mayoría de los PUFAs en las familias n-3 (ω3), n-6 (ω6) y n-9 (ω9) de los ácidos grasos. De forma mayoritaria los dobles enlaces, interrumpidos por grupos metileno (-CH2-), están dispuestos en configuración *cis* (los dos átomos de hidrógeno unidos a los carbonos adyacentes se encuentran en el mismo lado del doble enlace). Los FAs se encuentran ampliamente distribuidos en la naturaleza y son de enorme importancia en los organismos vivos como nutrientes y como fuente de sus metabolitos.

Los FAs existen en su forma libre, aunque la mayoría forman parte de moléculas más complejas tales como aceites, grasas, ceras, ésteres de colesterol, acilgliceroles, lipopolisacáridos, lipoproteínas, esfingolípidos, glicerofosfolípidos y glicoglicerolípi-dos.

Las plantas y muchos microorganismos tienen la capacidad de sintetizar los ácidos linoleico (LA) y α-linolénico (ALA), que pueden, a su vez, ser metabolizados por los microorganismos. A diferencia de las plantas y los microorganismos, el ser humano carece de las desaturasas necesarias para la síntesis de LA y de ALA, pero tiene la capacidad de metabolizar estos ácidos. Para producir metabolitos n-6 y n-3 de los PUFAs el ser humano debe obtener primero los ácidos grasos poliinsaturados n-6 (ácido 18:2n-6, LA) y n-3 (ácido 18:3n-3, ALA) mediante la alimentación. Por esta razón, los LA y ALA se conocen como ácidos grasos esenciales. La acción alternativa de enzimas como las elongasas y las desaturasas convierten los LA y ALA en nuevos n-3 y n-6 PUFAs de cadena más larga o de mayor nivel de insaturación, respectivamente.

6. Las subdisciplinas de la metabolómica: La nutrimetabolómica

Durante el siglo pasado se realizaron grandes avances por los científicos especialistas en alimentación para identificar los nutrientes esenciales necesarios para el crecimiento y la salud de la población humana. La mejora en la proposición de las dietas y las recomendaciones de suplementar la alimentación con minerales esenciales y vitaminas han sido realmente útiles para compensar la mayor parte de las deficiencias nutricionales. Actualmente los nutricionistas y dietistas se enfrentan al reto de encontrar las vías para tratar o prevenir las enfermedades derivadas de la "supersuficiencia" nutricional, tales como la obesidad, la diabetes, la inflamación crónica y las enfermedades cardiovasculares. Es por tanto de enorme importancia la identificación de componentes bioactivos de los alimentos que potencialmente incrementen la esperanza de vida, reduzcan el peso, mejoren el funcionamiento físico y mental y prevengan enfermedades tales como la ateroesclerosis, las enfermedades cardiacas, el cáncer y la artritis [25]. Estos problemas que tiene planteados la sociedad actual hace que los científicos confieran un especial interés a la nutrimetabolómica, en la que conviene resaltar las siguientes características:

a) La transformación química de los constituyentes de los alimentos durante su elaboración y digestión. Además, la ingestión de los alimentos puede tener un impacto en el metabolismo de los metabolitos endógenos (e.g. movilización de la grasa) que puede reflejarse en el perfil metabólico.

b) El efecto de la microflora bacteriana se asocia generalmente con el intestino grueso, pero dependiendo del biofluido utilizado para

el estudio la influencia de la microflora oral y la colonización gástrica por la *Helicobacter pylori* deberían también considerarse en la nutrimetabolómica. La microflora puede cambiar los constituyentes del alimento y hacerlos asequibles a ella o al resto de los componentes que actúan adicionalmente en el metabolismo.

c) El número de moléculas diferentes que se ingieren en el alimento que no son nutrientes sobrepasa al número de los nutrientes en varios órdenes de magnitud. Por ejemplo, las plantas acumulan metabolitos secundarios para su defensa, reproducción, etc., ninguno de los cuales es un nutriente esencial. En la nutrición tradicional estos compuestos fotoquímicos han sido ignorados hasta que recientemente se ha considerado su potencial efecto metabólico.

d) Se conoce que ciertos alimentos producen cambios obvios en la orina de ciertos individuos, lo que indica una interacción con el genotipo. Por ejemplo, en algunos individuos la ingestión de remolacha produce orina de color rojo; en otros, los espárragos dan mal olor a la orina.

e) El efecto de la ingestión del alimento persiste durante un cierto período de tiempo que puede ser diferente según los metabolitos. Por ejemplo, los metabolitos del café se detectan en la orina recogida a las 4 ó 5 h de la ingestión del café, mientras que las aminas heterocíclicas que se forman en la carne a la plancha persisten 48–72 h después de la ingestión [26].

Existen tres diferentes tipos de estudios a los que se puede orientar la investigación en nutrimetabolómica: (1) Análisis de los metabolitos que componen un alimento; (2) detección de la calidad/autenticidad de un

alimento; y (3) seguimiento de los cambios en el organismo causados por una dieta de intervención. El área de mayor interés y menos explotada de la nutrimetabolómica es, probablemente, la tercera, ya que el seguimiento de los cambios metabólicos relacionados con la administración de ciertos nutrientes es clave en la resolución de los problemas actuales de sobrealimentación. De hecho, las alteraciones del metabolismo debidas a los diferentes tratamientos dietéticos pueden seguirse a través de los cambios en fluidos biológicos tales como sangre u orina. Los perfiles metabolómicos se han utilizado para determinar los efectos en el metabolismo de la administración de fármacos y de las variaciones en la dieta. El esquema que se utiliza para estos estudios implica:

a) Selección del(los) alimento(s) para la dieta de intervención así como la población adecuada para participar en el estudio. Para obtener resultados estadísticamente representativos se requiere un tamaño de muestra suficiente y unos criterios preseleccionados tanto de inclusión/exclusión como de control de los grupos. Otro aspecto clave es establecer el patrón de muestreo adecuado, tanto antes como después de la intervención.

b) Análisis de los biofluidos recogidos antes y después de la ingestión del fármaco o la dieta, así como la de individuos no tratados (en caso de que se requieran como individuos control) en las mismas condiciones para todos ellos.

c) Análisis estadístico supervisado y no supervisado para encontrar las diferencias entre individuos relacionadas con la dieta o la

ingestión del fármaco que puede modificar el comportamiento del individuo frente a la dieta.

d) Determinación de las características analíticas responsables de las diferencias, que pueden consistir en una región del espectro de resonancia magnética nuclear o del infrarrojo cercano (NMR y NIR, respectivamente, o la relación m/z en el espectro de masas.

e) La elucidación de las posibles rutas metabólicas para proporcionar tanta información biológica como sea posible.

Los tres tipos de estudios —(1) análisis de los metabolitos que componen un alimento; (2) detección de la calidad/autenticidad de un alimento; y (3) seguimiento de los cambios en el organismo causados por una dieta de intervención— han formado parte de la investigación recogida en la presente Memoria: El análisis de los metabolitos que forman parte de la aceituna conforman la Parte I, la calidad de los aceites sometidos a fritura simulada componen el estudio que constituyen los Capítulos 4 y 5 (Parte II), mientras que los Capítulos 6–8, 11 y 12 (Partes II y IV, respectivamente) recogen investigación que corresponde al tercer tipo.

7. El proceso analítico en metabolómica

La detección, identificación y cuantificación de un número grande de metabolitos presentes en concentraciones muy diferentes requieren una optimización en profundidad de las condiciones de operación. Además, la amplia variedad de propiedades físicas y químicas hace imposible su análisis mediante una única plataforma analítica. La Fig. 3 muestra el esquema general que se sigue en química analítica, que también se aplica

en metabolómica con algunas peculiaridades que son función de cada caso particular.

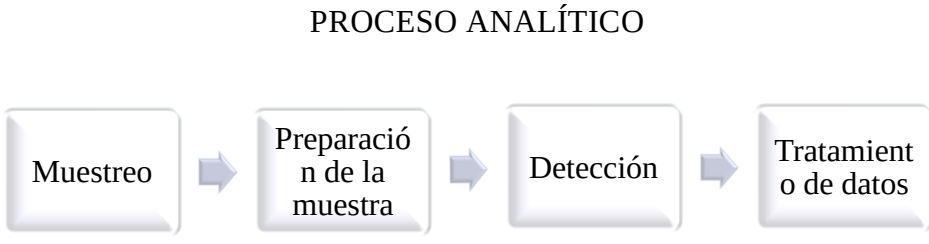


Figura 3. *Esquema general de un proceso analítico.*

7.1. El muestreo

La primera etapa del proceso analítico en general y de la metabolómica en particular es el muestreo, que implica la selección del material biológico más adecuado para el objetivo del análisis y debe perseguir la obtención de muestras representativas y asegurar su conservación hasta la siguiente etapa.

Las muestras más comunes utilizadas en metabolómica son los biofluidos, las células y tejidos animales y las plantas, que requieren protocolos de muestreo y conservación muy diferentes.

Entre los biofluidos, los dos más comunes que pueden obtenerse por medios prácticamente no invasivos son la sangre y la orina. Son muestras idóneas para la prognosis/diagnosis de enfermedades y para el seguimiento de dietas y terapias con fármacos. La orina contiene los productos finales del catabolismo/metabolismo, mientras la sangre refleja el catabolismo, metabolismo y anabolismo. Otros biofluidos utilizados en menor proporción son la saliva, el sudor, el aire exhalado, la leche, la bilis y los líquidos amniótico, cerebroespinal, seminal, digestivo, sinovial, etc.

Cuando se trabaja con células o tejidos, el objetivo en el primer caso puede ser el endometaboloma, el exometaboloma o ambos, mientras que en los tejidos el objetivo es comúnmente conocer los metabolitos formados en un comportamiento anormal del tejido en cuestión. Mención especial merece el muestreo mediante diálisis [27].

Las plantas ofrecen muy variados tipos de muestreo referidos a hojas, raíces, frutos, ramas, flores o materiales derivados (e.g. aceites, resinas, vino).

Sobre el tiempo máximo que puede transcurrir desde el muestreo y el almacenamiento, generalmente ultracongelación, y la temperatura de este último, así como el tiempo que la muestra se mantiene inalterada durante el almacenamiento a diferentes temperaturas existen numerosas y recientes revisiones bibliográficas [28,29] sobre los resultados obtenidos por diferentes autores, que a veces son contradictorios.

7.2. La preparación de la muestra

Puede considerarse que el proceso analítico propiamente dicho comienza tras el muestreo o la descongelación en el caso más común en el que la muestra se congela, y se inicia con la preparación, que varía enormemente dependiendo de que el análisis a realizar sea orientado o global, pero que tiene una etapa común si la muestra es sólida: Su disolución (salvo en el caso de la NMR, que permite el análisis de muestras sólidas). En el caso del análisis orientado la disolución es usualmente parcial, es decir, una etapa de lixiviación, mientras que para análisis global se requiere una disolución total (digestión, generalmente).

Cualquiera de estas etapas (lixiviación o digestión) se auxilia en la actualidad mediante algún tipo de energía para facilitarla, lo que implica su

aceleración, la capacidad de automatización o el aumento de su eficiencia. Los ultrasonidos, las microondas, o el uso de líquidos sobrecalentados son las formas más comunes de conseguir estos propósitos, una vez que los fluidos supercríticos han mostrado no poseer la aplicabilidad para la extracción que se predijo en la última década del siglo pasado [27].

Tras la disolución de al menos los compuestos de interés, las siguientes etapas son similares a las de cualquier muestra líquida y sólo difieren en función del análisis a realizar, global u orientado.

En análisis global las etapas anteriores a la introducción en el detector (o más comúnmente en un sistema de separación individual de los analitos, generalmente un cromatógrafo) son simples: Dilución, evaporación, ajuste de pH o desproteinización. Este considerado análisis directo minimiza las pérdidas de metabolitos, pero la complejidad de la matriz de la muestra puede causar supresión de la ionización y/o la formación de aductos en el caso de la detección mediante MS, o afectar al funcionamiento del instrumento por la formación de residuos no volátiles. Estos inconvenientes pueden obviarse introduciendo una etapa de extracción suficientemente efectiva, que es generalmente imprescindible en análisis orientado —e.g. extracción líquido–líquido (LLE) o extracción en fase sólida (SPE)— para limpiar, desalinizar la muestra y/o preconcentrar los compuestos de interés. La instrumentación que permite el análisis con mínima preparación de la muestra son los equipos de NMR (e.g. ajuste del pH mediante un tampón deuterado), si bien el tiempo de análisis puede ser muy largo si se pretende llevar a cabo identificación de metabolitos y la interpretación de los resultados es generalmente muy compleja [30].

La *LLE* se ha utilizado secularmente para el tratamiento de muestras líquidas. Las limitaciones bien conocidas como la formación de emulsiones,

el consumo de grandes volúmenes de disolventes orgánicos y la dilución de los analitos puede evitarse disminuyendo la escala de trabajo y alterando la relación de volúmenes de las fases inmiscibles. Las múltiples variantes de la LLE a escala micro (uso de membranas líquidas [31], extracción con gota colgante [32], o la más reciente de microextracción en fase líquida con capilares de fibra hueca [33,34], que viene a resolver algunos de los problemas de la de gota colgante [35]; así como la emulsión–microextracción asistida por ultrasonidos [36] resuelven el problema de la contaminación, dando lugar a una etapa que puede encuadrarse en la química analítica verde [37], pero tienen como aspecto negativo que su capacidad para la automatización es prácticamente nula.

La *SPE* es posiblemente la técnica de separación más utilizada en metabolómica, especialmente en la metabolómica clínica, dada la amplia gama de sorbentes que existe en la actualidad y su selectividad, la variedad de disoluciones para el lavado de los cartuchos y para la elución de los analitos, la posibilidad de miniaturización (SPME) y la capacidad para la automatización total de esta etapa [38]. Aunque existen variantes de la modalidad de SPE tales como la versión miniaturizada en jeringa (SPME), la extracción en fase sólida con dispersión de la matriz (MSPD) [39] y la realizada en barra agitada (SBSE), se van a considerar en esta introducción sólo aquellas modalidades utilizadas en la investigación que se ha realizado en esta Tesis. En los artículos correspondientes se justifica su uso.

Un sistema de bajo precio, como es el laboratorio en una válvula, lab-on-valve o LOV facilita la automatización de una amplia variedad de etapas del proceso analítico, entre ellas la SPE. El LOV permite utilizar una minicolumna fabricada ex profeso para un determinado método mediante la válvula multiposición. Esta válvula hace posible que se puedan seleccionar

secuencialmente las diferentes disoluciones para un desarrollo óptimo de la etapa (disoluciones para el transporte de la muestra hasta la minicolumna, para el lavado de ésta tras la retención de los analitos para eliminar los restos de la matriz de la muestra no retenidos y para la elución de los analitos retenidos) y el posterior transporte del eluato hasta el punto de desarrollo de la siguiente etapa. Otro aspecto que favorece el LOV es la selección de sólo una fracción del eluato (aquélla que contiene los analitos) evitando o minimizando la dilución mediante una válvula de desvío que permite llevar hacia el desecho la fracción no útil. La posibilidad de añadir un reactivo al eluyente de manera continua o discontinua para llevar a cabo una reacción simultánea con la elución o posterior a ella es otra de las posibilidades de la válvula multiposición.

Un mayor grado de automatización de la etapa de SPE, pero con un mayor coste de adquisición y mantenimiento, se consigue mediante los equipos comerciales Prospekt, en sus diferentes modelos, o Symbiosis (ambas de Spark Holland), utilizadas en la investigación realizada por la doctoranda. A diferencia del resto de plataformas semiautomáticas o automáticas, las comercializadas por Spark Holland realizan la extracción en fase sólida a alta presión, lo que facilita el acoplamiento con la etapa cromatográfica. En este sistema completamente cerrado no se producen pérdidas de analitos y, además, la elución en la etapa de extracción en fase sólida se realiza haciendo pasar la fase móvil cromatográfica por el cartucho, con lo cual la totalidad de los analitos retenidos en el sorbente se eluyen y pasan a la columna cromatográfica. Otra ventaja importante es la reducción del tiempo de extracción respecto a la opción manual, ya que disminuye el número de etapas. La Fig. 4 muestra un esquema generalizado de la automatización de la SPE mediante uno de estos equipos. Además, el

sistema comercial posibilita que los métodos desarrollados sean fácilmente trasladables al laboratorio clínico de análisis sistemático.

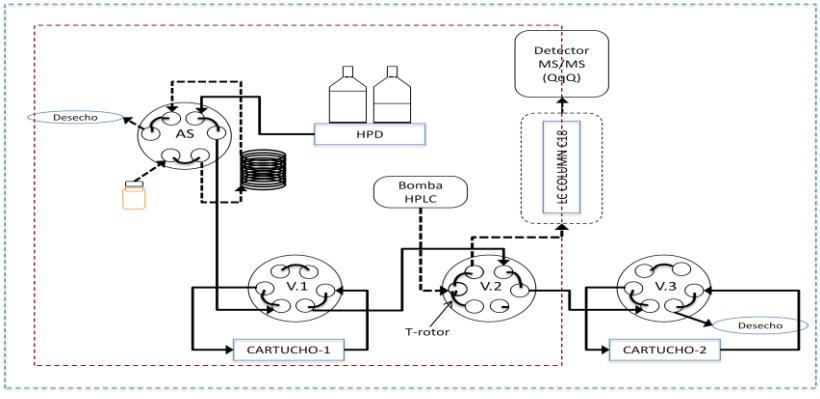


Figura 4. *Configuración instrumental para la extracción en fase sólida en línea.*

7.3. La separación–determinación: Herramientas analíticas

Las herramientas analíticas de las que se ha hecho uso para llevar a cabo la investigación que se recoge en esta Memoria han sido mayoritariamente espectrómetros de masas en diferentes versiones. En alguna ocasión se ha utilizado la fluorescencia molecular inducida por láser como fenómeno óptimo para conseguir una buena sensibilidad en la determinación de ciertos compuestos (ácidos siálicos). Por tanto, son estos tipos de detectores los que se comentarán, si bien se hará la comparación con la NMR, con la que generalmente compite la MS o a la que complementa. Los detectores de masas correspondientes han estado en todos los casos conectados a un cromatógrafo (de gases o líquidos, según las características de los analitos). Estos sistemas de separación de alta capacidad de resolución son sobradamente conocidos en química analítica para describirlos en esta introducción, por lo que se obvia su descripción.

La espectrometría de masas es una técnica de detección basada en el desplazamiento diferencial por aplicación de un campo eléctrico de moléculas ionizadas (o átomos ionizados) en vacío. En los estudios realizados se ha aplicado exclusivamente a moléculas. De forma simplista, un espectrómetro de masas consiste en una fuente de iones, un analizador de masas, un detector y un sistema de recogida de datos. Las moléculas de la muestra se insertan en la fuente de iones, donde se ionizan. Los iones así formados se encuentran en fase gaseosa y se separan entre sí en función de su relación masa/carga (m/z) en el analizador de masas, donde son detectados.

Son diferentes los tipos de fuentes de ionización que existen para generar los iones en fase gaseosa: Ionización por impacto electrónico (EI) e ionización química (CI) son las que se utilizan generalmente en el acoplamiento GC–MS, mientras que la ionización por electro-espray (ESI) y la ionización química a presión atmosférica (APCI) son más comunes en LC–MS [39].

En ESI la muestra se pulveriza en un metal o en un capilar de sílice fundida. El electro-espray se consigue aumentado el potencial en el capilar en el rango 2.5–4 kV tanto para el modo de ionización positivo como para el negativo. El espray resultante formado por gotitas cargadas se dirige al contraelectrodo a un potencial más bajo; en él las gotitas pierden el disolvente quedando como especies iónicas en la fase gaseosa. El contraelectrodo contiene un orificio a través del cual los iones se transportan a la cámara de vacío del espectrómetro, atravesando regiones bombeadas diferencialmente mediante lentes selectoras (“skimmers”) [40]. La muestra ionizada es transportada al analizador de masas mediante un

campo eléctrico o magnético. Puesto que la gama de analizadores de masas comercialmente asequibles es amplia, sólo se describirán de forma breve los utilizados para el desarrollo de esta Tesis.

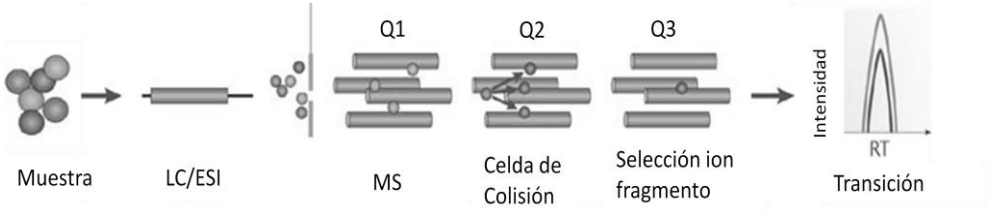


Figura 5. *Esquema general de un espectrómetro de masas de triple cuadrupolo.*

Un detector de masas de triple cuadrupolo (QqQ) consta de las unidades que se muestran en la Fig. 5 [40]: Una fuente de iones (generalmente una fuente de ESI) seguida de un conjunto de lentes para la transferencia de iones al primer cuadrupolo, formado por cuatro barras paralelas a las que se aplican voltajes específicos de corriente continua y de radiofrecuencia, que hacen que todos los iones excepto los de uno o varios valores de m/z se filtren a su través eliminándose. El voltaje aplicado es variable, por lo que secuencialmente son otros los iones que pasan entre ellos y otros los que siguen circulando hasta alcanzar la celda de colisión en la que se fragmentan. Esta celda, que generalmente recibe el nombre de segundo cuadrupolo, es en realidad un hexapolo relleno de un gas inerte, nitrógeno o argón, en el que se fragmentan los iones que se envían al tercer cuadrupolo para una segunda etapa de filtrado que permite aislar y examinar las múltiples transiciones desde el

precursor al ión producto. Éste es el modo llamado de seguimiento de la reacción seleccionada (selected reaction monitoring o SRM). Puesto que los fragmentos iónicos formados son partes de la molécula precursora, representan porciones de su estructura global.

Gracias a la seguridad que proporcionan los detectores QqQ en el caso de masas pequeñas, estos detectores se utilizan preferiblemente para análisis orientado, ya que permiten cuantificar con excelente sensibilidad y selectividad en el modo SRM. El modo de operación más sensible para el QqQ es fijar ambos cuadrupolos y hacer el seguimiento de un ión precursor específico a un ión producto también específico. Sin embargo, es deseable monitorizar varias transiciones para cada ión precursor con el fin de incrementar la selectividad del método.

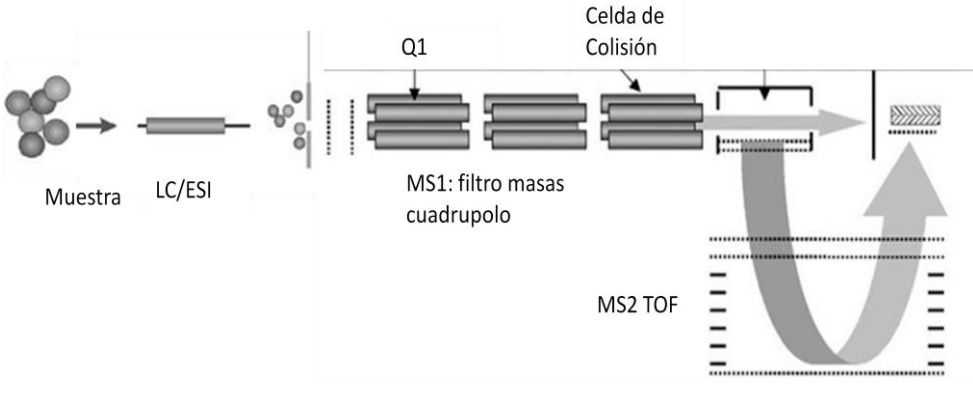


Figura 6. *Esquema general de un espectrómetro de masas de cuadrupolo–tiempo de vuelo.*

Un detector de masas del tipo cuadrupolo-tiempo de vuelo (QqTOF), también utilizado en la investigación recogida en esta Memoria, consta de las unidades que se muestran en la Fig. 6 [41]. Este instrumento tiene una configuración similar al *QqQ*, pero en él el último

cuadrupolo se ha reemplazado por un tubo de aceleración como analizador de masas (generalmente en configuración ortogonal) con el que se filtran para su eliminación los iones no analizados de acuerdo con su energía cinética. El analizador de masas QqTOF ofrece mayor selectividad que los QqQ, mientras que la sensibilidad es considerablemente menor. Por otra parte, gracias a su exactitud en la medida de masas (error por debajo de 2 ppm), se puede llevar a cabo una muy buena identificación; lo que lo hace muy adecuado para análisis global (obtención de perfiles metabolómicos).

Comparación de las características de la MS con la de NMR. Una forma de justificar el uso de los instrumentos en los que se ha basado la mayor parte de la investigación realizada (MS) es su comparación con los equipos con los que en ciertos casos compiten y a los que en otros complementan. El aspecto de mayor fortaleza de la MS es su sensibilidad. De hecho, la metabolómica basada en MS proporciona análisis cuantitativos que se caracterizan por una excelente selectividad y sensibilidad, y por la posibilidad de identificación de los metabolitos con una buena seguridad. A pesar de que la sensibilidad en MS se afecta por la ionización y el tipo de detector, puede decirse en términos generales que es más sensible que la NMR. Las fuentes de ionización ESI proporcionan sensibili-dades en el rango alto de los fentomoles o bajo de los picomoles; incluso algunas técnicas nanoESI tienen sensibilidades del orden de los zeptomoles. Las fuentes MALDI y APCI proporcionan sensibilidad en el rango de los fentomoles, mientras que las de CI y EI son menos sensibles (del orden de los picomoles) [87,88].

Por otra parte, la cuantificación requiere el uso de estándares internos con eficiencias en la ionización y en la fragmentación similares a las de los metabolitos en estudio. La información cuantitativa sobre un determinado metabolito puede obtenerse de tres formas: (i) Por su integración frente a una muestra de referencia del mismo compuesto (lo que requiere conocer la identidad del metabolito y compararla en diferentes análisis, lo que puede introducir error); (ii) por comparación de forma relativa de las relaciones de una serie de picos en una serie de espectros; y (iii) por adición a la muestra de una molécula del analito que contenga algún, o algunos isótopos estables de alguno de sus átomos (sustitución de algún átomo de hidrógeno por deuterio, de ^{12}C por ^{13}C, etc.). En cuanto al análisis cualitativo, los modelos de fragmentación mediante MS en tándem proporcionan información sobre la conectividad y la presencia de grupos funcionales específicos en un metabolito desconocido. La identificación de metabolitos se facilita frecuentemente por comparación con patrones internos y la búsqueda a través de bibliotecas espectrales. La fragmentación de compuestos sometidos a una determinada energía de colisión es muy reproducible, por lo que las medidas MS/MS son muy selectivas.

Aunque las medidas en MS son rápidas, el tiempo global del barrido depende principalmente de la etapa cromatográfica, que puede variar desde minutos a horas (por supuesto nunca llega a días, como puede ocurrir en el caso de la 2D-NMR). Sin embargo, el acoplamiento con un equipo cromatográfico o de electroforesis reduce la complejidad de los espectros de masas por la separación temporal de los analitos, además de proporcionar información adicional sobre las propiedades fisicoquímicas de los compuestos en estudio.

La Tabla 1 muestra las ventajas e inconvenientes del uso de la MS en metabolómica.

Tabla 1. Ventajas e inconvenientes de la MS para su uso en metabolómica

VENTAJAS	***INCONVENIENTES***
✓ Muy sensible en sus diferentes modos. ✓ Alta selectividad (especialmente en MS^n) ✓ Adecuada para análisis orientado, global y para la obtención de huellas y perfiles. ✓ Información estructural a través de modelos de fragmentación (MS^n). ✓ Medidas seguras de m/z, ideales para la identificación de metabolitos. ✓ Excelente resolución (mejorada mediante separación).	× La sensibilidad depende de la eficiencia de la ionización. × Más eficaz cuando se acopla a técnicas de separación. × La cuantificación requiere estándares internos semejantes a los metabolitos. × Técnica destructiva, especialmente complicado en muestras valiosas. × Limitada a muestras líquidas o extractos de muestras sólidas. × Protocolos de preparación de muestra laboriosos.

Entre los puntos débiles del uso de los equipos basados en MS están las etapas de preparación de la muestra, que causan pérdida de los metabolitos. La complejidad de estas etapas depende del tipo de

espectrómetro de masas, pero en general, son más tediosas que las requeridas en NMR. Así, los equipos MALDI–MS requieren mezclar la muestra con una matriz adecuada antes del análisis. En el caso de las fuentes de ionización mediante espray, la elección del disolvente es de enorme importancia: La ESI requiere disolventes orgánicos volátiles que ayuden a la evaporación; el acoplamiento GC–MS hace necesaria la derivatización previa a la detección en muchas ocasiones; mientras que el acoplamiento LC–MS es, por su propia naturaleza, inútil para análisis de muestras *in vivo*. Por otra parte, el análisis global también puede verse limitado por el tipo de ionización debido a que una determinada clase de metabolitos sea discriminada por el sistema de introducción de la muestra y/o la eficiencia de la ionización. A pesar de estos inconvenientes, la MS es actualmente la técnica más adecuada para identificación. Comparativamente puede decirse que los estudios sobre metabolómica basados en MS se caracterizan por un número de publicaciones por año que sobrepasan en mucho a los que utilizan NMR para sus investigaciones.

Las aplicaciones y perspectivas de la MS como técnica de detección en las diferentes plataformas analíticas con las que implantar las estrategias características de la metabolómica son amplias y variadas. De hecho, los perfiles metabolómicos, el análisis global y el orientado en esta disciplina se extienden a todas sus áreas de aplicación: Farmacéutica, clínica, forense, agricultura, alimentación, etc.

La MS es una técnica de gran poder de identificación molecular. La reciente introducción de los espectrómetros de alta resolución (Orbitrap y QqTOF) ha permitido su uso para obtener el perfil global

con una relativamente alta frecuencia de análisis, haciendo posible su aplicación para numerosos estudios sobre diagnosis de desórdenes metabólicos en diferentes biofluidos. Como ejemplo, el análisis del metaboloma de la orina para el diagnóstico de desórdenes metabólicos mediante GC–MS ha sido recientemente revisado por Buhara [89]. Más reciente es la revisión sobre la preparación de la muestra de orina para el análisis global u orientado mediante MS [29]. Además, la ESI con MS/MS se ha convertido en una excelente herramienta para el estudio de desórdenes en el metabolismo de aminoácidos, ácidos grasos y en la biosíntesis de ácidos orgánicos, entre otros. De ello dan fe varias revisiones bibliográficas recientes que describen con detalle la metodología y la interpretación bioquímica de los datos [41,42].

Una de las características más destacables del estudio de perfiles globales mediante MS es la posibilidad de caracterización de la fracción no polar, especialmente la fracción lipídica, que da lugar a la lipidómica. El lipidoma con sus diferentes clases, subclases y moléculas lipídicas señalizadoras, constituye un sub-compartimento del metaboloma que ha sido muy estudiado mediante MS [43]; lo que ha permitido profundizar en los mecanismos bioquímicos del metabolismo de lípidos y en las interacciones lípido–lípido y lípido–proteína. Como ejemplo, los perfiles metabólicos de los eicosanoides, una clase de moléculas señalizadoras lipídicas, se han englobado recientemente con el nombre de eicosanómica [44]. El análisis orientado generalmente requiere preparación de la muestra basada en etapas de limpieza, que se desarrollan de forma automática frecuentemente, tal como se ha llevado a cabo en la investigación que se recoge en esta Memoria.

La GC acoplada a MS y a otros detectores se ha utilizado extensamente en metabolómica para el análisis de un gran número de metabolitos en muestras muy variadas, especialmente en plantas [45]. Sin embargo, la naturaleza de la GC hace su uso posible sólo para compuestos volátiles o convertidos en volátiles tras la adecuada derivatización. De hecho, las moléculas no volátiles o termolábiles se analizan mejor mediante LC–MS con cualquiera de los modos de ionización a presión atmosférica, o mediante MALDI–MS. No obstante, la aplicación de reacciones de derivatización permite el análisis de una buena proporción del metaboloma mediante GC–MS. A pesar de la necesidad de derivatización, la GC–MS presenta una serie de ventajas sobre la LC–MS, a saber: Las columnas de GC ofrecen todavía mejor resolución que las columnas convencionales para LC, y los espectros obtenidos mediante ionización electrónica (EI) contienen una enorme cantidad de información estructural. La existencia de extensas bibliotecas de espectros EI (por ejemplo, http://www.nist.gov/srd/nist1a.htm) y los valores muy reproducibles de índices de retención hace más fácil la identificación de un compuesto mediante GC–MS que por LC–MS. Un inconveniente cuando se utiliza GC–MS con EI es que los iones moleculares pueden estar en muy pequeña proporción o ausentes en el espectro puesto que se trata de una técnica de ionización fuerte; lo que puede subsanarse mediante espectros obtenidos por ionización química, en los que los iones moleculares predominan en general.

La fluorescencia como fenómeno base de la detección da lugar al desarrollo de métodos con buena sensibilidad, tal como se pone de manifiesto en la investigación que se recoge en el Capítulo 9 de esta Memoria. Las ventajas clave para el uso de este tipo de detección son,

además de su sensibilidad, su adecuación para estudios cinéticos por la rápida respuesta analítica que proporcionan los instrumentos basados en fluorescencia, la posibilidad de utilizarla en formato sonda, su naturaleza no destructiva y la alta frecuencia de análisis. La principal limitación de la detección de la fluorescencia es su limitado campo de aplicación, ya que son pocos los analitos que pueden determinarse por fluorescencia intrínseca, haciéndola prácticamente inútil para análisis global. En la mayoría de los casos la detección fluorimétrica se ha utilizado para análisis orientado de moléculas pequeñas [46,47]. Por ejemplo, An et al. desarrollaron un método para el análisis cuantitativo de carotenoides intracelulares en células de levadura roja (*Phaffia rhodozyma*) basado en la fluorescencia intrínseca de estos compuestos [48]. Sin embargo, este tipo de detección generalmente requiere derivatización y separación cromatográfica o electroforética.

El uso de LIF está muy extendido en análisis orientado debido a su alta sensibilidad, requerida para la baja concentración de la mayoría de los metabolitos. Como ejemplos, Kennedy *et al.* analizaron aminoácidos en extractos de células neuronales individuales obtenidas de serpientes mediante CE–LIF tras derivatización química [49]; mientras que la cuantificación de gangliósidos (moléculas de esfingo-lípidos de peso molecular grande) en células tumorales de la pituitaria también se ha llevado a cabo con buena sensibilidad mediante este acoplamiento [50].

7.3. El tratamiento de datos en metabolómica

La cantidad de muestras generalmente manejadas en metabolómica junto con el número de datos que proporcionan los instrumentos utilizados para su análisis generan una enorme cantidad de datos que requieren diferentes

tipos de tratamientos para extraer de ellos toda la información posible que pueda resultar de interés. Esta información no sería útil sin el uso de los diferentes métodos multivariantes propuestos en la bibliografía para su tratamiento. Así, observar las posibles tendencias de los datos o utilizarlos para determinar un parámetro dado requiere el uso de herramientas multivariantes para clasificar o para cuantificar, respectivamente.

El análisis multivariante abarca los diferentes métodos estadísticos, matemáticos, o gráficos para analizar de forma simultánea los datos procedentes de las variables en estudio [51]. En el caso de las técnicas espectrométricas, el análisis multivariante tiene dos objetivos: (1) La determinación de perfiles de compuestos a partir de múltiples variables espectrales (valores de absorbancia a diferentes longitudes de onda); (2) la diferenciación o la clasificación de grupos de muestras con características comunes a partir de la información espectral. Estos objetivos son compartidos con las técnicas cromatográficas, si bien en este último caso en lugar de utilizar como datos para generar los modelos las variables espectrales se utilizan las áreas de los picos obtenidos en un determinado análisis.

El preprocesamiento de datos tiene como finalidad la transformación de los datos "brutos" (los que proporciona el instrumento analítico y que son exclusivos de su marca comercial) en datos "limpios" (aquéllos que poseen un formato universal) para su procesado con cualquier software de tratamiento de datos.

Algunos ejemplos representativos son:

1) *Deconvolución*: Método que permite resolver *in silico* picos solapados sin previo conocimiento de los compuestos en equipos de NMR, GC–MS o LC–MS. Existen muchos tipos de softwares para la deconvolución de los

datos obtenidos (por ejemplo, en MS, donde se utilizan los datos brutos y se obtienen los espectros de masas ya discriminados). Además, la deconvolución permite identificar exactamente el ión precursor para cada espectro de masas/masas.

2) *"Peak-picking"*: Etapa básica cuando se utilizan técnicas de análisis que generan un número grande de señales (de NMR o de MS) es la identificación de las señales y su presentación como tabla con los valores de los parámetros que los definen (desplazamiento químico en NMR, intensidad de señal para cada valor de m/z). Actualmente se lleva a cabo de forma automática debido a la gran cantidad de datos, generándose un archivo de datos (metadatos) que pueden ser posteriormente tratados para obtener información.

3) *Alineamiento*: Método para ordenar las señales que corresponden a los mismos metabolitos en registros NMR, en LC–MS, en GC–MS o en CE–MS. Los tiempos de retención en cromatografía y en electroforesis están sujetos a variabilidad entre análisis, por lo que es necesario corregir los tiempos de retención para alinear las señales correspondientes a los mismos metabolitos con el fin de combinar datos procedentes de diferentes muestras. La mayoría de los métodos de alineado trabajan con pares de muestras o bien con múltiples muestras respecto a una muestra de referencia. Estos métodos se dividen en dos categorías: (1) Los que utilizan datos brutos para generar un conjunto de mapas mediante la combinación de los tiempos de retención de cada análisis; y (2) los que agrupan características identificadas y producen una matriz en la que cada fila corresponde a un cluster (a un ión, por ejemplo) y las columnas contienen medidas (por ejemplo, área de pico) para cada muestra.

4) *Corrección de fase*: Método utilizado para corregir fases de señales en espectros NMR obtenidos por transformada de Fourier [52].

5) *Corrección de línea de base*: Los archivos de datos pueden contener ruido químico (como consecuencia del proceso de medida) y aleatorio (asociado al sistema de detección). Los métodos de reducción de ruido tienen como objetivo eliminar el ruido aleatorio y están basados en técnicas de filtrado tales como filtrado con ventana móvil, Savitzky–Golay o de ajuste polinómico. El ruido químico se manifiesta normalmente en la línea de base; por tanto, su eliminación se lleva a cabo por corrección de la línea de base mediante aplicación de los algoritmos adecuados [53].

Una vez obtenidos los datos "limpios" es habitual aplicar operaciones matemáticas con el fin de mejorar la comparación entre muestras, que es el objetivo del tratamiento de datos. Para ello es crítico eliminar los efectos de las variables biológicas, experimentales e instrumentales. Las operaciones que se aplican con mayor frecuencia con este fin son las siguientes:

a) *Normalización*: Método que permite estandarizar los datos para hacerlos comparables mediante el ajuste del ruido (biológico, experimental o instrumental).

b) *Escalado*: Operación que ordena las diferentes señales en función de la señal más intensa con el fin de facilitar la comparación entre ellas.

c) *Centrado*: Operación para trasladar el centro de gravedad de un archivo de datos y eliminar la importancia de determinadas señales.

d) *Detección de muestras o datos anómalos*: Descubrir aquellas variables, muestras o combinaciones de ambas que se desvían de la distribución de la mayoría de los datos.

El procesamiento de datos puede hacerse mediante métodos supervisados y no supervisados en función de la información del agrupamiento de datos de que se dispone a priori. En los métodos no supervisados —Análisis de Clúster y Análisis de Componentes Principales— no se aporta información de las muestras analizadas para el análisis estadístico o, si se dispone, es escasa. El objetivo es poner de manifiesto tendencias que están ocultas en la matriz de datos formada por los espectros de las muestras. Por el contrario, los supervisados sí cuentan con las categorías existentes en el colectivo de muestras; por lo que los modelos generados utilizan la información suministrada de partida para el análisis de muestras desconocidas.

Entre los métodos no supervisados se distinguen:

i) *El análisis jerárquico de clusters (HCA)*: En este grupo se encuentra una serie de métodos que permiten, a partir del cálculo de distancias entre objetos o muestras, agruparlos según las similitudes o diferencias entre ellos. La forma de ver los resultados de estos métodos suele ser un dendograma que muestra los agrupamientos de los objetos o muestras. El espacio multidimensional en el que se calculan las distancias es el formado, por ejemplo, en espectrometría óptica por *n* longitudes de onda, es decir, no se lleva a cabo ninguna reducción de variables [54].

ii) *El análisis de componentes principales (PCA)*: Este método de análisis no supervisado se define como el procedimiento matemático empleado para transformar un conjunto de datos en nuevas variables ortogonales, denominadas componentes principales (PCs), que son una combinación lineal de las variables de partida (cada longitud de onda en el caso de las técnicas espectrométricas o el valor de área para cada pico en las cromatográficas). Es decir, en el PCA se realiza una síntesis de nuevas

variables, mediante cada una de las cuales se pretende explicar la máxima variabilidad de los datos. Además, como el número de variables finales es menor que el de partida, se les denomina métodos de reducción de variables. La matriz de datos se descompone en dos matrices: La matriz de cargas (loadings), que contiene los coeficientes de las transformaciones lineales de variables originales que dan lugar a la nueva componente principal; y la matriz de puntuaciones (scores), que expresa los datos originales transformados en los valores de las componentes principales [55].

En los métodos supervisados es necesario distinguir entre los discriminantes y los de modelado. Los primeros tienen como objetivo distinguir entre las categorías existentes de forma que una determinada muestra se clasifica dentro de alguno de los tipos existentes. Los métodos discriminantes que más se utilizan son el Análisis Discriminante Lineal (LDA) y su derivado Análisis Discriminante Cuadrático (CDA). En los métodos de modelado, a diferencia de los anteriores, se define un volumen de espacio para cada una de las clases, de tal forma que puede haber muestras que no pertenezcan a ninguna clase. La filosofía de este tipo de métodos es clasificar según la similitud dentro de una misma clase, y no por discriminación entre clases. La clasificación de las muestras desconocidas se basa en los valores de distancia a una referencia distinta según el modelo usado. Se emplean diferentes formas de medida de distancia, entre las que se encuentran la distancia euclídea y la de Mahalanobis [56].

Los métodos de modelado más empleados para la clasificación de muestras según datos espectroscópicos son el método SIMCA (de las siglas en inglés de Soft Independent Modelling of Class Analogies) y el PLS con

diferentes algoritmos tales como PLS-DA (Parcial Least Squares-Discriminant Analysis) o PLS-CM (Partial Least Squares-Class Modelling).
1) *SIMCA*: Se trata de un método de modelado [57] en el que se realiza un PCA para cada una de las clases que se pretende modelar. A la hora de clasificar una muestra usando un modelo generado mediante SIMCA existen tres posibilidades: (i) La muestra no pertenece a ninguna de las clases que componen el sistema de modelado; (ii) la muestra pertenece a una de ellas; o (iii) pertenece a dos o más clases. Esta última opción se dará en el caso en el que las clases se encuentren solapadas y la muestra a clasificar se ubique en la zona de solapamiento. Hay dos criterios para ubicar una muestra en una determinada clase: La distancia de la muestra al centro del modelo y la distancia de la muestra al modelo. En el primer caso, la distancia informa sobre la varianza que queda explicada por el modelo de una determinada clase cuando la muestra se proyecta sobre el espacio de componentes principales de esta clase. La segunda opción informa sobre la varianza que no ha podido explicarse por el modelo, denominándose ésta residual.
2) *PLS-DA*: Está basado en el algoritmo PLS univariante, en el que se analizan datos que se caracterizan por muchas variables predictoras con problemas de multicoli-nealidad y pocas unidades experimentales en estudio, de forma que se consigue reducir las dimensiones de la matriz, establecer las fronteras entre las clases y hacer un análisis discriminante que clasifica las nuevas muestras según el modelo confeccionado. El algoritmo PLS-DA proporciona en metabolómica una alternativa para filtrar informa-ción no correlacionada con las clases predefinidas. Los loadings PLS-DA, de igual forma que en PCA, proporcionan información sobre qué señales

espectrales están asociadas a los agrupamientos observados; lo que resulta muy útil para la interpretación de los resultados en metabolómica.

3) *PLS-CM*: Se trata de una técnica de discriminación, de modelado de clase técnicas que permiten la comprensión de las propiedades espectrales de la muestra y el desarrollo de modelos de clasificación, siendo posible estimar la sensibilidad y la especificidad para cada clase de modelo. La sensibilidad mide la capacidad computarizada del modelo para reconocer sus propios objetos, mientras que la especificidad mide la capacidad del modelo para rechazar objetos extraños. Por lo tanto, ambos parámetros conjuntamente describen el funcionamiento de los modelos calculados y permiten la evaluación de la posible confusión entre categorías o de detección de datos atípicos. De las definiciones resulta evidente que son necesarios clase-modelos con alta sensibilidad y alta especificidad. Sin embargo, estos parámetros muestran en general un comportamiento opuesto: Cuando se aumenta uno el otra disminuye por lo que es útil para evaluar la sensibilidad y especificidad de una familia de clases, tanto en colocación y en predicción [58,59].

Referencias

[1] E. P. Ryan, A. L. Heuberger, T. L. Weir, B. Barnett, C. D. Broeckling, J. E. Prenni, J. Agric. Food Chem. 59 (2011) 1862–1870.

[2] J. C. Lindon J. K. Nicholson, Trends Anal. Chem. 27 (2008) 194–204.

[3] D. S. Wishart, C. Knox, A. C. Guo, R. Eisner, N. Young, B. Gautam, D. D. Hau, N. Psychogios, E. Dong, S. Bouatra, R. Mandal, I. Sinelnikov, J. Xia, L. Jia, J. A. Cruz, E. Lim, C. A. Sobsey, S. Shrivastava, P. Huang, P. Liu, L. Fang, J. Peng, R. Fradette, D. Cheng, D. Tzur, M. Clements, A. Lewis, A. De Souza, A. Zuniga, M. Dawe, Y. Xiong, D. Clive, R. Greiner, A. Nazyrova, R. Shaykhutdinov, L. Li, H. J. Vogel, I. Forsythe, Nucleic Acids Res. 37 (2009) D603–D610.

[4] G. B. Jacob, P. D. Matthew, R. V. Mark, Metabolomics 5 (2009) 3–21.

[5] D. G. Robertson, P. B. Watkins, M. D. Reily, Toxicological Sci. 120 (2011) S146–S170.

[6] A. K. Savage, G. Tucker, J. P. M. van Duynhoven, F. Wulfert, C. A. Daykin, Bioanalysis 1 (2009) 1537–1549.

[7] A. Zhang, H. Sun, Z. Wang, W. Sun, P. Wang, X. Wang, Planta Med. 76 (2010) 2026–2035.

[8] M. Karen, J. Clin. Gastroenterol. 45 (2011) 389–390.

[9] R. Llorach, M. García-Aloy, S. Tulipani, R. Vázquez-Fresno, C. Andrés-Lacueva, J. Agric. Food Chem. 60 (2012) 8797–8808.

[10] M. J. Gibney, M. Walsh, L. Brennan, H. M. Roche, B. German, B. van Ommen, Am. J. Clin. Nutr. 82 (2005) 497–503.

[11] A. Ishihara, F. Matsuda, H. Miyagawa, K. Wakasa, Metabolomics 3 (2007) 319–334.

[12] G. A. N. Gowda, S. Zhang, H. Gu, V. Asiago, N. Shanaiah, D. Raftery, Expert Rev. Molecular Diagnostics 8 (2008) 617–633.

[13] T. Wu, M. Yang, H.-F. Wei, S.-H. He, S.-C. Wang, G. Ji, Evidence-Based Complementary Alternative Med. 2012 (2012) 11.

[14] L. W. Sumner, P. Mendes, R. A. Dixon, Phytochem. 62 (2003) 817–836.

[15] N. D. Yuliana, A. Khatib, Y. H. Choi, R. Verpoorte, Phytotherapy Res. 25 (2011) 157–169.

[16] J. K. Nicholson, E. Holmes, J. C. Lindon, Chapter 1 - Metabonomics and Metabolomics Techniques and Their Applications in Mammalian Systems, in The Handbook of Metabonomics and Metabolomics, C.L. John, *et al*., Editors. 2007, Elsevier, Amsterdam, p. 1–33.

[17] R. J. Bino, R. D. Hall, O. Fiehn, J. Kopka, K. Saito, J. Draper, B. J. Nikolau, P. Mendes, U. Roessner-Tunali, M. H. Beale, R. N. Trethewey, B. M. Lange, E. S. Wurtele, L. W. Sumner, Trends Plant Sci. 9 (2004) 418–425.

[18] R. Stocker J. F. Keaney, J. Thrombosis Haemostasis 3 (2005) 1825–1834.

[19] M. Jenab, N. Slimani, M. Bictash, P. Ferrari, S. Bingham, Hum. Genet. 125 (2009) 507–525.

[20] M. Almena I. Mérida, Trends Biochemical Sci. 36 (2011) 593–603.

[21] E. Fahy, S. Subramaniam, H. A. Brown, C. K. Glass, A. H. Merrill, R. C. Murphy, C. R. H. Raetz, D. W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M. S. VanNieuwenhze, S. H. White, J. L. Witztum, E. A. Dennis, J. Lipid Res. 46 (2005) 839–862.

[22] D. E. Green A. Tzagoloff, J. Lipid Res. 7 (1966) 587–602.

[23] W. Cho R. V. Stahelin, Annual Rev. of Biophysics Biomolecular Structure 34 (2005) 119–151.

[24] J. B. German, L. A. Gillies, J. T. Smilowitz, A. M. Zivkovic, S. M. Watkins, Current Opinion Lipidol. 18 (2007) 66-71 10.1097/MOL.1090b1013e328012d328911.

[25] D. S. Wishart, Trends Food Sci. Technol. 19 (2008) 482–493.

[26] S. Watanabe, M. Yamaguchi, T. Sobue, Takahashi, Tosei, T. Miura, Y. Arai, W. Mazur, K. Wähälä, H. Adlercreutz, J. Nutrition 128 (1998) 1710–1715.

[27] M. D. Luque de Castro, J. L. Luque García, Acceleration and Automation of Solid Sample Treatment 2002, Elsevier, Amsterdam.

[28] B. Álvarez-Sánchez, F. Priego-Capote, M. D. Luque de Castro, Trends Anal. Chem. 29 (2010) 111–119.

[29] M. A. Fernández-Peralbo M. D. Luque de Castro, Trends Anal. Chem. 41 (2012) 75–85.

[30] D. Vuckovic, Anal. Bioanal. Chem. 403 (2012) 1523–1548.

[31] J. A. López-López, C. Mendiguchía, J. J. Pinto, C. Moreno, Trends Anal. Chem. 29 (2010) 645–653.

[32] H. Liu P. K. Dasgupta, Anal. Chem. 68 (1996) 1817–1821.

[33] M. Á. Bello-López, M. Ramos-Payán, J. A. Ocaña-González, R. Fernández-Torres, M. Callejón-Mochón, Anal. Letters 45 (2012) 804–830.

[34] M. Ghambarian, Y. Yamini, A. Esrafili, Microchim. Acta 177 (2012) 271–294.

[35] K. E. Kramer A. R. J. Andrews, J. Chromatogr. B 760 (2001) 27–36.

[36] M. D. Luque de Castro F. Priego Capote. Analytical Applications of Ultrasound. 2006. Elsevier, Amsterdam.

[37] M. D. Luque de Castro M. Alcaide Molina, Green Sample Preparation with Non-Chromatographic Separation Techniques, in Handbook of Green Analytical Chemistry. 2012, John Wiley & Sons, Ltd. New York. p. 125–151.

[38] Y. Saito K. Jinno, J. Chromatogr. A 1000 (2003) 53–67.

[39] P. Viñas, N. Campillo, M. Hernández-Pérez, M. Hernández-Córdoba, Analytica Chim. Acta 611 (2008) 119–125.

[40] W. J. Griffith, Metabolomics, Metabonomics and Metabolite Profiling. 2008, Royal Society of Chemistry, Camgridge, UK.

[41]. H. L. Newmark, Cancer Epidemiol Biomarkers Prevention 6 (1997) 1101–1103.

[42]. T. J. Smith, G. Y. Yang, D. N. Seril, J. Liao, S. Kim, Carcinogenesis 19 (1998) 703–706.

[43]. A. Bendini, L. Cerretani, A. Carrasco-Pancorbo, A. M. Gómez-Caravaca, A. Segura-Carretero, A. Fernández-Gutiérrez, G. Lercker, Molecules 12 (2007) 1679–1719.

[44]. K. L. Tuck, P. J. Hayball, J. Nutritional Biochem. 13 (2002) 636–644.

[45]. D. Boskou, Trends Food Sci. Technol. 17 (2006) 505–512.

[46]. S. C. Patil, V. P. Singh, P. S. V. Satyanarayan, N. K. Jain, A. Singh, S. K. Kulkarni, Pharmacol. 69 (2003) 59–67.

[47]. Q. S. Zheng, X. L.Sun, B. Xu, G. Li, M. Song, Biomedical Environmental Sci. 18 (2005) 65–70.

[48]. T. J. Tim, J. H. Kim, Y. R. Jin, Y. P. Yun, Archives Pharmacol. Res. 29 (2006) 67–72.

[49]. M. S. Fernández-Pachón, D. Villaño, A. M. Troncoso, M. C. García-Parrilla, Analytica Chimica Acta, 503 (2006) 101–108.

[50]. A. Zarzuelo, J. Duarte, J. Jiménez, M. González, M. P. Utrilla, Plant Med. 57 (1991) 417–419.

[51]. M. T. Khayyal, M. D. el-Ghazaly, D. M. Abdallah, N. N. Nassar, S. N. Okpanyi, M. H. Kreuter, Arzneimittelforschung 52 (2002) 797–802.

[52]. E. Bourquelot, J. Vintilesco, Compte Rendu 147 (1998), 533–555.

[53]. J. Mercier, Phytochemistry of Fruits and Vegetables. 1997 Claredon Press,UK.

54. K. Iwai, Y. Oi, F. Koyama, K. Watanabe, M. Hiraoka, T. Sekiguchi, Appl. JP 2004- 342612 20041126, Jpn Kokai Tokio Koho, pp 10.

[55]. A. Kubo, C. S. Lunde, I. Kubo, J. Agric. Food Chem. 43 (1995) 1629–1633.

[56]. N. Ucella, Trends Food Sci. Technol. 11 (2002) 315–327.

[57]. M. Raβ, C. Schein, B. Matthaus, European J. Lipid Sci. Technol. 110 (2008) 619–624.

[58]. F. N. Salta, A. Chiou, G. Boskou, N. K. Andrikopoulos, J. Food Sci. Technol. Internat. 75 (2007) 413–421.

[59]. R. Japón-Luján, M. D. Luque de Castro, J. Agric. Food Chem. 56 (2008) 2505–2511.

HERRAMIENTAS ANALÍTICAS UTILIZADAS

En este apartado de la Memoria se describen los diferentes instrumentos y equipos utilizados en el desarrollo experimental de la Tesis. En cada capítulo se incluye una explicación más detallada de los que se han utilizado en la investigación recogida en cada uno de ellos.

1. Sistemas no automáticos para la preparación de muestras

Dispositivo de ultrasonidos. Existen dos dispositivos básicos en el laboratorio analítico para aplicar ultrasonidos: Los baños y las sondas. Aunque los baños se utilizan con mayor frecuencia debido a su menor precio y omnipresencia en el laboratorio, las sondas tienen las ventajas de concentrar la radiación de ultrasonidos en una zona concreta, aumentando así su acción, y de no sufrir el fenómeno de fatiga con el tiempo, proporcionando, por tanto, una mayor reproducibilidad de los resultados, particular-mente en tratamientos largos.

La sonda de ultrasonidos utilizada fue una Branson 450 digital, que permite la selección de la amplitud de la radiación así como el modo de irradiación, continuo o discontinuo. Esta sonda se utilizó para, simultáneamente, acelerar el proceso de extracción, saponificación y derivatización de la fracción insaponificable del aceite, así como para acelerar la reacción de derivatización de la fracción no saponificable (ácidos grasos) en suero y, por último, para la hidrólisis y derivatización de los ácidos siálicos en matrices biológicas, que se detallan en las partes I, II y III de la Memoria. Para ello la muestra se mezcla con el disolvente en tubos de ensayos colocados en un recipiente que contiene agua como medio

de dispersión. La acción discontinua de la sonda de ultrasonidos situada en el baño de agua, según el dispositivo que se muestra en la Fig. 1, actúa sobre todas las muestras en las mismas condiciones de potencia y ciclo útil.

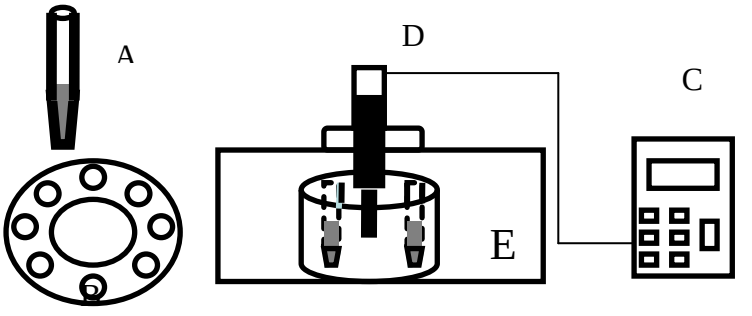


Fig. 1. Dispositivo para la lixiviación simultánea de 8 muestras con asistencia de una sonda de ultrasonidos. A, tubo de muestra; B, vista superior del dispositivo para la colocación de los tubos; C, controlador de la sonda de ultrasonidos; D, sonda; E, baño.

Para el análisis de ácidos siálicos (Capítulo 9) se utilizó un sistema de "laboratorio en válvula" (lab-on-valve, LOV) para la preconcentración y limpieza de la muestra basado en extracción en fase sólida (SPE). Los sistemas LOV constituyen la tercera generación de las técnicas de inyección en flujo (progresivamente FIA, SIA y LOV), y están basados en la inyección secuencial de muestra y reactivos en el sistema, utilizando volúmenes entre 100 μL y 1 mL. Por este motivo el sistema se denomina "meso-fluídico" por utilizar volúmenes a niveles "meso" de muestra, entre micro- y mililitros. El sistema LOV utiliza los seis puertos de una válvula multiposición como canales de entrada y salida del flujo, que pueden usarse de forma secuencial, permitiendo al usuario programar de forma sencilla todas las etapas requeridas para la preparación de muestra; lo que lo dota de una particular versatilidad. El sistema está constituido por una microjeringa de un volumen máximo de 1 mL, que permite aspirar, dispensar, parar,

acelerar y ralentizar el flujo; una válvula de 6 canales de plexiglás, en la que los puertos están conectados entre sí y con la posición central de la válvula; un bucle de llenado entre la bomba y la válvula y una válvula de selección de dos vías para la introducción de un flujo adicional de disolvente portador o "carrier". La secuencia de pasos está completamente automatizada y optimizada mediante el software FIAlab 5.0 para Windows. Una válvula externa adicional, también controlada por el software FIAlab, se usó para la recolección del eluido después de la SPE en el sistema. Las columnas de extracción se fabricaron en el laboratorio a partir de tubo Peek y material sorbente y se conectaron a uno de los puertos del LOV.

Las distintas muestras analíticas obtenidas con los dispositivos descritos anteriormente se centrifugaron mediante una centrífuga Selecta Mixtasel-BL para separar los residuos sólidos en cada muestra tratada que se llevó a sequedad en un Rotavapor Büchi R-200 para la posterior reconstitución del residuo en el disolvente adecuado.

2. Sistemas automáticos para la preparación de muestras

La preparación de la muestra mediante extracción en fase sólida de los compuestos de interés se realizó con equipos automáticos comerciales. De estos equipos, el denominado Prospekt-2 está compuesto por dos unidades: Un dispensador que trabaja a alta presión (high-pressure dispenser, HPD) y que, con auxilio de un conjunto de válvulas, permite realizar todas las etapas de que consta el proceso de extracción en fase sólida; y un sistema automático de cambio de cartucho (automatic cartridge exchange, ACE). Todo el proceso de extracción se realiza a alta presión; lo que lo diferencia

de otros sistemas automáticos de extracción en fase sólida (Capítulos 8 y 10).

3. Equipos cromatográficos

Durante el desarrollo experimental de esta Tesis Doctoral se desarrollaron métodos basados en separación cromatográfica de gases y de líquidos, y en detección mediante espectrometría de masas, fotometría de absorción en la zona de ultravioleta-visible utilizando un espectrofotómetro de diodos en fila (DAD), mientras que en emisión molecular (fluorescencia) se utilizó un láser (LIF) como fuente de excitación.

Así, en los Capítulos 2 y 3, dedicados al análisis de esteroles y alcoholes alifáticos en muestras vegetales, en los Capítulos 4 y 6, para la cuantificación de metabolitos insaponificables incluyendo los alcoholes triterpenicos, y en el Capítulo 5, para compuestos no insaponificables (ácidos grasos), la separación por cromatografía de gases se llevó a cabo con un cromatógrafo Varian 2000 con un software de Varian para la adquisición y tratamiento de datos.

En los Capítulos 8 y 10, el análisis de fenoles y ácidos siálicos en muestras bilógicas se realizó por cromatografía líquida y posterior detección por espectrometría de masas de triple cuadrupolo. El equipo utilizado fue un cromatógrafo Agilent modelo 1200 equipado con una bomba binaria, un desgasificador, un automuestreador y un compartimento de columna termostatizados y un espectrómetro de masas de triple cuadrupolo Agilent 6410 con una fuente de ionización por electrospray (ESI). El software Agilent MassHunter Workstation se usó para la toma de datos así como para el análisis cuali- y cuantitativo. En el Capítulo 5, la cuantificación de fenoles se realizó con un cromatógrafo líquidos

empleando un detector fotométrico de absorción molecular de diodos en fila (DAD) en la zona ultravioleta-visible, modelo HP 1100.

En los métodos puestos a punto para el estudio de los perfiles metabolómicos (metabolomic profilings) de muestras de plasma (Capítulos 11 y 12) se utilizó un cromatógrafo de líquidos (en todos los casos la separación cromatográfica se realizó en fase reversa) acoplado a un detector de masas de tiempo de vuelo de alta resolución, Agilent 6540. En todos los casos se usó el software MassHunter para la adquisición de datos, el análisis cualitativo y semicuantitativo y para la identificación de metabolitos utilizando los valores de relación masa/carga para los iones precursores.

En el Capítulo 9, dedicado al análisis de ácidos siálicos en cuatro biofluidos humanos, se utilizó un cromatógrafo a escala micro (μ-LC) modelo Agilent 1100, compuesto por una bomba binaria capilar, una columna micro en fase reversa (C-18), una válvula de inyección automática y un detector DAD. El sistema estaba conectado por tubos capilares y se usaron microvolúmenes de muestra y de fase móvil. Tras la salida de la célula de flujo del DAD, el sistema se conectó a un detector de fluorescencia inducida por láser (Zetalif 2000 con un láser de He-Ne, longitud de onda de emisión de 325 nm). El control del sistema cromatográfico y los detectores, y la integración de las señales se llevaron a cabo mediante el software Chemstation de Agilent.

PARTE EXPERIMENTAL

PARTE I

Desarrollo y aplicación de plataformas orientadas: Metabolómica del olivo

En esta primera parte de la Memoria se recoge la investigación realizada sobre metabolómica del olivo, en la que la estrategia analítica fue siempre orientada.

El Capítulo 1 lo constituye una revisión bibliográfica sobre la fracción insaponificable que ha venido a cubrir un vacío existente en la bibliografía sobre este tipo de compuestos, a los que la doctoranda ha dedicado una buena parte de su investigación, alguna de la cual se muestra en los Capítulos 2 y 3. En el Capítulo 2 se recoge la plataforma diseñada para la determinación de esteroles y alcoholes de cadena larga en hojas de olivo y en el fruto, y en la que previa a su separación individual mediante cromatografía de gases y su detección mediante espectrometría de masas en tándem, la preparación de la muestra (tanto la etapa de lixiviación como la de derivatización) se ha mejorado y acelerado con auxilio de ultrasonidos. La aplicación del método a la caracterización de estas dos familias de la fracción insaponificable en hojas y frutos de diferentes variedades de olivo, a diferentes tiempos de maduración del fruto y diferentes tiempos de recolección de las hojas, ha puesto de manifiesto la dependencia que tiene la concentración de estos compuestos de las variables en estudio, tal como se muestra en el Capítulo 3.

CAPITULO 1

Compuestos insaponificables: Características químicas, propiedades y análisis

Unsaponifiable compounds: chemistry, properties, and analysis

M.I. Orozco-Solano[a,b], M.D. Luque de Castro[a,b*]

[a]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain

[b]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain

Enviado a:

Journal of Agricultural and Food Chemistry para su publicación

Unsaponifiable compounds in virgin olive oil: chemistry, properties and analysis

M.I. Orozco-Solano, M.D. Luque de Castro

[a]*Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain*

[b]*Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain*

ABSTRACT

Minor components in virgin olive oil (VOO) seem to play a key role in the beneficial effects of VOO on several diseases; effects that stand from a prominent and well-balanced chemical composition, which is a blend of major (98% of total oil weight) and minor compounds including antioxidants. The main minor compounds constitute the unsaponifiable fraction, which can be divided into two groups: those non–chemically related to fatty acids such as hydrocarbons, alcohols, sterols, phenols, volatile compounds and antioxidants, and fatty acid derivatives such as phospholipids, waxes and sterol esters, as the most important. While those non-chemically related to fatty acids such as tocopherols and sterols can be found in other vegetable oils, minor compounds in olive oil are exclusive of the *Olea europaea* species endowing it with a chemotaxonomic interest. This review is focused on VOO unsaponifiable profile and, particularly, on sterols, aliphatic and triterpenic alcohols, phenols, some of which are present in several fats as well as in VOO. Analytical methods for qualitative and/or quantitative determination of these compounds are assessed. The implementation of efficient sample preparation protocols, separation techniques such as high performance liquid chromatography (HPLC) and gas chromatography (GC), as well as

detection systems by-mas spectrometry, and, are also here reported. The low content of unsaponifiable compounds and the presence of interfering compounds require an adequate treatment and purification of the sample. The advantages and drawbacks of conventional and accelerated methods for extraction of the target compounds, chemical reactions and different cleanup steps are also discussed in the light of stability of unsaponifiable components to heating, exposure to light and oxidation.

KEYWORDS: *Sterols, fatty alcohols, triterpenic alcohols, virgin olive oil,*olive, phenolic antioxidants, *ultrasounds energy, GC–MS, HPLC-DAD*

INTRODUCTION

The Mediterranean diet is mainly constituted by virgin olive oil (VOO) as primary source of fat intake replacing all types of commonly used animal fats that are detrimental to human health.[1] Several clinical and epidemiological studies have confirmed that people who closely follow the Mediterranean diet live longer than other Europeans and Americans thanks to the lower incidence of degenerative diseases. [2,3]

The chemical composition of VOO is divided into two main groups: major components (≈98%), and minor components (≈2%).[4] The first fraction mainly constituted by esterified fatty acids (FA), predominantly in the form of triacylglycerols. The key aspect of the fatty fraction is the high concentration of monounsaturated FA (MUFA), especially oleic acid, to the detriment of polyunsaturated FA (PUFA) that reduces the risk of atherosclerosis, [5] and protects from different kinds of cancer. [6] There are two types of minor compounds which are soluble in the lipid fraction:

glycerolipids and non-glycerolipids. Upon reaction with alkali, the unsaponifiable fraction of VOO and other vegetable oils (*i.e.* non-glycerolipids) does not form soap and can be extracted from the saponified mixture with lipophilic solvents such as diethyl ether, hexane, or cyclohexane (Figure 1). Over the past decades, these unsaponifiable compounds have been of interest for researchers for different reasons. In the first instance, the interest in the unsaponifiable fraction stemed from the observation that many components, especially sterols, tocopherols but also phenolic and other compounds, have antioxidant properties of utmost importance for the oxidative stability of VOO as well as of several edible oils. Moreover, much research is currently focusing on the biological and physiological activities of various unsaponifiable constituents and their possible contribution to the oil quality and the improvement human health.[7]

THEIR OCCURENCE AND SIGNIFICANCE IN VOO

Minor components of the unsaponifiable fraction include a number of heterogeneous compounds (more than 230).[8] These components are divided into two groups: those non-chemically related to FA such as hydrocarbons, alcohols, sterols, volatile compounds and antioxidants, and FA derivatives such as phospholipids, waxes and sterol esters, as the most important.[9] Despite the small concentration of minor components (only 2% of VOO weight), they play a key role in the quality and behavior of the different VOOs and in their characterization.[9] Antioxidants in VOO are represented by tocopherols, pigments (carotenoids, chlorophylls) and phenolic compounds. These compounds provide a defense mechanism that delays aging and prevents carcinogenesis, atherosclerosis, obesity, liver diseases and inflammations, among other health disorders.[10]

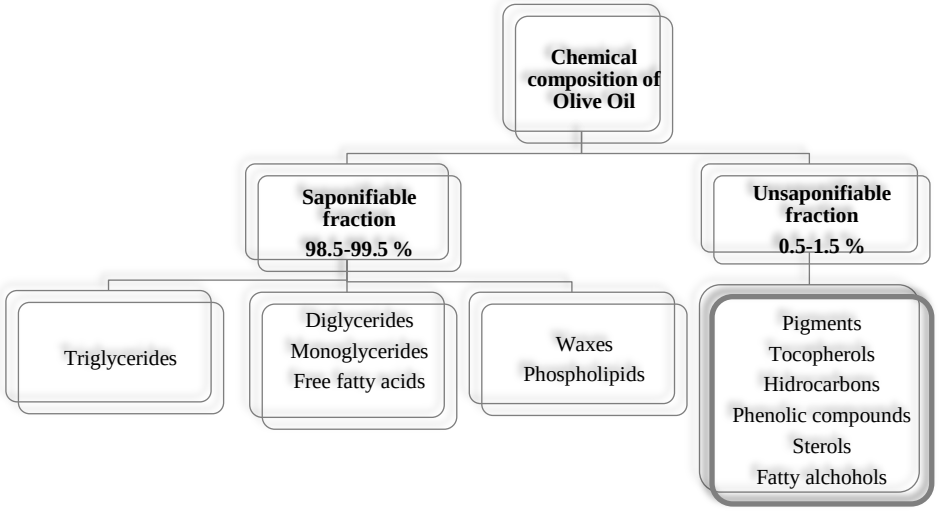


***Figure 1**. Scheme of chemical composition of olive oil (saponifiable and unsaponifiable fractions).*

Different reviews have been reported on the antioxidant capacity, stability and wholesomeness of VOO describing their benefits and analytical methods for their characterization [8] but none has been devoted or has emphasized the role of the unsaponifiable fraction, its determination employing alternatives methodologies endowed with similar analytical characteristics to the conventional methods which has been used in operational time more shorts and less wasteful obtaining best results than with the conventional methods. Lerker *et al.* described the use of conventional methodologies for the analysis and posterior identification of each family of compounds by gas chromatography with flame ionization detection (GC-FID) or mass spectrometry.[10] These techniques have been developed and widely used in recent years with some modifications in either sample preparation step and the number of families analized. This

review attemps to fill this gap by providing wide and up to dated information on the unsaponifiable composition of VOO: traditional and recent methods for determination and quantification of the different families of metabolites which constitute this fraction, and the results of recent studies about its biological properties, organoleptic characteristics and effects on human health.

Regarding the benefits of the unsaponifiable fraction in VOO on human health, the key role of minor compounds mainly attributed to sterols, the most abundant constituents of this fraction, which are known to have a wide range of biological activities. Clinical studies have demonstrated that plant sterols ─also known as phytosterols─ ingested in the normal diet or as dietetic supplements decrease cholesterol levels in blood, inhibiting their absorption in the thin intestine.[11] Also, it is recognized that phytosterols are biologically active substances in cancer prevention, although it has still not been demonstrated by epidemiological studies. [3]

Sterols. Sterols are widely occurring natural substances and make up the greatest proportion of the unsaponifiable fraction of lipids [8] (100–220 mg/100 g of VOO[8]). The most important natural sources of plant sterols in the human diet are oils and margarines, although these metabolites are also found in a range of seeds, legumes, vegetables and unrefined vegetable oils. [12] Their composition depends on the plant species [9] and, in oils, it may vary according to agronomic and climatic conditions, the quality of the fruits or seeds, extraction and refining procedures and storage conditions. Phytosterols can be classified on a structural or biosynthetic basis as 4-desmethyl sterols, 4α-monomethyl sterols and 4,4-

dimethyl sterols. In addition, 4-desmethyl sterols may be subdivided into Δ5-sterols, Δ7-sterols and Δ5,7-sterols, depending on the position of the double bonds in the β ring.[7,8] The predominant phytosterol in VOO is β-sitosterol, being minor components campesterol, stigmasterol, Δ5-avenasterol, Δ7-avenasterol and brassicasterol. Sterols, including precursors of cholesterol and their metabolites, seem to be biologically active, although many of their biological effects have to be clarified and assessed.

The potential of sterols to bind to nuclear receptors and activate target gene transcription has been one of the interesting properties of these metabolites that has encouraged much new research in this area.[8] Sterols are membrane components and, as such, they regulate its fluidity and permeability. In plants, where they are always present in mixtures, sterols act as substrates for the production of a wide variety of secondary metabolites, such as cardenalids, glycoalcaloids, pregnane derivatives and saponins (see Figure 2).

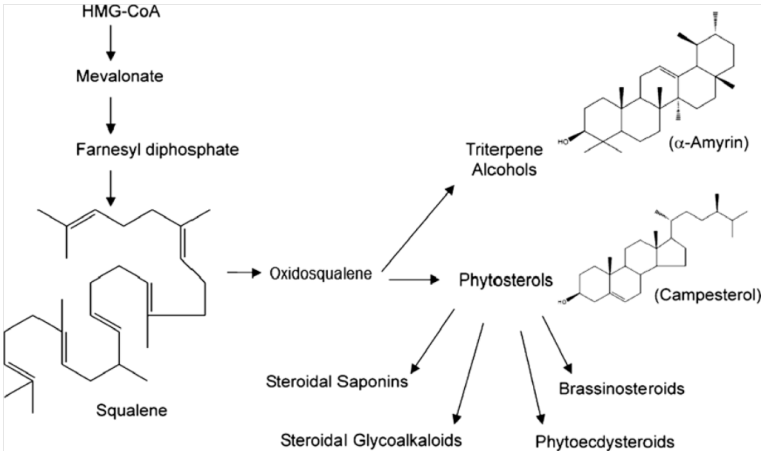


Figure2. *Biosynthesis of sterols and triterpenoids via the acetate–mevalonate pathway.*

The similar structure of plant sterols and cholesterol led to an erroneous identification of sterols as cholesterol in peas by Beneke.[13] This error was extensively demonstrated as such by the research developed on the subject since the early fifties of the last century. The similarity of sterols and cholesterol can be visualized in Figure 3. In fact cholesterol is a specific type of sterol that contains 27 carbon atoms, 46 hydrogen atoms and one oxygen atom in a ring structure. The nutritional interest derives from the fact that the sterols have a structure similar to cholesterol, and have the capacity to lower plasma cholesterol and LDL cholesterol. Since the morbidity and mortality from cardiovascular disease have been dramatically reduced using cholesterol-lowering drugs (statins), the interest in sterols lies in their potential to act as a natural preventive dietary product.

The chemical structure of sterols are very similar to that of cholesterol, with a Δ5 double bond and a 3β-hydroxyl group, but with structural modifications of the side chain. Campesterol and sitosterol, 24-methyl and 24-ethyl cholesterol analogues are the most abundant phytosterol occurring at low concentrations in human plasma. They are a part of the diet and are exclusively taken up from the intestine. Other sterols such as brassicasterol, avenasterol, ergosterol, stigmasterol, and even shellfish sterols could also be identified in human plasma, but at lower levels. Although the dietary intake of plant sterols is comparable to that in plasma cholesterol, plant sterols levels in plasma are usually less than 10^{-3} mg/L. The reasons for low plasma concentrations of sterols are poor intestinal absorption rates and high biliary elimination rates as compared to cholesterol.[2] Some of the main sterols present in plant and food are listed in Table 1.

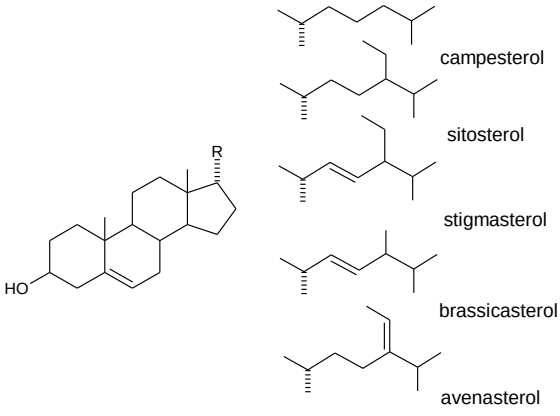


***Figure 3**. Chemical structure of main plant sterols.*

Their chemical structure is very similar to that of cholesterol with a Δ5 double bond and a 3β-hydroxyl group, but with structural modifications of the side chain. Campesterol and sitosterol, 24-methyl and 24-ethyl cholesterol analogues are the most abundant phytosterols occurring at low concentrations in human plasma. They are a part of the diet and are exclusively taken up from the intestine. Other sterols such as brassicasterol, avenasterol, ergosterol, stigmasterol, and even shellfish sterols could also be identified in human plasma, but at lower levels. Although the dietary intake of plant sterols is comparable to that in plasma cholesterol, plant sterols levels in plasma are usually less than 10^{-3} mg/l. The reasons for low plasma concentrations of sterols are poor intestinal absorption rates and high biliary elimination rates as compared to cholesterol.[2] Some of the main sterols present in plant and food are listed in Table 1.

Tabla 1. Main sterols present in food analyzed by GC

Sterols	Food	Internal standard
Brassicasterol, campesterol, stigmasterol, β-sitosterol, Δ^{-5} avenasterol	Milk, margarine	Betulin
Brassicasterol, campesterol, stigmasterol, β-sitosterol, Δ^{-7} avenasterol	Vegetable oils	Cholesteryl heptadecanoate
Cholesterol, brassicasterol, campesterol, stigmasterol , sitosterol, Δ-5-avenasterol, others (stanols: campestanol, sitostanol)	Whole- wheat flour and diet composite	
Cholesterol, 24-norderhydrocholesterol, trans-22-dehydrocholesterol, brassiscasterol.	Oils, margarines, cereal products, vegetables, fruits, berries	
Brassicasterol, campesterol, stigmasterol, β-sitosterol,	Plant matrices	Dihydrocholesterol
Sitosterol, campesterol, stigmasterol, brassicasterol, Δ^{-5}-avenasterol , cholesterol, others (sitostanol, campestanol)	Edible oils and fats	Epicholesterol
B-sitosterol, campesterol, stigmasterol, Δ^{-5}-avenasterol, sitostanol, campesterol	Nuts and seed	Epicholesterol

Aliphatic alcohols. Fatty alcohols are aliphatic compounds derived from natural fats and oils, originated in plants, but also synthesized in animals and algae. Their historically overlooked significance in nutrition and health is at present recognized, as they are closely related to fatty acids including

the well-documented omega-3 fatty acid. Their counterparts are fatty aldehydes. Production from fatty acids yields normal-chain alcohols, in which the alcohol group is attached to the terminal carbon; other metabolic pathways can yield iso-alcohols, in which the alcohol group attaches to a carbon in the interior of the carbon chain.

The smallest alcohol molecules are used in cosmetics and food, and as industrial solvents. Some of the largest molecules are simply seen as biofuels, but little research had been done until 2006 regarding many of them, despite they had shown to be endowed with anticancer, antiviral, antifungal, and anti-HIV properties, and thus, with potential in medicine and also as health supplements.[13]

Due to their amphipathic nature, fatty alcohols behave as non-ionic surfactants. They find use as emulsifiers, emollients and thickeners in cosmetics and foods, and are common components of waxes, mostly as esters of fatty acids, but also as free alcohols.

Octacosanol, present in carrots and ginseng, was found in 1994 to lower cholesterol levels. Following this, a major paper was released in 2006 and other in 2007, in which information on hundreds of previous studies on fatty alcohols was reported. VOO contain linear alcohols,[14] which are constituted by primary fatty alcohols generally having 20–32 carbon atoms in the alkyl chain. Triterpene alcohols, also known as 4,4-dimethylsterols, have a steroid structure and are present at different levels in all vegetable lipids.[15] Other alcohols, as phytol, are generated as artifacts in lipid saponification.

Very long-chain fatty alcohols (VLCFA) obtained from plant waxes and beeswax have been reported to lower plasma cholesterol in humans [13]. They can be found in unrefined cereal grains, beeswax, and many

plant-derived foods. Reports suggest that 5–20 mg per day of mixed C24–C34 alcohols, including octacosanol and triacontanol, lower low-density lipoprotein (LDL) cholesterol by 21–29% and raise high-density lipoprotein (HDL) cholesterol by 8–15%. Wax esters are hydrolyzed by a bile salt-dependent pancreatic carboxyl esterase, releasing long-chain alcohols and fatty acids that are absorbed in the gastrointestinal tract. Studies of fatty alcohol metabolism in fibroblasts suggest that very long-chain fatty alcohols, fatty aldehydes, and fatty acids are reversibly inter-converted by a fatty alcohol cycle.[15]

The main alcohols present in olive oil are hexacosanol, octacosanol, tetracosanol and docosanol (always at higher concentration in the VOO from second press than in virgin olive oil).[16]

Triterpenes. Commonly, triterpenic compounds are components of natural herbal preparations used for the treatment of human diseases. Chemically, pentacyclic triterpenes are all based on a 30-carbon skeleton comprising five six-membered rings (ursanes and oleananes) or four six-membered rings and one five-membered ring (lupanes) (see Figure 4), and as lipophilic molecules may also penetrate the blood-brain barrier, as it has already been demonstrated for some of them .[17,18] The therapeutic properties of extracts from leaves, flowers and fruits of *Olea europaea*, used traditionally for medicinal purposes,[19,20] can in part be attributed to the major components in the unsaponifiable fraction of olive-pomace oil (*viz.* triterpenic acids, oleanolic and maslinic acids and the triterpenic diols, uvaol and erythrodiol)[21]. Erythrodiol and uvaol are in the ranges from 10 mg/100 to 280 mg/100 g of VOO.

Triterpenes are also important bioactive secondary metabolites thanks to the wide range of their biological activities. They are common constituents of plants, fruits and vegetables for human diet. They occur widely in the plant kingdom in the form of free acids or aglycones of triterpenoid saponins. Remarkably, the literature contains relatively little information on the distribution of triterpenic compounds in the plant kingdom, despite their pharmacological properties, which include anti-inflammatory, hepatoprotective, anti-tumor, anti-viral, anti-HIV, anti-microbial,[6] anti-fungal, anti-diabetic, gastroprotective, and anti-hyperlipidemic effects, antifeedant, and insecticidal activities.[23-25] In addition, *in vivo* studies have demonstrated some benefits in preventing hypertension . Indeed, oleanolic acid, as well as uvaol and the maslinic acid derivative methyl maslinate have also shown to possess vasodepressor, cardiotonic, and antidysrhythmic properties.[26]

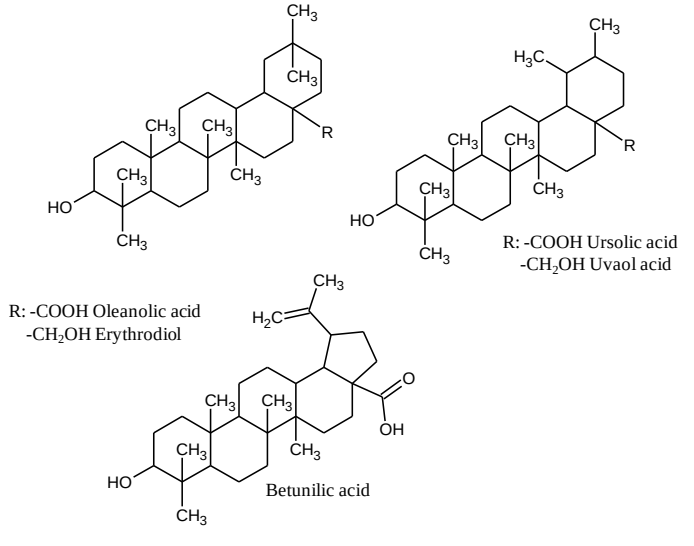


Figure 4. *Chemical structure of main plant triterpenic alcohols.*

Other studies have also shown that a relationship between triterpenes and vasorelaxation in the aorta of hypertensive rats can exist.[27] Recently, it has been suggested that erythrodiol derivatives can prevent the harmful effects of ultraviolet rays that lead to skin aging or skin cancer.[28] These compounds are relatively non-toxic and have been used in cosmetic and health products. In addition, other studies have suggested that both acidic and alcoholic triterpenes present anti-tumor activities, although they markedly differ in their cytotoxic activity and the precise mechanism of action is still unclear, especially for the triterpenic diols. Thus, oleanolic and maslinic acids are powerful pro-apoptotic agents in human colon cancer cells, while uvaol shows weak activity against an array of human cancer cell lines from different tissues.[23-24]

Triterpenics compounds have also been used as markers to characterize and to detect mixture of vegetable oils.[8] The most representative and commercially available triterpenic compounds found in olive-tree leaves and drupes are erythrodiol uvaol, oleanolic, ursolic, and maslinic acids. Oleanolic acid [(3β)-3-hydroxyolean-12-en-28-oic acid], one of the best known bioactive pentacyclic triterpenoids, also exists widely in medicinal herbs and plants.

Phenolic compounds. Phenolic compounds make up one of the major families of secondary metabolites in plants and represent a diverse group of compounds. Phenols can be broadly divided into non-soluble compounds such as condensed tannins, lignins, and cell-wall bound hydroxycinamic acids, and soluble phenols such as phenolic acids, phenylpropanoids, flavonoids and quinines. All these groups are involved in many processes in

plants and animals. Figure 5 shows the structure of the main groups of phenolic compounds

Soluble phenols are also known as hydrophilic phenols, which are specially important to establish olive oil quality due to their antioxidant activity and their effect on the organoleptic characteristics of VOO. Among these secondary metabolites, secoiridoids and lignans are the most abundant.

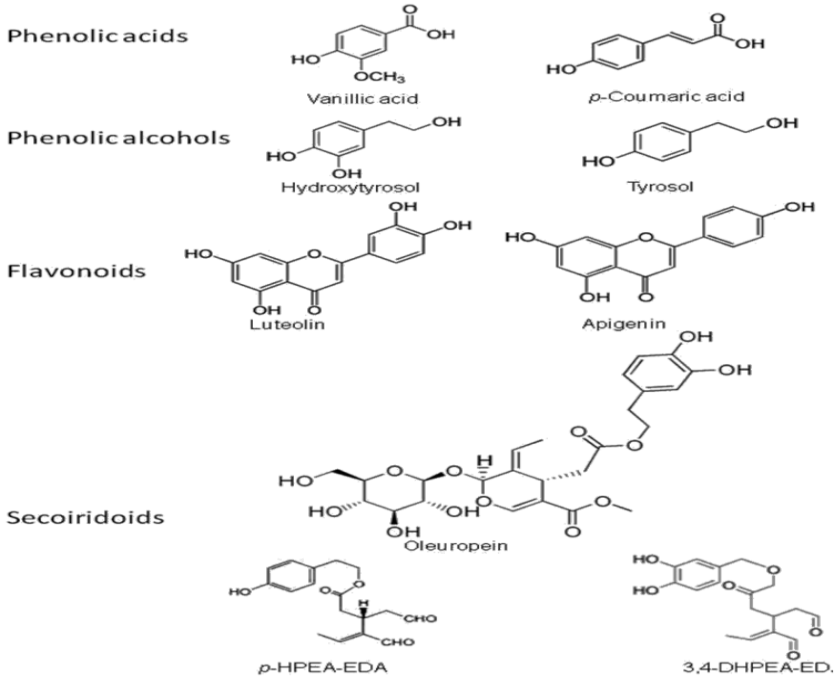


Figure 5*. Structures of the most representative hydrophilic phenols.*

Within this selected group, has been found the phenolic acids — including caffeic, vanillic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, *p*-hydroxybenzoic and gallic acids; also ferulic and cinnamic acids which

have been quantified, but in lower quantities (less than 1 mg analyte/Kg olive oil). Phenolic acids are secondary aromatic plant metabolites spread in a wide range of plants which have been associated with color and sensory properties of foods. They are also used as potential markers of geographical originof olive cultivars.[25] The main phenolic alcohols in VOO are 3,4-dihydroxyphenyl ethanol, also known as 3,4-DHPEA or hydroxytyrosol, and *p*-hydroxyphenyl ethanol p-HPEA or tyrosol. These alcohols are in fresh oils at low concentration which increases during storage as they result from the hydrolysis of olive oil secoiridoids containing 3,4-DHPEA and *p*-HPEA in their molecular structures.[18] Among these secondary metabolites, secoiridoids and lignans are the most concentrated.

Phenolic compounds are considered as the most important components in unsaponifiable fraction. Their interest has increased in the last decades, thus stimulating multidisciplinary research on their composition, histological distribution and histochemical enzymatic localization to determine their biomolecular functions. [4] Nevertheless, the main reason of the growing research in this field lies in the healthy properties of these compounds.

The excellent properties of phenols compounds are a consequence of their function in the olive tree (namely, reactivity against pathogens attack and response to insect injury.[7] Olive phenols include a major group of secondary metabolites that display a wealth of both structural variety and diversity of key activities. The healthy effects of olive oil —mainly due to the presence of these particular phenols— have been widely studied in the last decades.[8,9] Oleuropein, the most abundant phenol in olive leaves and also at high concentrations in pomace, has been used in a number of medical treatments since its first reference in the literature; thus, this phenol

prevents cardiovascular diseases by protecting membrane lipid oxidation acting on coronary dilation and by antiarythmic action, improves the lipid metabolism to mitigate obesity problems,[25] protects both enzymes and against hypertensive cell death in cancer patients,[26] and presents antiviral properties.[27] Hydroxytyrosol, an oleuropein derivative, also get better cardiac and tumoral diseases with effects similar to those of oleuropein; in addition, it protects against atherosclerosis[15] and prevents diabetic neuropathies (16). Also, the nutraceutical utility of other phenols present in olive leaves and pomace at high amounts such as verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside has been studied. Thus, verbascoside has been used to repair brain's oxidative damage caused by heroin consumption, apigenin-7-glucoside to fight against Alzheimer's disease or liver diseases, and luteolin-7-glucoside to avoid the abnormal proliferation of aortic vascular smooth muscle cells that is a common cause of pathogenesis such as atherosclerosis and restenosis.[20] However, among these olive phenols, only oleuropein is present in VOO at high amounts. Verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside are generally detected at low concentration in VOO.[21] The continuous research on phenolic compounds has led to the isolation of compounds such as oleocanthal, found in olive oil and whose interesting ibuprofen-like activity has been reported.[22]

Olive phenols have promoted active research on raw materials for their obtainment. The two main sources have been olive leaves and the pomace or waste from VOO production. In the case of two-phase production system for the pomace (presently the most frequent used in this industry) is a polluting semisolid residue. It is a cheap source of natural antioxidants in concentrations up to 100-times higher than in olive oil,[23]

which results from the polar nature of both pomace and olive phenols and the low polar nature of oil; nevertheless, olive leaves have the highest antioxidant and scavenging power between the different parts of the olive three (*e.g.* taking oleuropein as an olive phenol model, its content in olive oil ranges between 0.005 and 0.12%; in pomace up to 0.87% and in olive leaves between 1 and 14%).[24,25]

Tocopherols. These compounds (see Table 2) [26,27] display antioxidant properties and are active as vitamins (vitamin E), thus making them particularly important for human health. Tocotrienols are present in small amounts in food lipids, except for palm oil and, grapeseed oil and the annatto lipid fraction, which have a relatively high content of these active components.

Tocopherols are compounds that contribute to the stability of olive oil, and have a very important biological role as antioxidants. They are also involved in the mechanisms of oil oxidation and preserve oil quality during storage.[28] Tocopherols and tocotrienols are present in small amounts in olive oils between 100 and 300 ppm, but their content in vegetable oils of high acidity has been found above of 5 ppm. α-Tocopherol representing approximately 95% of total tocopherols in VOO.

It is worth noting that in preparative thin-layer chromatography (TLC) for unsaponifiable fractionation tocopherols and tocotrienols coelute with other compounds of similar polarity. Co-eluted substances are epoxy-squalenes, which are structurally similar to squalene but they have an oxyranic ring.[28] Epoxy-squalenes derive from the oxidation of squalene, which can be of enzymatic or chemical origin, as in olive husk oil. [29] In oils

extracted from olives harvested at different ripening stages, the level of epoxy-squalenes vary. Since they were practically absent in oils obtained from mature olives, it can be stated that the epoxy-squalenes are precursors to the steroid biosynthesis (*i.e.* lanosterol in the animal systems and cycloarthenol in the vegetal ones).

Tabla 2. Main IUPAC names of tocopherols

Name	IUPAC name	Abreviaiton
5-Methyltocotrienol	2,5-Dimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	5-T
7-Methyltocotrienol	2,7-Dimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	7-T
δ-Tocotrienol (8-methyltocotrienol)	2,8-Dimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	Δ-T-3
5,7-Dimethyltocotrienol	2,5,7-Trimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	5,7 – T-3
β-Tocotrienol (5,8-dimethyltocotrienol)	2,5,8-Trimethyl-2-(4′,8′,12′-trimethyl 3′,7′,11′-tridecatrienyl)-6-chromanol	β-T-3
γ-Tocotrienol (7,8-dimethyltocotrienol)	2,7,8-Trimethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	5-T-3
α-Tocotrienol (5,7,8-trimethyltocotrienol)	2,5,7,8-Tetramethyl-2-(4′,8′,12′-trimethyl-3′,7′,11′-tridecatrienyl)-6-chromanol	

Hydrocarbons. Hydrocarbons are present in the natural lipid systems in quite small amounts (≤0.2% of the total lipids); the only exception being VOO, which contains about 0.5%, mainly constituted by squalene. Hydrocarbons are formed by a homologous series of linear compounds that are mainly saturated chains of 15–33 carbon atoms; in food matrices, most of hydrocarbons have an odd number of carbon atoms. There are also small amounts of ramified compounds present, which exhibit isostructures.[29,31]

Squalane, a natural lipid belonging to the terpenoid family and a precursor of cholesterol biosynthesis, is at present a metabolite of great and growing interest. It is synthesized by humans and also by a wide array of organisms and substances, from sharks to olives and even bran, among others. Because of its significant dietary benefits, biocompatibility, inertness, and other advantageous properties, squalene is extensively used as an excipient in pharmaceutical formulations for disease management and therapy. In addition, squalene acts as a protective agent and has been shown to decrease chemotherapy-induced side-effects. Moreover, squalene alone exhibits chemopreventive activity. Although squalene is a weak inhibitor of tumor cell proliferation, it contributes either directly or indirectly to the treatment of cancer due to its potentiation effect. In addition, squalene enhances the immune response to various associated antigens, and is therefore being investigated for vaccine delivery applications. Since this triterpene is well absorbed orally, it has been used to improve the oral delivery of therapeutic molecules. All of these qualities have rendered squalene a potentially interesting excipient for pharmaceutical applications, especially for the delivery of vaccines, drugs, genes, and other biological substances.

During the refining process, unsaturated hydrocarbons as squalene isomerize, generating a large number of isomers, such as squelene. The presence of these isomers can be detected by CG.[30]

Squalene, a biochemical precursor of sterols, is an important hydrocarbon of VOO and refined olive oil. Virgen olive oil contain the major amount of squalene (2500–9259 µg/g) as compared other vegetable oils (16–370 µg/g) (45). Other hydrocarbons as polycyclic aromatic hydrocarobons (PAHs) such as phenanthrene and pyrene, among others, have also been found inVOO.

Small quantities of pollutants (*e.g.* pesticides, volatile organic compounds, chlorinated hydrocarbons, aromatic hydrocarbons, etc.) can be found in some unrefined squeezed oils. In fact, small quantities of benzene (about 1 ppm) have been detected in VOO by using GC.[31] On the other hand, polycyclic aromatic hydrocarbons (PAHs), which originate from environmental pollution, together with the other hydrocarbons in TLC fractionation. However, when these substances are analyzed by cGC, the peaks of PAHs overlap with those of the high-molecular weight hydrocarbons, thus rendering impossible to obtain a reliable quantification of both types of components. High-performance liquid chromatography (HPLC) would be, in this case, a suitable analytical choice, since it has proven successfully to separate PAHs from the other hydrocarbons .[32]

METHODS FOR DETERMINATION OF STEROLS, PHENOLS, AND TRITERPENIC AND ALIPHATIC COMPOUNDS

Preparation of crude extracts from plants is a key step prior to qualitative and/or quantitative analysis of chemical constituents present in vegetable tissues. The traditional leaching techniques of plant materials are mostly based on the use of heat and/or agitation to increase the rate of mass transfer to the suitable leachant. Thus, the extraction of sterols, phenols and aliphatic and triterpenic alcohols is traditionally performed by maceration, hydrodistillation or Soxhlet extraction.[31-35] These techniques are often low-efficient, time-consuming and require large volumes of organic solvents, which means additional costs and environmental hazards. In addition, many natural products are thermally unstable and may degrade during extraction if the temperature is high enough. In the last years, alternative techniques such as ultrasound- and microwave-assisted extraction, pressurized liquid extraction and supercritical fluid extraction[8,1] have increased the efficiency of extraction processes and shortened the leachant volume and procedure time. An ideal leaching step should be exhaustive with respect to the constituents to be analyzed —if quantitative determination is pursued—, fast, simple, inexpensive, environmental friendly and amenable to automation for routine analysis.

Sample drying and grinding. The vegetal sample must be dried at temperatures above 40 ºC to avoid degradation of thermolabile compounds. Then, the usually dried solid sample crushed to a homogeneous and small grain size to facilitate transfer of the analytes to a liquid phase. The device used for this task is a function of the hardness of the given sample. Mortars plus pestles, and mills of different types depending on both the sample and the desired grain size have been used.

Sample preparation. Sample preparation can involve a number of steps of different complexity and difficulty depending on the type of the sample–analyte(s) binomial. Between the most common steps in sample preparation are solid–liquid extraction (more correctly named as leaching or lixiviation), for solid samples, solid-phase extraction or liquid–liquid extraction and selective derivatization for liquid samples.[36] Less frequent steps are thin-layer chromatography (mainly used for separation of aliphatic alcohol, triterpenic alcohols, hydrocarbons and sterol families in the official method) and preparatory chromatography.[37]

Obtainment of in-solution analytes (either by digestion or by leaching) from vegetal samples has traditionally been carried out by conventional methods, generally slow, but the effectiveness of which has never been put in doubt by the users who have applied them without validation. The growing involvement of analytical chemists in the development of analytical platforms for "omics" (an area in which these professionals should have a high involvement), has led to: (i) assess the effectiveness and accepted characteristics of conventional methods for sample preparation; (ii) promote acceleration of sample preparation by using auxiliary energies and automation by the design and optimization of dynamic systems.

(a) *Separation of analytes from the sample matrix*. The isolation technique to be applied depends on the nature of the matrix, its physical state (liquid or solid), and also on the form (free, esterified or glycosylated) in which sterols and aliphatic alcohols, triterpenic alcohols and some hydrocarbons occur in the matrix.

Sample leaching is the most usual step for analyte extraction in dealing with solid samples, usually carried out by traditional methods based

on the solubility of sterols and aliphatic and triterpenic alcohols in different organic solvents such as dicholorometane, chloroform, hexane or benzene subjected to stirring to facilitate mass transfer to the liquid phase and, in some cases, with the help of heating devices. In the case of phenols, the leaching step is similar but the leachant for hydrophilic antioxidants must be polar; therefore, pure methanol, ethanol or mixtures between them or with other solvents is usually the best choice. Phytosterols and triterpenes in plant tissues and seed oils can be isolated by leaching using chloroform–methanol [18] or chloroform–methanol–water mixtures,[19] or pure hexane,[20] methylene chloride[21] or acetone.

A chloroform–methanol–water mixture was applied by Thompson and Merola to extract cholesterol from multicomponent foods as an alternative method to the AOAC method, and the usefulness of the mixture for determining major non-glycosidic plant sterols.[18,20] A modification of this method has been applied to the extraction of plant sterols from special foods used in nutritional research.[36]

Supercritical CO_2 has also been used for extraction of lipids from vegetables, with the characteristic concentration resulting from extractant decompression, after which sterols can be enriched and isolated following saponification or supercritical fluid fractionation (SFF) together with additional sample cleanup by different chromatographic techniques.

Nowadays, the use of alternative energies for accelerating leaching (or the less common digestion step) is a growing trend. Ultrasound energy and microwaves proved to be useful to shorten the time required for the given step, to use smaller amount of sample and decreased volumes of organic solvents, thus approaching to green methods.[21.23]

Solid-phase extraction (SPE) is usually applied for separation of target analytes from liquid matrices. Cunha S. *et al.* developed this step using neutral alumina cartridges for the extraction of free and esterified sterols and fatty alcohols from oils and fats.[24] Tocopherols and hydrocarbons were previously separated and cleaned by SPE before its determination. Also SPE but using silicagel cartridges has been used to isolate hydrocarbons, tocopherols, sterols and sterol esters from a slightly more polar triacylglycerol matrix.[25] Prior to SPE, the free hydroxyl group of sterols and tocopherols was silylated to reduce their polarity, and the eluted fraction was then analyzed by GC. As compared to solvent extraction, SPE is an environmentally more friendly technique which provides a more convenient way for sample extraction.

(b) *Saponification*. Direct saponification methods have been applied prior to determination of plants sterols in foods. Matrices such as vegetables oils, plants, and fat food require acid or basic hydrolysis. Acid hydrolysis, alkaline saponification and lipid extraction with toluene have been applied, but a combination of both proved to be superior to alkaline hydrolysis alone.[26] Saponifications are quite laborious and not appropriated for routine analysis as a result. On the other hand, acid hydrolysis is not applicable to compounds such as D^{-7}-phytosterol, D^{-7}-stigmastenol, etc, because they decompose or isomerize by subjection to a short time of acidic hydrolysis.[27] Saponifications with ethanolic potassium hydroxide, either at room temperature or under heating (hot saponification) have been described, and, when in the presence of an internal standard, this step can be applied to most matrices (*e.g.* cereals, milk, yoghurt). Saponification at room temperature using 1 M ethanolic potassium hydroxide to promote hydrolysis in lipid extracts or oils requires stirring overnight.[27,28] Acid

hydrolysis has to be applied to obtain total free, esterified and glycosidic sterols from lipid extracts, which also apply in the case of fatty alcohols. Although isolation of unsaponifiable material containing phytostanols and phytosterols, among others, after saponification by organic solvent extraction can be rather time consuming, most methods use multiple extractions in a separatory funnel with an organic solvent such as diethyl ether, ether, light petroleum or heptane, followed by extractant evaporation to dryness. A typical undesirable phenomenon ocurring during washing and shaking (step performed after hydrolysis) is the formation of difficult to break emulsions. The use of small columns of aluminum oxide (alumina) for solid-phase extraction can replace solvent extractions, thus avoiding emulsions and saving time during serial analyses. In addition, reproducibility can be improved. The soap solution can be applied to the alumina column after dilution with ethanol. Potassium salts of free fatty acids are strongly bound to the aluminum oxide surface by the formation of highly insoluble aluminum soaps; the unsaponifiable component being then eluted from the column with ether.[29] Sterol extracts usually require a further cleanup step to remove interferents and/or to be enriched in the case of very diluted extracts, as commented below.

(c) *Extract cleanup*. A cleanup step is most times mandatory following extraction, particularly when the identification–detection step is different from mass spectrometry (to remove co-extracted interferent compounds), but also in mass spectrometry detection because of ionic suppression, which can sometimes be very strong in the absence of this step. For this reason, cleanup has undergone substantial development in the last few years .[30]

The use of accessible and affordable separation techniques such as column chromatography (CC) or TLC can be required depending on the amount of sample to be processed. Both techniques are appropriate for sample cleanup, purification, qualitative assays and preliminary sterol estimation studies. TLC on silica gel has been used to fractionate lipid or unsaponifiable lipid extracts.[8] Spots can be visualized with a UV lamp on a silicagel plate impregnated with a fluorescence indicator such as dichlorofluorescein. However, fractionation of sterols with CC or TLC is time consuming and, therefore, not suitable for routine analysis. That is why in the purification and/or fractionation of lipid extracts or unsaponifiable fractions these techniques have often been replaced by SPE, which provides faster fractionation and requires smaller volumes of solvent.[3]

SPE has been used in reverse-phase mode (octadecylsilica) to isolate the sterol and fatty alcohol fractions from the unsaponifiable extract, and in normal-phase mode to separate trimethylsilyl (TMS) derivatives from non-saponified vegetable oils.[31] Solid-phase microextraction (SPME) is widely used at present in separation–preparation methods for biological samples.[32]

(d) *Derivatization*. Although separation of sterols and fatty alcohols as such without derivatization is possible, the resolution between peaks is not good enough for individual quantitation of the analytes. Derivatization of the target analytes improves peak shape, resolution and sensitivity, and promotes a higher stability for the thermally labile unsaturated sterols in subsequent GC analysis.[33,34] Sterols are commonly analyzed as their TMS or acetate derivatives, the former being more suitable for GC–MS characterization and quantitation. Usual derivatizing agents are *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) in anhydrous pyridine and

bis(trimethyl-silyl)-trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) (1:1, v/v) added to a dried sample. Simultaneous derivatization with BSTFA and extraction (by SPME) was carried out successfully [9]. In this case, the fiber (polyacrylate) was coated with BSTFA in the vapor phase prior to use, and, on sampling, the analytes (saponified sample) were converted into derivatives that have a high affinity for the coating.

Ultrasound assistance to sample preparation. Concerning improvement of existing sample preparation methods, one of the most accepted strategies has been the assistance with auxiliary energies such as microwaves (MW) or ultrasound (US). Both auxiliary energies are in competition as the most efficient to accelerate sample preparation. Although the use of US to assist sample preparation has so far been limited in relation to its potential, few analytical chemists use US devices (or at least proper analytical devices to help, improve or accelerate steps of the analytical process.There are many sample preparation steps which can be assisted by US.[32]

There are two common US devices for sample-preparation applications: bath and probe units. Although US baths (usually designed for cleanup and degassing) are more frequent in analytical laboratories and thus more widely used, they have two main disadvantages that substantially decrease experimental repeatability and reproducibility[7,6]: (a) lack of uniformity in the distribution of US energy (only a small fraction of the total liquid volume in the immediate vicinity of the US source experiences cavitation); and, (b) decline in power with time. In addition, most US-assisted sample-preparation steps are developed in discrete systems, despìte

continuous approaches usually involve automation and availability to be interfaced to other also automated steps.

US-assisted steps involving solid samples, most times a leaching step, are by far the most numerous applications of this type of energy with clear advantage over US-assisted liquid–liquid steps. The reason of this frequent use is because US-assisted leaching combines several effects, namely:

(a) relatively low temperatures (usually 50 ºC is not surpassed) of great interés in dealing with thermolabile compounds;

(b) high localized pressures because the cavitation phenomenon, which favor penetration of the leachant and transport of the target species to the liquid phase; and,

(c) presence of free radicals created during sonolysis of the solvent (hydroxyl and hydrogen peroxide for water) with drastically favored oxidation reactions.

INDIVIDUAL DETECTION–QUANTITATION OF THE TARGET ANALYTES

The great diversity of the ‘‘minor components’’ in the unsaponifiable fraction makes the analysis of this fraction difficult. Once saponification has taken place the unsaponifiable matter can be fractionated into several classes of compounds by any type of chromatography —*e.g.* TLC (AOCS Official methods, 1991) or liquid chromatography (HPLC)—; or the families of compounds be individualy separated with identification/quantitation purposes by capillary zone electrophoresis (CZE) or capillary electrochromatography (CEC)[32]; or derivatized to TMS derivatives for subsequent separation by GC with either FID or MS

detection .[19] Major disadvantages of GC for this task are the requirement of both stable columns and chemical derivatization prior to analyses; therefore, in the last years methods for these compounds based on either LC or nano-LC with MS detection have been reported.[21,24] The existing methods are applied according the unsaponifiable family under study. For instance, hydrocarbons, present in a 0.5% in VOO and constituted mainly by squalene, have been determined by non-polar cGC with FI detection after separation from other unsaponifiable families by TLC. Tocopherols and tocotrienols have been converted into TMS derivatives and individually separated on a fast GC of medium polarity.[31] TLC for this unsponifiable fraction is not feasible because of coelution with compounds with similar polarity such as epoxy-squalenes.[32]

Fatty alcohols are more polar than tocopherols and are often poorly separated in silica TLC —no analytical problems, however, are observed for their determination by cGC with FID or MS detection.[32] Sterols, the most studied fraction of unsaponifiable matter in VOO, have been usually analyzed by GC with packed columns [libro nutraceutical], as well as with short and long capillary columns [grasas y aceites]. In addition, sterols are so efficiently separated on a polar cGC column (65% phenyl–35% dimethylpolysiloxane) that it is possible to detect a series of them that cannot be separated by other cGC columns.[33] Sterols are mainly present in olive oils in free and esterified forms, the combined determination of both providing a very useful information for checking olive oil authenticity.[34] An LC–GC method has been described by Grob *et al.* for the rapid determination of free sterols, esterified sterols and wax esters in oils, thus avoiding triglycerides saponification as they are removed by LC together with to other interferents. In a more recent research from the same authors

the esterification step was replaced by TMS derivatization, thus allowing the simultaneous determination of squalene, epoxy squalene and tocopherols. Biedermann *et al*. have proposed a method to substitute saponification by transesterification followed by online LC–GC .[33]. Further on, Señoráns *et al.*have used a non automatable reverse phase chromatographic method with a programmed temperature vaporizer (PTV) as interface for the determination of nonpolar unsaponifiable compounds such as sterols and aliphatic alcohols.[36] A Through Oven Transfer Adsorption Desorption (TOTAD) interface allowed Cortés *et al.* to couple online reversed phase liquid chromatography (RPLC) and GC, for fully automation of the method. However, the method thus developed does not permit quantitation of esterified sterols, but only of free sterols.[37] Recently, Toledano *et al.* have developed a new RPLC–GC method which allows analysis of free sterols, esterified and total sterols.[35] On the other hand, Zarrouk *et al.*, and Martínez-Vidal *et al.* have reported methods based on LC–MS based on atmospheric pressure chemical ionization mass spectrometry combined with tandem ion trap (APCI–IT) for quantitative multicomponent analysis of sterols, tocopherols and triterpenic dialcohols for identification and quantitation of a great number of compounds belong to these three families.[34.37]

In the case of phenols, the methods for individual separation and determination are similar to those for the sterols and aliphatic and triterpenic alcohols. The great diversity of phenolic compounds, the scant selectivity of the traditional methods and the present and growing importance of phenolic compounds require the replacement of overall quantitation methods by others providing individual information about each phenol. The development of methods based on LC, GC and, in a lesser

extent, capillary electrophoresis (CE) has enabled to elucidate the profile of phenolic compounds in VOO. The combination of any of the high-resolution separation techniques with MS has increased the selectivity of analytical methods for identification purposes as compared to conventional methods based on spectrophotometric or fluorimetric detection.

CONCLUSIONS

Methods for analysis of sterols, tocopherols, phenols, hydrocarbons, triterpenic and aliphatic alcohols require long and tedious sample preparation steps (*i.e.* extraction, isolation, saponification and purification); thus making sample preparation the most critical part of the overall analysis, since it is the mainly responsible for losses, the elimination of which —or that of the interferences appeared in the absence of the corresponding step in sample preparation— is difficult. A proved solution is the use of auxiliary energies as US to shorten the time required for these steps.

TLC and GC remains the most important tools for separation into families/individual separation for identification and/or quantification purposes of the unsaponifiable fraction. Among the overlay techniques, while quantification is carried out by mass spectrophotometry and MS--TOF. The latter is the technique of choice, thanks to its specificity, greater sensitivity and capacity to generate structural information.

Methods based on LC–UV and CG–MS remain useful, not only for identification of unsaponifiable compounds with good sensitivity and reproducibility. —this being of interest not only for determining in vegetable oils, biological samples could also be used.

ACKNOWLEDGEMENTS

The Spanish Ministerio de Ciencia e Innovación (MICINN) is thanked for financial support through project CTQ2009-07430.

REFERENCES

1. Cunha S, Fernandes J, Oliveira M. B. Quantification of free and esterified sterols in Portuguese olive oils by solid-phase extraction and gas chromatography–mass spectrometry. Journal of Chromatography A 1128:220-227 (2006).

2. Conde C, Delrot S and Gerós H, Physiological, biochemical and molecular changes occurring during olive development and ripening. Journal of Plant Physiology 165:1545-1562 (2008).

3. Ercisle S, Barut E and Ipek A, Molecular characterization of olive cultivars using amplified fragment length polymorphism markers. Genetics and Molecular Research GMR 8:414-419 (2009).

4. Yazaki K, Sugiyama A, Morita M and Nobukazu S, Secondary transport as an efficient membrane transport mechanism for plant secondary metabolites. Phytochemistry Reviews 7:513–524 (2008).

5. Ostlund R and Lin X, Regulation of cholesterol absorption by phytosterols. Current Atherosclerosis Reports 8:487-491 (2006).

6. Berger A, Jones P and Abumweis S, Plant sterols: factors affecting their efficacy and safety as functional food ingredients. Lipid in Health and Disease 3: 1-19 (2004).

7. Angerosa F, Camera L, Cumitini S, Gleixner G and Reniero F, Carbon Stable Isotopes and Olive Oil Adulteration with Pomace Oil. Journal of Agricultural and Food Chemistry 45:3044–3048 (1997).

8. REGLAMENTO nº 702/2007 de la Comisión, de 21 de junio de 2007, por el que se modifica el Reglamento nº 2568/91 relativo a las características de los aceites de oliva y de los aceites de orujo de oliva y sobre sus métodos de análisis. Diario Oficial de la Unión Europea L161 (22 de junio de 2007)".

9. Segura Carretero A, Carrasco-Pancorbo A., Cortacero S, Gori A, Cerretani L and Fernandez-Gutierrez A, A simplified method for HPLC-MS analysis of sterols in vegetable oil. European Journal of Lipid Science and Technology 110: 1142-1149 (2008).

10. Lagarda MJ, García-Llatas G and Farré R, Analysis of phytosterols in foods. Journal of Pharmaceutical and Biomedical Analysis 41:1486-1496 (2006).

11. Giacomelli L, Mattea M and Ceballos C, Analysis and characterization of edible oils by chemometric methods. Journal of the American Oil Chemists' Society 83:303-308 (2006).

12. Hargrove JL, Greenspan P and Hartle DK, Nutritional Significance and Metabolism of Very Long Chain Fatty Alcohols and Acids from Dietary Waxes. Experimental Biology and Medicine 229:215-226 (2004).

13. Cercaci L, Rodriguez-Estrada MT and Lercker G, Solid-phase extraction-thin-layer chromatography-gas chromatography method for the detection of hazelnut oil in olive oils by determination of esterified sterols. Journal of Chromatography A 985:211-220 (2003).

14. Volin P, Analysis of steroidal lipids by gas and liquid chromatography. Journal of Chromatography A 935:125-140 (2001).

15. Kim YK, Dohnalek Z, Kay BD and Rousseau R, Competitive Oxidation and Reduction of Aliphatic Alcohols over (WO3)3 Clusters. The Journal of Physical Chemistry 113:9721-9730 (2009).

16. Lerma-García MJ, Ramis-Ramos G, Herrero-Martínez JM, Gimeno-Adelantado JV and Simó-Alfonso EF, Characterization of the alcoholic fraction of vegetable oils by derivatization with diphenic anhydride followed by high-performance liquid chromatography with spectrophotometric and mass spectrometric detection. Journal of Chromatography A 1216:230-236 (2009).

17. Hyötyläinen T and Riekkola M-L, Solid-phase extraction or liquid chromatography coupled on-line with gas chromatography in the analysis of biological samples. Journal of Chromatography B 817:13-21 (2005).

18. Ruiz-Gutiérrez V and Pérez-Camino MC, Update on solid-phase extraction for the analysis of lipid classes and related compounds. Journal of Chromatography A 885:321-341 (2000).

19. Lin L-J, Tai SSK, Peng C-C and Tzen JTC, Steroleosin, a Sterol-Binding Dehydrogenase in Seed Oil Bodies, Plant Physiology 128:1200-1211 (2002).

20. Gutiérrez A, del Río JC, González-Vila FJ and Martín F, Analysis of lipophilic extractives from wood and pitch deposits by solid-phase extraction and gas chromatography. Journal of Chromatography A 823:449-455 (1998).

21. Abidi S, List G and Rennick K, Effect of genetic modification on the distribution of minor constituents in canola oil. Journal of the American Oil Chemists' Society 76:463-467 (1999).

22. Naengchomnong W, Pinho PM, Kijjoa A, Sawangwong P, Gonzalez MJ, Silva AMS, Eaton G and Herz W, Clerodanes and other constituents of Cleidion spiciflorum. Phytochemistry 67:1029-1033 (2006).

23. Dinh TTN, Blanton Jr JR, Brooks JC, Miller MF and Thompson LD, A simplified method for cholesterol determination in meat and meat products. Journal of Food Composition and Analysis 21:306-314 (2008).

24. Toivo J, Phillips K, Lampi A-M and Piironen V, Determination of sterols in foods: recovery of free, esterified, and glycosidic sterols. Journal of Food Composition and Analysis 14:631-643 (2001).

25. Phillips KM, Ruggio DM, Toivo JI, Swank MA and Simpkins AH, Free and esterified sterol composition of edible oils and fats. Journal of Food Composition and Analysis 15:123-142 (2002).

26. Lechner M, Reiter B and Lorbeer E, Determination of tocopherols and sterols in vegetable oils by solid-phase extraction and subsequent capillary gas chromatographic analysis. Journal of Chromatography A 857:231-238 (1999).

27. Pörschmann J, Plugge J and Toth R, In situ derivatisation using pressurized liquid extraction to determine phenols, sterols and carboxylic acids in environmental samples and microbial biomasses. Journal of Chromatography A 909:95-109 (2001).

28. Whitaker BD and Gapper NE, Ripening-specific stigmasterol increase in tomato fruit is associated with increased sterol C-22 desaturase (CYP710A11) gene expression. Journal of Agricultural and Food Chemistry 56:3828–3835 (2008).

29. Weissenberg M, Schaeffler I, Menagem E, Barzilai M and Levy A, Isocratic non-aqueous reversed-phase high-performance liquid chromatographic separation of capsanthin and capsorubin in red peppers (Capsicum annuum L.), paprika and oleoresin. Journal of Chromatography A 757:89-95 (1997).

30. Kundu MK and Deb AT, Column Chromatographic Determination of Unsaponifiable Matter in Fats and Oils. Fette, Seifen, Anstrichmittel 83:73-76 (1981).

31. López-Feria S, Cárdenas S and Valcárcel M, One step carbon nanotubes-based solid-phase extraction for the gas chromatographic–mass spectrometric multiclass pesticide control in virgin olive oils. Journal of Chromatography A In Press, Corrected Proof, doi:10.1016/j.chroma.2009.02.060.

32. Azadmard-Damirchi S and Dutta PC, Novel solid-phase extraction method to separate 4-desmethyl-, 4-monomethyl-, and 4,4'-dimethylsterols in vegetable oils. Journal of Chromatography A 1108:183-187 (2006).

33. Fu X, Liao Y and Liu H, Sample preparation for pharmaceutical analysis. Analytical and Bioanalytical Chemistry 381:75-77 (2005).

34. Clifton P, Plant sterol and stanols-comparison and contrasts. Sterols versus stanols in cholesterol-lowering: is there a difference? Atherosclerosis Supplements 3:5-9 (2002).

35. Päivi L, Analysis of sterols from various food matrices, European Journal of Lipid Science and Technology 107:402-410 (2005).

36. Riddle LA and Guiochon G, Separation of free sterols by high temperature liquid chromatography. Journal of Chromatography A 1137:173-179 (2006).

37. Lerma-García MJ, Simo-Alfonso EF, Ramis-Ramos G and Herrero-Martínez JM, Rapid determination of sterols in vegetable oils by CEC using methacrylate ester-based monolithic columns.Electrophoresis 29:4603-4611 (2008).

38. Yan L, Zhang Q, Zhang J, Zhang L, Li T, Feng Y, Zhang L, Zhang W and Zhang Y, Hybrid organic-inorganic monolithic stationary phase for

acidic compounds separation by capillary electrochromatography. Journal of Chromatography A 1046:255-261 (2004).

39. Lerma-García MJ, Simo-Alfonso EF, Ramis-Ramos G and Herrero-Martínez JM, Determination of tocopherols in vegetable oils by CEC using methacrylate ester-based monolithic columns. Electrophoresis 28:4128-4135 (2007).

40. Careri M, Elviri L and Mangia A, Liquid chromatography-UV determination and liquid chromatography-atmospheric pressure chemical ionization mass spectrometric characterization of sitosterol and stigmasterol in soybean oil. Journal of Chromatography A 935:249-257 (2001).

41. Rozenberg R, Ruibal-Mendieta NL, Petitjean G, Cani P, Delacroix DL, Delzenne NM, Meurens M, Quetin-Leclercq J and Habib-Jiwan JL, Phytosterol analysis and characterization in spelt (Triticum aestivum ssp. spelta L.) and wheat (T. aestivum L.) lipids by LC/APCI-MS. Journal of Cereal Science 38:189-197 (2003).

42. Headley JV, Peru KM, Verma B and Robarts RD, Mass spectrometric determination of ergosterol in a prairie natural wetland. Journal of Chromatography A 958:149-156 (2002).

43. Palmgrén JJ, Töyräs A, Mauriala T, Mönkkönen J and Auriola S, Quantitative determination of cholesterol, sitosterol, and sitostanol in cultured Caco-2 cells by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. Journal of Chromatography B 821:144-152 (2005).

CAPÍTULO 2

Extracción y derivatización asistidas por ultrasonidos de esteroles y alcoholes grasos de hojas y frutos del olivo previas a su determinación mediante cromatografía de gases–espectrometría de masas en tándem

Ultrasound-assisted extraction and derivatization of sterols and fatty alcohols from olive leaves and drupes prior to determination by gas chromatography–tandem mass spectrometry

*M. Orozco-Solano, J. Ruiz-Jiménez, M.D. Luque de Castro**

Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

Journal of Chromatography A, 1217 (2010) 1227–1235

Ultrasound-assisted extraction and derivatization of sterols and fatty alcohols from olive leaves and drupes prior to determination by gas chromatography–tandem mass spectrometry

M. Orozco-Solano, J. Ruiz-Jiménez, M.D. Luque de Castro

Abstract

A method for the simultaneous determination of sterols and fatty alcohols in olive leaves and drupes based on ultrasound-assisted extraction and derivatization prior to individual identification–quantitation by chromatographic separation and mass-spectrometry detection (single ion monitoring mode) is here reported. The sample preparation procedure involves the following steps: (i) leaching of the raw material accelerated by ultrasound; (ii) saponification of the leachate, also accelerated by ultrasound, and separation of the unsaponifiable; (iii) cleaning of the extract by solid-phase extraction; (iv) silylation of the target analytes —also assisted by ultrasound— ; (v) injection into the gas chromatograph for identification–simultaneous quantitation of the two families of compounds. Individual separation–determination of the fatty alcohols and sterols provide limits of detection (LOD) in the range $9.8x10^{-2}$ – 2 μg/l and 5.0 – 6.0 μg/l, respectively. The LOQs range from 0.3 to 0.9 μg/l and 17.0 to 21.0 μg/l, and the linear dynamic ranges are between LOQ–25.0 μg/ml. The between-day precision, expressed as relative standard deviation (RSD), ranges between 3.6–6.1% and the within laboratory reproducibility, also expressed as RSD, between 6.4 and 9.2%. Within the study of the metabolomic profile of the unsaponifiable fraction in olive tree, the method has been applied to the determination of the target analytes in different varieties of olive trees cultivated in the same zone, so that differences in this unsaponifiable fraction can be attributed to characteristics of the target varieties. As compared with its European Union counterpart, the method is endowed with similar analytical characteristics and drastic shortening of the operational time.

Keywords: *Sterols, fatty alcohols, ultrasound-assisted steps, GC–MS, olive fruit, olive leaves*

1. Introduction

Information on the unsaponifiable matter of olive oil and its components is essential for oil classification into categories [1]. These compounds have been widely studied because of their healthy properties, mainly phytosterols, with recognized biological effects. Clinical research has shown that phytosterols reduce biliary cholesterol absorption in the intestine [2], thereby increasing faecal excretion of cholesterol [3]. Decrease of plasma cholesterol levels is important for prevention of cardiovascular diseases, which are the main causes of death in Europe [4]. Fatty alcohols in olive oil were found to be minor components of the unsaponifiable matter as compared to phytosterols. The main alcohols present in olive oil are hexacosanol, octacosanol, tetracosanol and docosanol (always at higher concentration in the oil from second press than in extra virgin olive oil —first, cold press).

Sterols and alcohols profiles are used to characterize virgin olive oils and to detect adulteration of olive oil with hazelnut oil [5] or virgin olive oil with olive-pomace oil [6]. Chemometric studies have been used to characterize varietal olive oils based on their sterols and other fatty components (fatty acids, tocopherols, diacylglycerols and/or triacylglycerols) [7] to classify Portuguese olive oils according to the Protected Denomination of Origin [8] and discriminate between virgin olive oils from different olive-tree varieties [9].

Fatty aliphatic alcohols and phytosterols in vegetable oils have been usually analyzed by GC with flame ionization detection (FID) [10–19] and with MS detection [10,12,18,19]. HPLC coupled to mass detectors has been scarcely used to analyze the alcoholic fraction of oils [22] due to low ionization efficiency [23,24]. Atmospheric-pressure chemical-ionization mass-spectrometry has gained interest for sterol characterization in the last few years [25], despite its price.

Analytical applications of ultrasound, particularly sample preparation, have experienced a significant increase in the last decade [26]. In addition to assistance to leaching [27], a number of chemical reactions including both enzymatic [28] and non-enzymatic steps [29], and physical processes such as emulsification [30], have evidenced the usefulness of this energy to improve and/or shorten analytical methods.

In this work, an MS-GC method to characterize the unsaponifiable fraction of drupes and olive leaves from different varieties, with individual identification–quantitation of sterols and fatty alcohols has been developed. Prior to CG separation, a fast method for sample preparation, in which the different steps have been efficiently assisted by ultrasound, has also been developed. The potential influential variables on these steps were studied by a multivariate approach, and the contents of these alcohols and sterols in different samples were established.

2. Experimental

2.1. Samples

Samples used in this research —leaves and drupes from 3 varieties of olive trees (*i.e.* manzanilla, picual and gordal)— were collected in the region of Guadalquivir valley, Encinarejo (Córdoba). All samples (5 olive leaf samples and 5 drupe samples) were taken from the same geographical area to avoid variations caused by soil characteristics and environmental factors. The samples were homogeneized and kept at –20 °C until use. Leaves previously dehydrated were milled, sized (60 μm) and kept at –20 ºC until use.

2.2. Reagents

Alcohols —docosanol, tetracosanol, hexacosanol and octacosanol— and sterols — campesterol, stigamasterol and stigmastanol— obtained from Sigma–Aldrich (St. Louis, MO, USA), with purity ≥ 98%, were used as standards. Eiocosanol and cholesterol also from Sigma–Aldrich were used as internal standards (IS) in the determination step. Solutions at concentrations 1000 μg/ml were prepared in chloroform. All solutions were stored at −20 ºC in glass vials and kept in the dark at room temperature until use. Aminopropyl-phase cartridges (500 mg) from Waters (Millipore, Milford MA, USA) and silica cartridges from Supelco (Bellefonte, PA, USA) were used for solid-phase extraction. Bis-(trimethylsilyl)-fluoroacetamide (BSTFA) and trimethylchloro-silane (TMCS) from Sigma–Aldrich were used as silylation reagents for derivatization. Pyridine from Merck (Darmstadt, Germany) was used as derivatization solvent.

2.3. Apparatus and instruments

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium–alloy probe (12.70 mm diameter), which was immersed into a lab-made stainless steel container with eight compartments to place test tubes [31]. A Varian CP-3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable-temperature injector and coupled to a Saturn 2200 ion-trap mass-spectrometer (Sunnyvalley, TX, USA) was used for the determination of sterols and fatty-alcohols profiles in the target unnsaponifiable fraction. The chromatograph was furnished with a Varian CP 8400 autosampler and a Factor Four VF-5 ms fused silica capillary column (30 m × 0.25 mm I.D., 0.25-μm film thickness) provided by Varian.

2.4. Conventional extraction method

Two g of olive drupes or leaves and 10-ml of a 2:1 dichloromethane–hexane mixture were placed in a flask and agitated vigorously for 24 h; then, the extract was centrifuged for 10 min at 3000 rpm, the solid particles were separated from the liquid phase and the extract was suject to the saponification step. All the experiments were carried out in duplicate [32].

2.5. Proposed method

The proposed method can be divided into five steps: (1) leaching of fatty alcohols and sterols from the target sample; (2) saponification step; (3) preconcentration and cleanup of the solution from the saponification step by solid-phase extraction; (4) silylation of the target analytes to a more volatile products; (5) individual separation of the silylated sterols and fatty alcohols and determination by GC–MS.

2.5.1. Leaching step

Two g of olive drupes or leaves was placed in test tubes to which 10-ml portions of a 2:1 dichloromethane–hexane mixture was added. The tube was placed in the stainless-steel container, which was immersed into the water bath at 20 ºC, where ultrasound under the optimal working conditions —duty cycle 10% (*viz.* ultrasound application 0.1 s/s), output amplitude 10% of the converter, applied power 50 W, position of the ultrasonic probe-tip 1 cm from the bath bottom, and irradiation time 10 min— was applied. The extract (oleaginous fraction) was centrifuged for 10 min at 3000 rpm to separate solid particles both from the liquid phase and extract.

2.5.2. Saponification step

The clean extract from the previous step was mixed with 2-ml 2 M KOH, and 10-µl internal standards for sterols and fatty alcohols (0.1% chloroform solutions of cholesterol and 1-eicosanol, respectively) were added. The mixture was subjected to ultrasound (output amplitude 45% of the converter, applied power 200 W, duty cycle 50%) for 10 min, the unsaponifiable was extracted with 2-ml hexane, and this phase separated by centrifugation for 10 min at 3000 rpm. A gentle N_2 stream was used to dry the unsaponifiable fraction.

2.5.3. Solid-phase extraction

200 µg of the residue from the previous step was reconstituted into 0.5-ml 4:1 hexane–chloroform and the resulting solution circulated through an aminopropyl column. The sterols and fatty alcohols were bonded to the functional groups of the sorbent and the compounds not retained by the column were disposed off. The column was rinsed with 10-ml 1:1 hexane-ethyl ether to remove matrix remainings, dried with a nitrogen stream and the analytes eluted with 8-ml hexane and then with 6-ml 5:1 hexane–chloroform.

2.5.4. Derivatization procedure

Conversion of sterols and fatty alcohols into their more volatile derivatives is a necessary step prior to gas-chromatography individual separation. 200 µl of the clean extract were subjected to dryness by a nitrogen stream and the residue reconstituted with 100-µl N-pyridine and homogeneized in a vial for 1 min; then, 98-µl BSTFA was added and the mixture shaken vigorously in the vial for 1 min. Finally, 2-µl chloride trimethylsilyl was added, the mixture was shaken vigorously in the vial for 2 min more and then subjected to ultrasound (output amplitude 40% of the converter, applied power 180 W, duty cycle 50%) for 10 min to favor derivatization.

2.5.5. Individual sterols and fatty alcohols separation and determination by GC–MS

After derivatization, 1 µl of the analytical sample was injected into the chromatograph. The injector temperature was fixed at 250 ºC, and the injection was in the split–splitless mode. The splitter was opened (50:1) for 0.5 min, closed for 3.5 min and then opened at 100:1 split ratio for 10 min. The oven temperature program was as follows: initial temperature 50 ºC (held for 2 min), increased at 8 ºC/min to 250 ºC, followed by a second gradient at 3 ºC/min to 260 ºC (held for 20 min); and, finally, increased at 3

ºC/min to 300 ºC (held for 10 min). The total analysis time was 70 min, and 5 min extra time was necessary for re-establishing and equilibrating the initial conditions.

The ion-trap mass spectrometer was operated in the electron impact ionization (EI) positive mode, for which the instrumental parameters were set at the following values: filament emission current 80 μA; transfer line, ion trap and manifold temperatures were kept at 220, 200 and 50 ºC, respectively. The recording window was set between 40 *m*/*z* and 650 *m*/*z* and the data were acquired using total ion current (TIC) scan mode. Digital selected ion monitoring (SIM) was applied as data treatment in order to remove the chromatographic background.

2.6. Statistical analysis

The variables potentially influencing the leaching procedure were studied by a multivariate approach. Statgraphics Centurion XV, Statpoint technologies, Inc. (Warrenton, VA, USA) was used as statistical software with this purpose.

3. Results and discussion

Both raw materials under study, olive-tree leaves and drupes, were used in the optimization study, the sequence of which consisted of four steps. First, individual separation of the silylated analytes by gas chromatography and mass-detection variables were optimized for proper monitoring of sample preparation. In this experiments silylation was performed without ultrasound assistance, as described in the literature [33]. Then, the optimal working conditions for ultrasound-assisted extraction of the target compounds were established; those to accelerate saponification by ultrasound as well as those for solid-phase extraction were also optimized. The two last steps were optimized both with standards and extracts in order to detect potential interferents in the extracts. Finally, silylation was optimized.

3.1. Optimization of the determination step

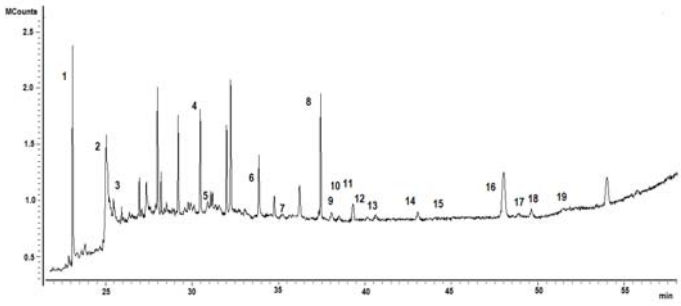
The experimental chromatographic variables were optimized resulting the operating conditions described in the experimental section. Splitless and split injections were tested to check the influence of the split ratio on the GC–MS analysis. An 1:50 split ratio was found optimum. Optimization was carried out both with standard solutions and extracts derivatized by the conventional procedure [33]. Cholesterol and 1–

eicosanol were used as internal standards for sterols and alcohols, respectively, because of their physical and chemical behaviors similar to those of the target analytes and after checking their absence in the samples.

Concerning detection, the emission current of the filament was optimized with two purposes: (1) selection of the most characteristic ions to identify each analyte with the highest selectivity, and (2) selection of the ion with the highest sensitivity for quantitation of each alcohol and sterol in SIM mode. The optimum filament current was 80 μA for all analytes.

Identification of target analytes with commercially available standards (*viz.* docosanol, tetracosanol, hexacosanol, octacosanol, campesterol, stigmasterol and stigmastanol) was based on comparison of the retention times and mass spectra. Those compounds with no commercial standards (*viz.* tricosanol, pentacosanol and some sterols as cholestanol, brassicasterol, 24-methylene cholesterol, campestanol, cholesterol, Δ^5-avenasterol and Δ^7-avenasterol) were identified by comparing their retention times and mass spectra with those in the literature. These values and the principal ions for quantitation are listed in Table 1. Complete separation of the target compounds was achieved within 70 min, as shows Fig. 1.

A)



B)

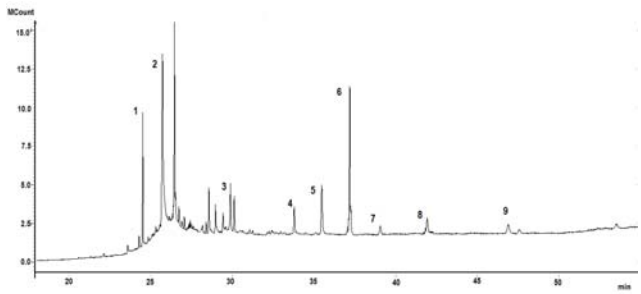


Fig. 1. (A) *Chromatogram from an alcohol+sterol extract of olive drupes. Peak identification: 1, eicosanol (internal standard); 2, docosanol; 3, tricosanol; 4, tetracosanol; 5, pentacosanol, 6, hexacosanol;7, heptacosanol, 8, cholesterol (internal standard); 9, octacosanol; 10, brassicasterol; 11, 24-methylen cholesterol; 12, campesterol; 13, campestanol; 14, stigmasterol; 15, clerosterol; 16, β-sitosterol; 17,*

sitostanol; 18, Δ-5-avenasterol and Δ-7-avenasterol. (B) *Chromatogram from a 25 μg/ml standard solution of sterol and alcohol compounds. Peak identification: 1, eicosanol (internal standard); 2, docosanol; 3, tetracosanol; 4, hexacosanol; 5, cholesterol (internal standard) ; 6, octacosanol; 7, campesterol; 8, stigmasterol; 9, sitostanol.*

Table 1. Ions monitored in SIM mode for identification–quantitation of fatty alcohols and sterols

Sterol	**Retention time (min)**	***m/z*[a]**
Eicosanol (IS)	24.5	80, **435**
Docosanol	24.7	41, 75, 207, 281, **355** (89), 384
Tricosanol	25.9	353, 280, 103(45), 73
Tetracosanol	30.2	80, 226, **412** (91), 491
Pentacosanol	33.0	80(90), 95, 226 , 374, 464
Hexacosanol	33.3	80, **440** (75), 519
Heptacosanol	32.6	69, 8 (92), 147
Octacosanol	38.2	52, 80, 147, 226, **468** (90), 547
Cholesterol (IS)	37.9	44, 75, 147, 207, 330, 368(83)
Brassicasterol	38.3	73, 121, 265, **380** (42)
24-Methylene cholesterol	38.5	57, **147**(75), 192, 355
Campesterol	38.6	281, 344, **382** (83), 473, 503
Campestanol	38.6	185, 218, 343, **503**(89)
Stigmasterol	42.2	83, 129, 256, **395** (89), 485
Clerosterol	44.8	73, 170, **221**(42), 341
ß-Sitosterol	45.1	73, 229, 255, 382, **396** (93)
Sitostanol	45.3	79, 147, 216, **384**(91), 474
Δ-5-Avenasterol	48.2	69, **80**(95), 161, 393, 408
Δ-7-Avenasterol	53.9	**89**(92), 203, 380, 379

[a] In brackets, third column the percentages of the most abundant fragments are

3.2. Optimization of ultrasound-assisted leaching

This study was focused on the eight more influential variables on the leaching step in order to obtain the best values of them to remove the target analytes with the highest

efficiency and without degradation. The eight variables with potential inter-relation in the leaching of the unsaponifiable fraction from the two raw materials —and therefore studied by a multivariate design —a Plackett-Burman design 2^8 x 3/64 type III resolution allowing six degrees of freedom and involving 12 randomized runs plus three centre points— were ultrasound radiation amplitude, percent of ultrasound exposure (duty cycle), probe position (*viz.* distance between the bottom of the water-bath and the tip probe), irradiation time, water-bath temperature, extractant volume, extractant composition and number of leaching cycles. The upper and lower values given to each variable were selected from the experience gathered in preliminary experiments and are shown in Table 2A. The response variable was the extraction efficiency expressed as the ratio between the peak areas of each compound and that of the IS.

Table 2A. Optimization of the ultrasound-assisted leaching of the target compounds from olive drupes and leaves

Variable	Tested range (1st screening)	Tested range (2nd screening)	Optimum value
Radiation amplitude (%)	10–50		10
Duty cycle (%)	10–50		10
Irradiation time (min)	10–20	5–10	10
Extractant composition (dichloromethane–hexane)	33.3–66	66–100	66
Extractant volume	5–10		10
Cycles number	1–3	3–5	5*
Water-bath temperature (ºC)	15–25		20
Probe position (cm)	1–3		1

*From univariate optimization

The conclusions of this screening study were that the irradiation amplitude, duty cycle, extractact volume, probe position and water-bath temperature were not statistically influential factors within the ranges under study. However, the results showed higher extraction efficiencies with the lowest values tested for the irradiation amplitude (10%), duty cycle (10%), probe position (1 cm from the bath bottom and water bath temperature (20 ºC); and the highest value tested for the extractant volume (10 ml). The extractant composition, the number of extraction cycles and irradiation

time were influential factors within the range under study. The first and second factors had positive influence on the process and the influence of the third was negative.

A second experimental design involving higher values for the extractant composition and the number of extraction cycles, and lower values for the irradiation time was carried out using the optimum conditions for the rest of the variables. The tested and optimum values obtained for each variable are shown in Table 2A. The conclusions for this screening study were that the extractant composition was not a statistical influential factor in the range under study. However, the best results were obtained with a 66:33 dichloromethane–hexane mixture. The number of extraction cycles and irradiation time were influential factors, positive influence, within the range under study. Taking into account that the irradiation time had also a significant but negative influence on the first design, 10 min was selected for further experiments. Finally, the number of cycles was studied to know the number of extraction cycles necessary for exhaustive extraction of the target compounds. Figure 2 shows that 5 cycles were necessary for extraction of the two target families in the case of olive drupes; and 3 and 5 cycles for alcohols and sterols, respectively, in the case of leaves.

3.3. Saponification step

Saponification developed in homogeneous media which proceed via radical or radical–ion intermediates, so it is sensitive to ultrasound energy [26]. For this reason, ultrasound energy was also used to accelerate saponification of the target analytes. In this case, the ultrasonic variables (duty cycle, irradiation amplitude and irradiation time) were optimized giving to the chemical variables (concentration and volume of the KOH solution) the optimum values proposed by Cunha *et al.* [*34*]. The temperature was fixed at 30 ºC, as temperatures close to (or higher than) 40 ºC are non recommended. This step was optimized with extracts from the two raw materials.

The ultrasound variables were optimized by a response surface using a central composite design 2^3 + star, involving 16 plus 3 centre points. The tested ranges and the optimum values obtained from the design are shown in Table 2B. Comparison of the results obtained with the proposed method —assisted by ultrasound— and those provided by the conventional method showed that the reaction yield was nine times higher for the proposed method for a reaction time shortened from 50 to 10 min.

Table 2B. Optimization of the ultrasound-assisted saponification step

Variable	Tested range	Optimum value
Duty cycle (% s/s)	10–50	50
Irradiation time (min)	10–50	10
Irradiation amplitude (%)	10–50	45

3.4. Solid-phase extraction

The isolation and cleanup of fatty alcohols and sterols using normal-phase sorbents —silica or aminopropyl— and appropriate eluting solutions —hexane or hexane–chloroform mixtures— have been reported [35].

Five variables —sorbent type, and volumes of: (a) analytical sample as obtained after applying the previous step; (b) solvent circulated through the column after sample application to eliminate non retained species; (c) fatty alcohols eluent; (d) sterols eluent— were tested by a univariate design to obtain the optimum conditions for cleanup of the target analytes. The results obtained are shown in Table 2C.

Table 2C. Optimization of the solid-phase extraction step

Variable	Tested range	Optimum value
SPE sorbent	Aminopropyl, silica	Aminopropyl
Weight of sample (μg)	100–300	200
Volume of cleaning solvent (ml 1:1 hexane–ethyl ether)	5–15	10
Volume of eluant for sterols (ml 5:1 hexane–chloroform)	3–12	6
Volume of eluant (ml hexane)	5–20	8

3.5. Derivatization step

Ultrasound energy was also used to accelerate silylation of fatty alcohols and sterols. Ultrasound variables, irradiation amplitude and duty cycle, were studied in a multivariate mode using 5 min as irradiation time using a 5-μg/ml standard solution and leachates from olive leaves and drupes. The best conditions were 40% irradiation amplitude (180 W, applied power) and 50% of duty cycle (that is, 0.5 s/s), which were

used to check the reaction time from 1 to 15 min. Only 10 min was required for maximum silylation of the target analytes; drastic decrease as compared with 120 min proposed by Janicsak *et al.* [33].

3.6. Characterization of the individual separation–detection method

Calibration plots were run for the seven analytes with commercial standards (peak area *versus* standard concentration —see Table 3). Compounds with no commercial standards were quantified by the calibration curve of the most similar alcohol or sterol and their typical characterization parameters also appear in Table 3. Eicosanol and cholesterol were used as IS for quantitation of fatty alcohols and sterols, respectively.

Calibration equations were established by using the ratio between the peak area of each compound and that of its family as a function of concentration of each compound. The regression coefficients ranged between 0.9991 and 0.9998 for the linear dynamic range tested for each analyte, which was LOQ–25 μg/ml. The characterization of the method was completed with the limits of detection (LODs) and quantification (LOQs), which were calculated from the GC–MS chromatograms obtained with natural samples. The peak height to averaged background noise ratio was calculated, for which the background noise was estimated by the peak to peak baseline near the analyte peak. LODs and LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise (S/N) ratio of 3 and 10, respectively.

The LODs for each analyte ranged between 9.8 10^{-2} and 2.0 μg/l for fatty alcohols and between 5.0 and 6.0 μg/l for sterols. The LOQs ranged from 0.3 to 0.9 μg/l and from 17.0 to 2.0 μg/l for fatty alcohols and sterols, respectively. LODs and LOQs were estimated for standard solutions and are shown in Table 3.

3.7. Assessment of precision

Within-laboratory repeatability was evaluated in a single experimental setup with duplicates with natural samples (leaves and drupes) subjected to the proposed method. Two analysis of these samples per day were carried out on 7 days.

Equation (1) was used to determine the variance due to inter-day repeatability:

$$s_{between}^2 = (MS_{between} - MS_{within})/ n_j \qquad (1)$$

Table 3. Calibration curves, limits of detection (LODs), limits of quantitation (LOQs) of sterols and fatty alcohols found (intercept and slope errors in brackets)

Compound	Calibration curve	Linear range	LOD (mg/kg)	LOQ (mg/kg)
Docosanol	Y=11.8(0.1)X + 0.9(0.1)	LOQ–50	1.3	4.5
Tricosanol	As docosanol			
Tetracosanol	Y=44.2(1.3)X + 6.4(0.9) Y=5.2(0.1)X + 65.1(2.3)	LOQ–0.5 0.5–25	0.1	0.3
Pentacosanol	As tetracosanol			
Hexacosanol	Y=181.7(4.2)X + 1.3(0.1) Y=4.5(0.1)X + 38.4(0.6)	LOQ–0.25 0.25–25	2.8	9.5
Heptacosanol	As hexacosanol			
Octacosanol	Y=338.9(2.8)X + 1.5(0.1) Y=3.5(0.1)X + 47.8(1.2)	LOQ–0.25 0.25–25	0.2	0.7
Brassiscasterol	As campesterol			
24-methylene cholesterol	As campesterol			
Campesterol	Y=7.9(0.1)X + 1.3(0.1)	LOQ–25	5.3	17.9
Campestanol	As campesterol			
Stigmasterol	Y=10.0(0.2)X + 2.9(0.2)	LOQ–25	6.3	21.0
Clerosterol	As stigmasterol			
β-Sitosterol	As stigmasterol			
Sitostanol	Y=68.0(3.2)X + 0.1(0.1) Y=9.1(0.2)X + 41.9(3.2)	LOQ–0.5 0.5–25	6.1	20.5
Δ^{-5}-Avenasterol	As sitostanol			
Δ^{-7}-Avenasterol	As sitostanol			

where MS is the mean square (residual sum of squares rated by the freedom degrees) and *nj* is the number of replicates per day. The within-laboratory reproducibility, s^2_{WR}, was calculed by Eq (2).

$$s^2_{WR} = s_r^2 + s_{between}^2 \qquad (2)$$

where s_r^2 is the residual mean squares within-days, and $s_{between}^2$ is the variance due to the inter-day effect.

The results obtained are listed in Table 4. Between-day precision, expressed as relative standard deviation (RSD), ranges between 3.6 and 6.1% and the within laboratory reproducibility, also expressed as RSD, between 6.4 and 9.2%.

Table 4. Repeatability (s_r) and within-laboratory reproducibility (s_{wr}), expressed as relative standard deviation, of the proposed method

	Leaves		**Drupes**	
Compound	**(s_r)**	**(s_{wr})**	**(s_r)**	**(s_{wr})**
Docosanol	4.0	6.8	4.4	7.5
Tricosanol	3.6	6.4	4.3	7.2
Tetracosanol	3.7	6.6	4.2	7.8
Pentacosanol	3.8	6.9	4.5	8.1
Hexacosanol	4.0	7.0	5.1	8.5
Heptacosanol	4.2	7.2	5.2	8.3
Octacosanol	5.1	8.0	6.1	9.2
Brassiscasterol	3.8	6.6	4.0	6.9
24-methylene	3.7	6.9	4.2	7.3
Campesterol	3.9	7.1	4.7	7.2
Campestanol	4.0	6.6	4.9	7.4
Stigmasterol	4.2	7.2	5.1	7.9
Clerosterol	4.1	7.7	5.0	8.2
β-sitosterol	4.4	7.8	4.0	8.0
Sitostanol	3.9	6.4	4.8	8.1
Δ^{-5}-Avenasterol	3.8	6.5	4.7	7.3
Δ^{-7}-Avenasterol	4.2	7.8	5.1	8.4

3.8. Characterization of alcohol and sterol fractions

Ten samples (drupes and leaves) obtained from different varieties were used to assess the proposed method. The chromatogram from an alcohol+sterol extract of olive drupes is shown in Fig. 2A, and the results for each analyte and sample are summarized in Table 5.

3.8.1. Fatty alcohols fraction

In general terms, the concentration of the alcohols in the drupes ranged between 154.3 and 1434.9 µg/g, which is higher than in leaves, where the content of these compounds ranged between 21.2 and 298.5 µg/g. The concentration of each compound in a target raw material depends on the variety.

The study of the composition of the alcoholic fraction shows that all the studied alcohols are present in the samples. The concentration of each analyte depends on the sample, but in general, the alcohols present at higher concentrations are hexacosanol

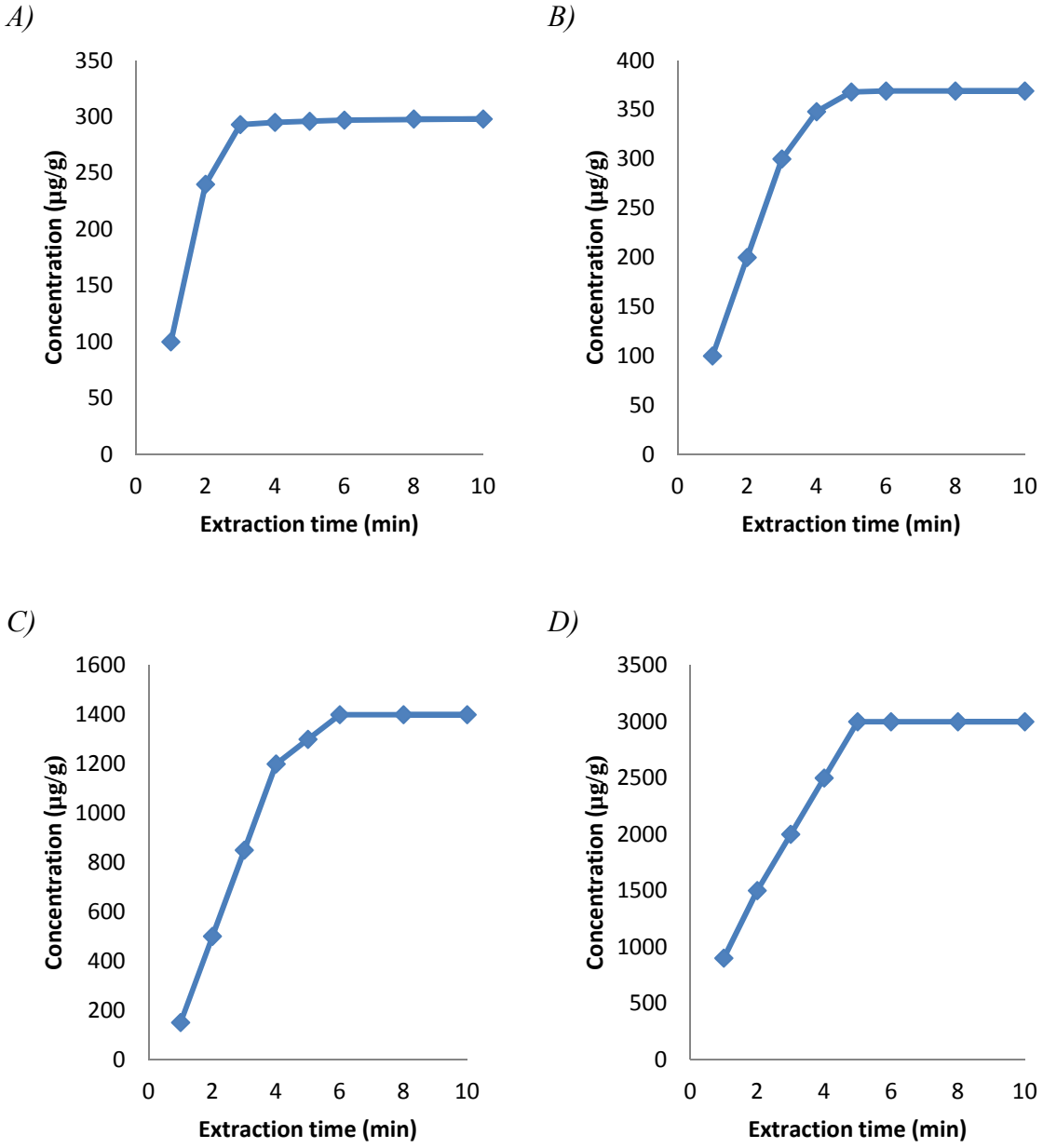


Fig. 2. *Leaching kinetics of olive materials. A) fatty alcohols from leaves; B) sterols from leaves; C) fatty alcohols from drupes; and D) sterols from drupes.*

followed by tetracosanol; the alcohols at lower concentrations are tricosanol and docosanol. The concentration of octacosanol, one of the most important bioactive alcohols according to the literature, ranges between 7.3 and 144.1 μg/g.

Concerning leaves, the study of this fraction shows that alcohols with an impair number of carbon atoms are not in the sample and hexacosanol is the alcohol at the highest concentration; meanwhile the concentration of octacosanol ranges between 2.8 and 73.9 μg/g.

3.8.2. Sterols fraction

The concentration of sterols in drupes and leaves is higher than that of alcohols. Furthermore, their concentration in drupes (between 1187.1 and 3064.3 μg/g) is higher than in leaves (between 39.2 and 369.1 μg/g).

The results obtained are in agreement with those found in the literature on the composition of sterols fraction: the predominant sterol is β-sitosterol, and minor

Table 5. Analysis of sterols and fatty alcohols in olive leaves and drupes using the proposed method (all the results are expressed as μg/g — errors, in brackets, expressed as %, n= 3 replicates)

	DRUPES*																			
	1				2				3				4				5			
Compound	**CONV**		**US**		**CONV**		**US**		**CONV**		**US**		**CONV**		**US**		**CONV**		**US**	
Docosanol	3.0	(1.9)	7.8	(1.8)	11.5	(1.9)	83.7	(2.0)	4.1	(2.1)	7.3	(2.3)	14.2	(2.1)	22.5	(2.0)	6.7	(2.0)	2.4	(2.0)
Tricosanol	<LOD		22.1	(2.1)	<LOD		21.7	(2.0)	0.3	(1.8)	5.5	(1.9)	1.3	(1.9)	7.0	(1.6)	0.1	(2.1)	14.4	(2.2)
Tetracosanol	22.3	(1.6)	218.9	(1.8)	34.5	(1.8)	339.9	(2.3)	20.4	(2.9)	46.3	(1.8)	25.5	(1.7)	40.5	(1.6)	50.8	(1.7)	48.2	(1.7)
Pentacosanol	5.6	(2.0)	372.8	(1.9)	4.8	(2.9)	370.2	(2.1)	4.9	(2.8)	10.3	(2.4)	3.2	(2.7)	9.1	(2.5)	<LOD		38.7	(1.9)
Hexacosanol	52.0	(1.9)	403.9	(1.4)	35.6	(2.6)	386.7	(2.7)	55.0	(1.7)	99.6	(1.6)	19.1	(1.9)	56.8	(1.8)	95.2	(1.5)	36.4	(2.3)
Heptacosanol	0.9	(2.3)	104.2	(2.5)	0.5	(2.3)	102.1	(2.6)	0.1	(2.9)	1.0	(2.9)	0.1	(2.8)	0.5	(2.9)	0.0	(3.2)	2.5	(2.5)
Octacosanol	20.3	(3.3)	144.1	(3.2)	11.8	(3.5)	130.6	(3.3)	17.6	(3.4)	33.8	(3.6)	1.8	(3.2)	17.9	(3.1)	18.5	(3.3)	7.3	(3.4)
Brassiscasterol	0.2	(1.8)	4.0	(2.3)	0.1	(2.5)	3.7	(2.2)	0.3	(2.8)	2.1	(2.2)	0.2	(2.4)	1.8	(2.8)	0.2	(2.8)	0.2	(2.9)
24-methylene	<LOD		0.2	(2.8)	<LOD		<LOD		<LOD		0.9	(2.7)	<LOD		0.9	(2.5)	<LOD		<LOD	
Campesterol	3.9	(1.6)	6.5	(2.1)	21.0	(2.5)	258.2	(1.6)	7.5	(1.9)	16.1	(1.8)	28.6	(1.7)	38.4	(1.5)	5.0	(1.9)	2.9	(2.6)
Campestanol	0.1	(1.9)	0.8	(2.0)	0.6	(2.3)	7.8	(1.9)	0.2	(2.7)	0.6	(3.0)	0.8	(2.1)	1.4	(1.9)	0.1	(2.3)	8.1	(1.8)
Stigmasterol	8.2	(2.0)	19.8	(2.3)	8.2	(2.4)	79.3	(2.7)	14.6	(2.9)	24.8	(2.3)	7.1	(2.2)	19.2	(1.8)	1.3	(2.3)	1.1	(2.8)
Clerosterol	0.1	(2.2)	1.9	(2.4)	0.1	(2.9)	6.7	(2.6)	0.8	(2.6)	2.5	(2.1)	0.4	(2.1)	2.2	(2.1)	2.0	(1.9)	3.1	(2.6)
β-sitosterol	229.7	(1.5)	2399.7	(1.8)	137.1	(1.8)	2594.6	(1.8)	475.1	(2.4)	1407.0	(1.5)	141.5	(1.7)	1205.4	(1.5)	272.3	(1.8)	1138.8	(1.5)
Sitostanol	0.7	(2.3)	71.7	(2.7)	0.6	(2.6)	71.7	(2.9)	0.4	(2.7)	2.1	(2.4)	0.4	(2.9)	2.1	(2.3)	1.3	(2.5)	18.2	(1.8)
Δ^{-5}-avenasterol	2.0	(2.2)	8.5	(2.5)	2.5	(2.8)	19.4	(2.6)	9.0	(2.0)	15.5	(2.5)	10.3	(2.4)	29.9	(1.9)	1.0	(2.4)	6.3	(1.9)
Δ^{-7}-avenasterol	2.3	(2.3)	10.4	(2.1)	4.2	(2.9)	21.9	(2.8)	2.2	(3.0)	3.8	(2.3)	2.8	(2.2)	6.8	(2.0)	1.2	(2.1)	8.4	(1.8)
Alcohols	104.0		1273.9		98.7		1434.9		102.4		203.9		65.2		154.3		171.2		149.8	
Sterols	247.1		2523.5		174.5		3063.3		510.0		1475.4		192.1		1308.1		284.1		1187.1	

	LEAVES																			
	1				2				3				4				5			
Compound	**CONV**		**US**		**CONV**		**US**		**CONV**		**US**		**CONV**		**US**		**CONV**		**US**	
Docosanol	1.4	(1.7)	2.4	(1.9)	1.7	(1.5)	3.0	(1.8)	0.2	(1.9)	0.7	(2.1)	0.3	(2.2)	2.8	(2.3)	0.7	(2.1)	15.3	(2.0)
Tricosanol	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		0.1	(3.2)	1.0	(2.3)
Tetracosanol	2.8	(1.5)	7.9	(1.8)	1.4	(1.9)	2.8	(1.6)	0.2	(2.0)	3.2	(1.9)	0.2	(1.8)	5.3	(1.6)	2.9	(1.8)	26.6	(1.5)
Pentacosanol	<LOD		<LOD		<LOD		<LOD		0.1	(2.2)	1.2	(1.9)	0.1	(2.8)	1.6	(1.9)	0.7	(1.9)	12.6	(1.6)
Hexacosanol	13.9	(1.6)	27.3	(1.4)	10.5	(1.8)	16.0	(1.7)	2.4	(1.8)	13.7	(1.6)	2.1	(1.9)	8.6	(1.7)	9.1	(1.6)	169.0	(1.6)
Heptacosanol	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		0.1	(1.7)
Octacosanol	3.4	(3.3)	6.4	(3.1)	2.7	(3.0)	4.2	(3.4)	1.1	(3.0)	4.8	(3.1)	1.0	(3.1)	2.8	(3.6)	3.5	(3.4)	73.9	(3.3)
Brassiscasterol	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LDO		<LOD		0.1	(2.4)	0.5	(2.0)
24-Methylene	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD	
Campesterol	1.4	(3.0)	2.5	(1.7)	1.7	(1.7)	2.9	(1.7)	0.3	(2.0)	1.0		0.4	(1.7)	3.2	(1.5)	1.4	(1.9)	32.7	(2.0)
Campestanol	<LOD		<LOD		<LOD		0.1	(2.6)	<LOD		<LOD		<LOD		0.1	(1.8)	<LOD		0.8	(2.4)
Stigmasterol	0.4	(1.8)	0.7	(1.7)	0.4	(2.4)	0.6	(2.2)	0.7	(1.8)	2.2	(1.8)	0.7	(2.1)	1.1	(1.5)	2.7	(1.8)	66.5	(1.9)
Clerosterol	0.6	(2.8)	1.1	(2.8)	0.6	(2.8)	0.9	(3.0)	<LOD		<LOD		<LOD		<LOD		0.1	(3.0)	3.8	(1.9)
β-Sitosterol	75.7	(1.7)	129.9	(1.6)	67.1	(1.6)	101.0	(1.5)	17.8	(1.5)	77.3	(1.6)	16.7	(1.6)	32.6	(1.4)	85.7	(1.6)	204.6	(1.5)
Sitostanol	0.3	(2.7)	0.5	(2.4)	0.2	(3.0)	0.4	(2.9)	<LOD		0.2	(2.8)	<LOD		0.2	(2.0)	<LOD		<LOD	
Δ^{-5}-Avenasterol	0.4	(2.9)	0.2	(1.9)	0.5	(2.0)	0.2	(1.8)	0.2	(1.8)	0.6	(2.2)	0.2	(2.1)	0.9	(1.7)	1.8	(2.6)	49.0	(1.8)
Δ^{-7}-Avenasterol	0.5	(2.9)	0.9	(2.0)	0.6	(2.0)	0.9	(1.7)	0.3	(1.9)	0.7	(2.1)	0.3	(2.0)	1.1	(1.7)	0.4	(1.9)	11.4	(1.7)
Alcohols	21.4		44.0		16.3		26.0		4.0		23.6		3.6		21.2		16.9		298.5	
Sterols	79.3		135.9		71.1		107.0		19.3		82.0		18.3		39.2		92.0		369.1	

components are campesterol, stigmasterol, Δ5-avenasterol, Δ7-avenasterol and brassicasterol.

All sterols under study were quantifiable in drupe samples. In the case of leaves, 24-methylene cholesterols was under the LOD in all cases and brassicaterol was detected only in one sample (sample number 5) and at low concentration.

3.9. Comparison of the efficiency of the proposed method versus that of the conventional method

The efficiency of the conventional method based on maceration extraction applied for 24 h was calculated taking as 100% the efficiency of the proposed method. In this way, 22% and 36% were the efficiencies provided by the former method for the fatty alcohol and sterol fractions, respectively. In addition to the high efficiency of the proposed ultrasound-assisted extraction, no degradation of target compounds was detected, as demonstrated by the plateaux in Fig. 2 when the number of extraction cycles increased and thus, the subjection of the extracted compounds to longer times of ultrasonic irradiation.

4. Conclusions

The method here reported is a clear demonstration of the enormous capability of ultrasound to favor and/or accelerate a number of steps of the analytical process [34]. Solid–liquid extraction and free-radical involved reactions, such as saponification or silylation, are drastically shortened and also their yields improved by ultrasonic assistance.

In short, the contributions of the method here proposed are as follows:

(1) It is the first time that a method allows the simultaneous determination of sterols and fatty alcohols from olive drupes and leaves in a single chromatogram, thus saving time and resources.
(2) The leaching step of these compounds accelerated by ultrasonic irradiation is also reported for the first time (the time required for this step was shortened from 24 h to 10 min).
(3) The use of aminopropyl sorbent for solid-phase extraction provides a clean extract.
(4) Saponification of the target analytes, also assisted by ultrasound, shortens the time for this step from 2 h to 10 min with no degradation of analytes.
(5) The extraction efficiency of the proposed method is 5 and 3 times higher than that provided by the conventional maceration method in the case of alcohols and sterol fractions, respectively.

The results obtained for the composition of the alcohol and sterol fractions are in agreement with those provided by the literature.

5. Acknowledgements

The Spanish Comisión Interministerial de Ciencia y Tecnología (CICyT) is thanked for financial support (Project No. CTQ 2009-07430). M. O.-S. expresses her gratitude to Carolina Foundation for a scholarship.

6. References

[1] Directive (CEE) No. 1989/2003 de la Comisión por la que se modifica la Directiva (CEE) No. 2568/91 relativa a las características de los

aceites de oliva y de los aceites de oliva y sobre sus métodos de análisis.

[2] A. Conchillo, L. Cercaci, D. Ansorena, M. T. Rodríguez-Estrada, G. Lercker, I. Astiasanan. J. Agric. Food Chem. 53(2005) 7844–7850

[3] K. B. Hicks, & R. A. Moreau. Food Technol. 50 (2001) 63–67.

[4] R. Santos, E. Limas, M. Sousa, M. da Conceição Castilho, F. Ramos, & M. I. Noronha da Silveira. Food Chem. 102 (2007) 113–117.

[5] S. Vichi, L. Pizzale, E. Toffano, R. Bortolomeazzi, L. Conte. J. of AOAC International 84(5) (2001) 1534–1541.

[6] R. J. Reina, K. D. White, E. G. E. Jahngen. J. AOAC Internat. 80 (1997) 1272–1280.

[7] L. C. Matos, S. C. Cunha, J. S. Amaral, J. A. Pereira, P. B. Andrade, R. M. Reabra. Food Chem. 102 (2007) 406–414.

[8] M. Rui Alves, S. C. Cunha, J. S. Amaral, J. A. Pereira, M. B. Oliveira. (2005). Anal. Chim. Acta 549 (2005) 166–178.

[9] J. Sánchez Casas, E. Osorio Bueno, A. M. Montaño García, M. Martínez Cano. Food Chem. 87 (2004) 225–230.

[10] A. M. F. Abou Hadeed, A. R. Kotb, C. E J. Daniels, Food Chem. 35 (1990) 167–174.

[11] D. Chryssafidis, P. Maggos, V. Kiosseoglou, D. Boskou, J. Sci. Food Agric. 58 (1992) 581–583.

[12] A. Lanzon, T. Albi, A. Guinda, J. Am. Oil Chem. Soc. 76 (1999) 1421–1423.

[13] E. Stefanoudaki, F. Kotsifaki, A. Koutsaftakis, J. Sci. Food Agric. 80 (2000) 381–389.

[14] A. Ranalli, L. Pollastri, S. Contento, G. Di Loreto, E. Lannucci, L. Lucera, F. Russi, J. Sci. Food Agric. 82 (2002) 854–859.

[15] P.L. Benítez-Sánchez, M. León-Camacho, R. Aparicio, Eur. Food Res. Technol. 218 (2003) 13–19.

[16] R. M. Rivera del Álamo, G. Fregapane, F. Aranda, S. Gómez-Alonso, M. D. Salvador, Food Chem. 84 (2004) 533–537.

[17] J. Giacometti, Analyst 126 (2001) 472–475.

[18] S.S. Cunha, J.O. Fernandes, M. Beatriz, P.P. Oliveira, J. [19] S. Azadmard-Damirchi, G.P. Savage, P.C. Dutta, J. Am. Oil Chem. Soc. 82 (2005) 717–725.

[20] S. Vichi, L. Pizzale, E. Toffano, R. Bortolomeazzi, L. Conte, J. Assoc. Offic. Anal. Chem. Int. 84 (2001) 1534.

[21] A. Lazzez, E. Perri, M.A. Caravita, M. Khlif, M. Cossentini, J. Agric. Food Chem. 56 (2008) 982–988.

[22] N. Cortesi, M.G. Fusetti, E. Fedeli, Riv. Ital. Sostanze Grasse 64 (1987) 513–519.

[23] A. Micó-Tormos, C. Collado-Soriano, J.R. Torres-Lapasió, E. Simó-Alfonso, G. Ramis-Ramos, J. Chromatogr. A 1180 (2008) 32–41.

[24] A. Micó-Tormos, E. Simó-Alfonso, G. Ramis-Ramos, J. Chromatogr. A 1203 (2008) 47–53.

[25] J.L. Martínez-Vidal, A. Garrido-Frenich, M.A. Escobar-García, R. Romero- González, Chromatographia 65 (2007) 695–699.

[26] M. D. Luque de Castro, F. Priego-Capote, Analytical Applications of Ultrasound, Elsevier, Amsterdam, 2006.

[27] M.D. Luque de Castro, F. Priego-Capote. Trends Anal. Chem. 26, No. 2 (2007), 154–162.

[28] B. Álvarez-Sánchez, F. Priego-Capote, M. D. Luque de Castro. Analyst 134 (7) (2009) 1416–1422.

[29] N. Sánchez Ávila, F. Priego Capote, M. D. Luque de Castro. J. Chromatogr A 1165 (2007) 158–65.

[30] J. A. Pérez-Serradilla, F. Priego-Capote, M. D. Luque de Castro. Anal. Chem. 79(17) (2007) 6767–6774.

[31] O.F. Mijangos Ricardez, M.D. Luque de Castro, J. Ruiz-Jiménez, L. Rivera Lagunez. Sent to Food Chem. for publication.

[32] Ruiz-Gutiérrez, M. C. Pérez-Camino. J. Chromatogr. A 14 (2000) 321–341.

[33] G. Janicsak, K. Veres, A.Z. Kakasy, I. Mathe. Biochem. Systematics and Ecology 34 (2006) 392–396.

[34] S. Cunha, J. Fernandes, M. B. Oliveira. J. Chromatogr. A 1128 (2006) 220–227.

[35] J.C. Del Río, G. Marques, IM. Rodríguez and A. Gutiérrez. Industrial Crops and Products 30 (2009) 241–249.

CAPÍTULO 3

Caracterización de las fracciones de alcoholes grasos y esteroles en olivo

Characterization of fatty alcohol and sterol fractions in olive tree

Mara I. Orozco[†], *José Ruiz-Jimenez*[†], *María D. Luque de Castro*[†] *

[†]*Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain*

Journal of Agricultural and Food Chemistry, 58 (2010)7539-46

Characterization of fatty alcohol and sterol fractions in olive tree

Mara I. Orozco, José Ruiz-Jimenez, María D. Luque de Castro

ABSTRACT

The determination of sterols and fatty alcohols is a part of the study of the metabolomic profile of the unsaponifiable fraction in olive tree. Leaves and drupes from 3 varieties of olive tree (*viz.* arbequina, picual and manzanilla) were used. The content of the target compounds was studied in 5 ripeness states and three harvesting periods for olive drupes and leaves, respectively. A method based on ultrasound-assisted extraction and derivatization for the individual identification–quantitation of sterols and fatty alcohols, involving chromatographic separation and mass-spectrometry detection by selected ion monitoring, was used. The concentrations of alcohols and sterols in the drupes ranged between 0.1–1086.9 and 0.1– 5855.3 μg/g, respectively, which are higher than in leaves. Statistical studies were developed to show the relationship between concentration of the target analytes and variety, ripeness state and harvesting period.

Keywords: ***Olive tree,*** leaves, drupes, fatty alcohols, sterols, GC-MS, statistical analysis**.**

INTRODUCTION

Olive drupes are one of the most extensively cultivated fruit crops in the world. The cultivation area of olive trees has tripled in the past 44 years, from 2.6 to 8.6 million hectares *(1)*. Hundreds of olive-tree varieties have been selected over centuries for their adaptation to different microclimates and soil types. Among them, some cultivars are characteristic of a given zone, whereas others can be found in several countries. With regard to the names of the different varieties, the same name is sometimes given to clearly different varieties and different names are used for identical varieties *(2)*.

Olive trees produce a wide variety of metabolites that includes from relatively simple primary compounds to very complex products of the secondary metabolism and with a great chemical diversity *(3)*.

Sterols and aliphatic alcohols are secondary metabolites, the determination of which is of great interest as the content of these compounds influences the quality of vegetal oils, including olive oil. Clinical studies have demonstrated that plant sterols ─also known as phytosterols─ ingested in the normal diet or as dietetic supplements, decrease cholesterol levels in blood, inhibiting their absorption in the thin intestine *(4)*. Also, it is recognized that phytosterols are biologically active substances in cancer prevention, although it has still not been demonstrated by epidemiological studies *(5)*. In addition, sterols and alcohol profiles are used to characterize virgin olive oil, and, specially, to detect adulteration of olive oil by hazelnut oil *(6)*. The content of these compounds, present in the unsaponifiable fraction of olive oils and other vegetal oils, has been regulated by Regulation 2568/91/EEC and latter amendments *(7)*.

Sterols are widely occurring natural substances in plant fats and make up the greatest proportion of the unsaponifiable fraction of lipids *(8, 9)*. Their composition depends on the plant species *(10)* and, in oils, composition may vary according to agronomic and climatic conditions, the quality of the fruits or seeds, extraction and refining procedures, and storage conditions. The predominant phytosterol is β-sitosterol; minor components are campesterol, stigmasterol, Δ5-avenasterol, Δ7-avenasterol and brassicasterol. Sterols, including precursors of cholesterol and their metabolites, can be biologically active, although many of these activities have to be clearly defined. However, the potential of sterols to bind to nuclear receptors and activate target gene transcription has encouraged much new research in this area *(11)*. These compounds are

membrane components and, as such, they regulate its fluidity and permeability. In plants, where they are always present in mixtures, sterols act as substrates for the production of a wide variety of secondary metabolites, such as cardenalids, glycoalcaloids, pregnane derivatives and saponins.

Concerning fatty alcohols, the smallest molecules are used in cosmetics and food, and as industrial solvents. Some of the largest molecules are simply seen as biofuels, but little research had been done until 2006 regarding many of them, despite they had shown to be endowed with anticancer, antiviral, antifungal, and anti-HIV properties, and thus, with potential in medicine and as health supplements *(12)*. Due to their amphipathic nature, fatty alcohols behave as non-ionic surfactants. They find use as emulsifiers, emollients and thickeners in cosmetics and food industries, and are common components of waxes, mostly as esters of fatty acids but also as free alcohols. Very long-chain fatty alcohols (VLCFA) obtained from plant waxes and beeswax have been reported to lower plasma cholesterol in humans *(13)*. They can be found in unrefined cereal grains, beeswax, and many plant-derived foods. Reports suggest that 5–20 mg per day of mixed C24–C34 alcohols, including octacosanol and triacontanol, lower low-density lipoprotein (LDL) cholesterol by 21–29% and raise high-density lipoprotein (HDL) cholesterol by 8–15%. Wax esters are hydrolyzed by a bile salt-dependent pancreatic carboxyl esterase, releasing long-chain alcohols and fatty acids that are absorbed in the gastrointestinal tract. Studies of fatty alcohol metabolism in fibroblasts suggest that very long-chain fatty alcohols, fatty aldehydes, and fatty acids are reversibly inter-converted by a fatty alcohol cycle *(14)*. The main alcohols present in olive oil are hexacosanol, octacosanol, tetracosanol and docosanol (always at higher concentration in the oil from second press than in extra-virgin olive-oil) *(15)*.

In the present research, the study of the profile of sterols and fatty alcohols was planned to be extended to 3 olive-tree varieties —drupes collected during five maturation states and leaves collected during three different seasons— grown in the same agricultural zone. Therefore, the differences found in the insaponificable fraction can be attributed to the characteristics of the referred varieties, the sampling period or the maturation state, taking into account that climate, type of soil and other environmental conditions are common to all three.

MATERIALS AND METHODS

Samples, chemicals and instruments. The samples (leaves and drupes) used in this research were collected in the region of the Guadalquivir valley, Encinarejo (Córdoba). Three genetic varieties of olive trees, ***Olea europaea*** (*i.e.* manzanilla, picual and gordal) were used in this study. The olive drupes were harvested from September 2008 to January 2009 in each of the five maturity states of the fruit (1, green; 2, green-yellow; 3, yellow-purple; 4, purple; and 5, black); meanwhile leaves were harvested in three seasons of the year: autumn (October 2008), winter (January 2009) and spring (April 2009). All samples were taken from the same agricultural zone to avoid variations caused by soil characteristics and environmental factors. For representativeness, the samples were taken from four different trees of each variety. The drupes were, milled, homogeneized by agitation in a vortex, and kept at –20 °C until use. The leaves, previously dehydrated, were milled, sized (60 μm) and kept at –20 ºC until use.

Chemicals weredocosanol, tetracosanol, hexacosanol and octacosanol as alcohols— and sterols —campesterol, stigmasterol and stigmastanol— obtained from Sigma–Aldrich (St. Louis, MO, USA). These were used as standards. Eiocosanol and cholesterol, also from Sigma–Aldrich, were used as internal standards (IS) in the determination step. The stock standard solutions of alcohols and sterols were prepared at 1000 μg/mL in chloroform, while campesterol was prepared at 100 μg/mL also in chloroform. The standard solutions, which contained 4 alcohols and 3 sterols, were prepared by dilution of the appropriate volume of the stock solution. All solutions were stored at −20 ºC in glass vials and kept in the dark at room temperature before use.

Aminopropyl-phase cartridges (500 mg) from Waters (Millipore, Milford MA, USA), and silica-phase cartridges from Supelco (Bellefonte, PA, USA) were used in the solid-phase extraction step. Bis-(trimethylsilyl)-fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich were used as silylation reagents in the derivatization step. Pyridine from Merck (Darmstadt, Germany) was used as derivatization solvent.

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium–alloy probe (12.70 mm diameter), which was immersed into a lab-made stainless steel container with eight compartments to place test tubes *(16)*. A Selecta Mixtasel (Barcelona, Spain) centrifuge was used to separate solid particles from the extract. A Büchi R-200 (Postfach, Switzerland) rotary evaporator furnished with a B-490 heating bath was used to

concentrate the sterol and fatty alcohol extracts after ultrasound-assisted extraction (UAE). A mechanical electrical stirrer MS2 minishaker from Ika (Wilmington, USA) was used to assist the derivatization step. A dry nitrogen stream was used to remove dichloromethane from the unsaponifiable fraction.

A Varian CP-3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable-temperature injector and coupled to a Saturn 2200 ion-trap mass-spectrometer (Sunnyvalley, TX, USA) was used for the determination of sterol and fatty alcohol profiles in the target unnsaponifiable fraction. The chromatograph was furnished with a Varian CP 8400 autosampler and a Factor Four VF-5 ms fused silica capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness) provided by Varian.

Sample preparation. The sample preparation step was similar to that proposed by Orozco-Solano *et al. (17)*. Briefly, 2-g sample —either, drupes or leaves— was placed in test tubes to which 10-mL portions of a 2:1 dichloromethane–hexane mixture was added. The tube was immersed in the water bath at 20 ºC, where ultrasonic irradiation —duty cycle 10%, output amplitude 10% of the converter, applied power 50 W, position of the ultrasonic probe tip was 2 cm from the bath bottom, and irradiation time 10 min— was applied.

After extraction, the extract was centrifuged for 10 min at 3000 rpm for separation of the solid particles from the liquid phase. The clean extract was mixed with 2-mL 2 M KOH, and 10-µL internal standards for sterols and fatty alcohols (0.1% chloroform solutions of cholesterol and 1-eicosanol, respectively). The mixture was subject to ultrasound —output amplitude 45% of the converter, applied power 200 W, duty cycle 50%— for 10 min, and, finally, the unsaponifiable fraction was extracted with 2-mL hexane, and the immiscible organic phase separated by centrifugation for 10 min at 3000 rpm. A gentle N_2 stream was used to dry the unsaponifiable fraction.

200-µg of the residue from the previous step was dissolved in 0.5-mL 4:1 hexane–chloroform. The resulting solution was passed through an aminopropyl column, the sterols and fatty alcohols were bounded to the functional groups of the sorbent and the compounds not retained by the column were disposed off. The column was rinsed with 10-mL 1:1 hexane–ethyl ether to remove matrix rests, dried with a nitrogen stream and the analytes eluted from it with 8-mL hexane and then with 6-mL 5:1 hexane–chloroform.

Finally, conversion of sterols and fatty alcohols into their more volatile derivatives is a necessary step prior to GC individual separation. 200 µL of the clean

extract were subject to dryness with a nitrogen stream and the residue reconstituted with 100-µL N-pyridine and homogeneized in a vial for 1 min; then, 98-µL N,O-bis(trimetilsylil)trifluore-acetamide was added and the mixture shaken vigorously in the vial for 1 min. Finally, 2-µL chloride trimethylsilylane was added, the mixture was shaken vigorously in the vial for 2 min more, then subjected to ultrasound (output amplitude 40% of the converter, applied power 180 W, duty cycle 50%) for 10 min to favour reaction.

Individual separation and determination of sterols and fatty alcohols by GC–MS. The individual sterols and fatty alcohols separation and determination by GC–MS was similar to that previously proposed by the authors *(17)*. Briefly, 1-µL of analytical sample was injected into the chromatograph for GC–MS analysis. The injector temperature was fixed at 250 ºC, and the injection was in the split–splitless mode. The splitter was opened (50:1) for 0.5 min, closed for 3.5 min and then opened at 100:1 split ratio for 10 min. The oven temperature program was as follows: initial temperature 50 ºC (held for 2 min), increased at 8 ºC/min to 250 ºC, followed by a second gradient at 3 ºC/min to 260 ºC (held 20 min); and finally, increased at 3 ºC/min to 300 ºC (held 10 min). The total analysis time was 70 min, and 5 min extra time was necessary for re-establishing and equilibrating the initial conditions.

The ion-trap mass spectrometer was operated in the electron impact ionization (EI) positive mode, for which the instrumental parameters were set at the following values: filament emission current 80 µA; transfer line, ion trap and manifold temperatures were kept at 220, 200 and 50 ºC, respectively. The recording window was set between 40 *m*/*z* and 650 *m*/*z* and the data were acquired using total ion current (TIC) scan mode. Digital selected ion monitoring (SIM) was applied as data treatment in order to remove the chromatographic background

Identification of target analytes with commercially available standards (*viz.* docosanol, tetracosanol, hexacosanol, octacosanol, campesterol, stigmasterol and stigmastanol) was based on comparison of the retention times and mass spectra. Those compounds with no commercial standards, low purity or expensive standards, (*viz.* tricosanol, pentacosanol and some sterols as cholestanol, brassicasterol, 24-methylene cholesterol, campestanol, chlerosterol, Δ^5-avenasterol and Δ^7-avenasterol) were identified by comparing their retention times and mass spectra with those in the literature. Calibration plots were run for the seven analytes with commercial standards

(peak area *versus* standard concentration). Compounds with no commercial standards were quantified by the calibration curve of the most similar alcohol or sterol.

Statistical analysis. Models based on Principal Component Analysis (PCA) were developed in order to study the influence of the olive-tree variety and harvest period on the concentration of sterols and fatty alcohols. Those results were confirmed by means of models based on Hieratical Cluster Analysis (HCA). The concentrations obtained by the proposed method for these compounds, expressed as µg/g, were used as variables for development of the models. Statistical analysis was performed using the data obtained after normalization in the case of leaves, and both the raw data and those obtained after the normalization and differentiation processes —Mean Normalization X(i,k)=X(I,k)/Abs(Mean(X(i,*) and Norris derivative (Segment size for averaging equal to 3; and, difference (Xk-Xk-1) equal to 2 — in the case of drupes.

The Unscrambler 9.0 from CAMO (Oslo, Norway) was used as statistical software (15) in the case of the PCA models. Statgraphics Centurion XV, Statpoint technologies, Inc. (Warrenton, VA, USA) was used as statistical software for the development of the HCA models.

RESULTS AND DISCUSSION

Alcohol and sterol fractions of olive drupes and leaves have been characterized using the proposed method. Despite wet and dry material was used as sample for the extraction of the target analytes in the case of drupes and leaves, respectively, all the results were expressed as dry material in order to make possible the comparison between the different matrices. Humidity was calculated in the case of the drupes samples for correction of the results.

The properties of the target compounds —alcohol and sterols— in addition to the presence in the leaves of other added-value compounds —triterpene dialcohols, phenols, etc.— and the extensive amount of olive leaves (in Spain 110 tonnes approximately) which are annually generated from pruning and also after separation of olive fruit in mills prior to oil production *(18)* give an additional interest to the characterization of these compounds in leaves samples and their subsequent use in the industry as source of biological active compounds.

Five ripeness states —(1, green; 2, green-yellow; 3, yellow-purple; 4, purple; and 5, black) were studied in the case of the drupes and 3 harvesting periods —winter (drupes, ripeness state 5), spring (no drupes or flowers in the tree) and autumn (drupes, ripeness state 1)— were studied in the case of the leaves.

There is not information in the literature about the composition of alcohol and sterol fractions in olive leaves. Therefore all, the discussion of the results is based on the results obtained for olive drupes.

Characterization of the alcohol and sterol fractions. **Table 1** shows the concentrations of individual fatty alcohols and sterols found in each variety expressed as μg/g in dry material, and the precision of the method —calculated by triplicate analysis.

Drupes. The total concentration of alcohols in the drupes ranges between 40.8 and 3267.4 μg/g. These values are in agreement with the results provided by Ranalli *et al.* (2002) *(19)* for oil obtained from whole olive fruit using an organic solvent as extractant, and are ten times higher than in extra virgin olive oil *(20).* This difference can be easily explained taking into account the extraction procedure used for extraction of extra-virgin olive-oil.

A detailed study of the composition of this fraction reveals that the fatty alcohols present at higher concentration are hexacosanol, tetracosanol and docosanol, for all the varieties. This result is in agreement with the results obtained for extra-virgin olive-oil, but not with those provided by organic solvent extraction, in which docosanol is present at low concentrations. This can be explained by the ripeness state of the drupes. Drupes in state 4 are used for the extraction of extra-virgin olive-oil. As can be seen in **Table 1** the concentration of docosanol for these drupes is high for this ripeness state but not for the others, which could have been used in the case of the organic solvent extraction.

Two trends were observed in the case of drupes (**Figure 1A**) for the variation of the concentration of fatty alcohols as a function of the ripeness state. In one of them —found in gordal and manzanilla varieties (used for production of table olives)—, the concentration of alcohols in drupes in states 4 and 5 is higher than in those in states 1–3. Furthermore, the concentration of fatty alcohols in the drupes of the ripeness state 1 is higher than in states 2 and 3. This behavior was also found by Ranalli *et al*. in 1998 *(21)* for other varieties. In that case, samples in state 1 had the same concentration of total fatty alcohols as samples in states 4 and 5.

Table 1. Analysis of Sterols and Fatty Alcohols in Olive Leaves and Drupes (all the results are expressed as µg/g –errors, in brackets, are expressed as %, n= 3 replicates).

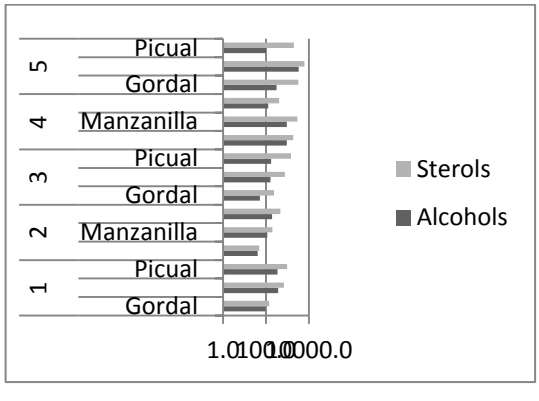
	Drupes																													
	Gordal								**Manzanilla**												**Picual**									
Compound	1		2		3		4		5		1		2		3		4		5		1		2		3		4		5	
Docosanol	3.4	(1.9)	14.3	(2.0)	3.7	(2.3)	180.1	(2.1)	0.1	(2.0)	9.9	(2.0)	33.9	(1.9)	7.1	(2.1)	210.7	(2.3)	6.5	(2.1)	10.1	(2.2)	55.5	(2.5)	9.6	(2.2)	29.1	(2.4)	2.2	(2.4)
Tricosanol	0.1	(2.1)	0.2	(2.0)	0.1	(1.9)	<LOD		43.0	(2.2)	0.1	(3.0)	0.1	(2.2)	<LOD	(2.3)	20.8	(2.4)	44.2	(2.3)	1.0	(2.1)	5.6	2.7)	0.6	(2.4)	2.9	(1.9)	14.9	(1.8)
Tetracosanol	28.3	(1.8)	17.4	(2.3)	2.7	(1.9)	466.5	(1.7)	113.4	(1.8)	74.6	(1.9)	37.4	(1.6)	7.6	(2.0)	407.6	(1.7)	574.7	(1.6)	91.7	(1.9)	74.3	(1.6)	7.5	(2.0)	56.3	(1.9)	39.9	(2.0)
Pentacosanol	0.1	(2.0)	0.1	(2.1)	<LOD		2.7	(2.6)	116.0	(2.0)	23.5	(1.7)	11.7	(1.9)	8.2	(2.0)	12.1	(2.0)	1086.9	(1.7)	18.0	(1.9)	14.4	(1.9)	8.3	(2.0)	1.7	(2.3)	4.5	(2.0)
Hexacosanol	50.5	(1.4)	8.1	(2.8)	35.2	(1.7)	212.6	(1.9)	23.4	(2.3)	189.2	(1.5)	24.8	(1.8)	90.1	(1.7)	203.2	(1.8)	932.2	(1.6)	162.8	(1.8)	34.3	(1.7)	99.9	(1.9)	28.1	(2.0)	36.2	(1.9)
Heptacosanol	<LOD	(2.6)	<LOD	(2.7)	<LOD	(3.0)	0.2	(3.0)	7.5	(2.6)	4.6	(2.0)	<LOD		1.5	(2.5)	<LOD		306.4	(1.8)	1.7	(2.0)	<LOD		0.4	(3.0)	<LOD		1.0	(2.4)
Octacosanol	9.9	(3.2)	0.8	(3.3)	9.8	(3.7)	78.8	(3.1)	2.3	(3.5)	69.8	(3.2)	5.3	(3.4)	46.0	(3.1)	69.9	(3.7)	316.6	(3.3)	53.9	(3.1)	6.2	(3.4)	47.6	(3.6)	9.7	(3.4)	<LOD	
Brassiscasterol	0.1	(2.3)	<LOD		<LOD		5.8	(3.0)	0.4	(3.0)	0.9	(2.9)	0.2	(3.0)	0.1	(3.0)	<LOD		11.0	(2.0)	2.2	(2.0)	1.4	(2.2)	<LOD		4.6	(2.3)	4.0	(2.0)
24-methylene	<LOD		<LOD		<LOD		<LOD		<LOD		0.2	(3.0)	<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		<LOD		7.1	(2.0)	2.7	(2.3)
Campesterol	2.4	(2.1)	10.8	(1.7)	4.3	(1.9)	489.3	(1.6)	1.9	(2.7)	10.7	(1.8)	38.3	(1.8)	<LOD		733.8	(1.6)	2.4	(2.0)	13.9	(1.9)	80.6	(2.0)	21.6	(1.9)	101.4	(1.8)	12.9	(2.0)
Campestanol	0.1	(2.1)	0.3	(2.0)	0.1	(3.0)	18.8	(2.0)	24.1	(1.9)	0.2	(3.0)	0.9	(2.7)	0.5	(2.9)	20.5	(1.9)	1.8	(2.4)	0.4	(3.0)	2.8	(2.3)	0.6	(2.9)	2.8	(2.0)	0.8	(3.0)
Stigmasterol	0.6	(2.3)	0.2	(2.8)	1.4	(2.3)	165.4	(1.9)	1.4	(2.9)	21.3	(1.8)	4.4	(2.2)	28.4	(1.9)	223.8	(1.6)	9.7	(2.0)	25.1	(1.9)	8.4	(2.0)	44.6	(1.9)	30.9	(2.0)	4.7	(2.0)
Clerosterol	1.0	(2.5)	0.3	(2.7)	2.1	(2.2)	4.1	(2.1)	6.3	(2.7)	0.2	(2.9)	0.1	(3.0)	0.5	(2.8)	15.2	(1.9)	4.9	(2.0)	1.4	(3.0)	0.5	(3.0)	2.8	(2.0)	2.1	(2.0)	3.3	(2.1)
β-sitosterol	134.5	(1.9)	34.3	(1.9)	224.9	(1.6)	1101.2	(1.6)	3057.0	(1.6)	634.1	(1.7)	131.9	(1.6)	709.6	(1.7)	1796.5	(1.5)	5855.3	(1.5)	910.7	(1.8)	305.8	(1.8)	1343.7	(1.7)	248.3	(1.8)	1966.8	(1.9)
Sitostanol	0.6	(2.8)	0.2	(3.0)	0.8	(2.5)	4.7	(2.3)	53.2	(1.9)	2.2	(2.5)	0.7	(3.0)	1.8	(2.9)	3.4	(2.0)	211.0	(1.7)	0.7	(3.0)	0.7	(2.9)	0.6	(2.8)	0.5	(2.2)	4.9	(2.0)
Δ^{-5}–Avenasterol	0.3	(2.5)	1.3	(2.7)	1.3	(2.5)	12.1	(2.0)	17.3	(2.0)	3.2	(2.0)	10.8	(1.9)	8.7	(2.2)	33.8	(1.8)	13.6	(1.9)	8.4	(2.1)	51.6	(1.9)	33.7	(1.9)	4.7	(2.0)	4.5	(2.0)
Δ^{-7}–Avenasterol	0.4	(2.1)	1.6	(2.9)	1.7	(2.4)	57.9	(2.0)	23.0	(1.9)	3.7	(2.0)	12.9	(1.8)	10.3	(2.2)	35.8	(1.8)	17.1	(1.8)	2.2	(2.3)	11.1	(2.0)	7.5	(2.0)	5.0	(2.0)	1.7	(2.3)
Alcohols	92.3		40.8		51.4		940.8		305.6		371.6		113.1		160.5		924.3		3267.4		339.1		190.3		173.8		127.7		98.7	
Sterols	140.2		48.9		236.6		1859.3		3184.6		676.8		200.3		760.0		2862.9		6126.8		964.8		462.8		1455.1		407.3		2006.3	

Analyte	Leaves																	
	Gordal						**Manzanilla**						**Picual**					
Compound	Winter		Spring		Autumn		Winter		Spring		Autumn		Winter		Spring		Autumn	
Docosanol	1.6	(2.1)	2.8	(2.0)	3.2	(2.0)	0.7	(2.9)	5.0	(2.0)	0.7	(2.9)	4.0	(2.0)	6.3	(2.0)	26.6	(2.0)
Tricosanol	0.1	(2.7	0.1	(3.0)	0.1	(2.6)	<LOD		<LOD		<LOD		0.4	(2.9)	0.6	(2.8)	1.5	(2.8)
Tetracosanol	13.5	(1.9)	3.4	(2.0)	2.2	(2.0)	1.2	(2.1)	5.4	(2.0)	5.3	(2.1)	35.8	(1.7)	8.3	(1.9)	17.4	(1.9)
Pentacosanol	<LOD		<LOD		<LOD		0.7	(2.4)	1.6	(2.3)	1.6	(2.2)	6.6	(2.0)	1.5	(2.6)	18.6	(1.9)
Hexacosanol	24.2	(1.9)	1.6	(2.1)	30.4	(1.9)	13.8	(1.9)	3.6	(2.2)	13.6	(1.9)	64.1	(1.8)	3.8	(2.1)	274.0	(1.7)
Heptacosanol	<LOD		<LOD		<LOD		0.1	(3.1)	<LOD	(3.1)	0.1	(2.8)	0.1	(3.0)	<LOD	(3.1)	0.1	(3.0)
Octacosanol	4.6	(3.1)	0.1	(3.4)	8.3	(3.1)	4.6	(3.2)	0.7	(3.4)	5.0	(3.2)	20.9	(3.1)	0.5	(3.3)	126.9	(2.9)
Brassiscasterol	0.1	(2.1)	<LOD		<LOD	(3.1)	0.1	(3.0)	0.1	(3.0)	0.1	(3.0)	0.8	(2.7)	0.1	(2.9)	0.1	(2.9)
24-Methylene	<LOD		<LOD		<LOD		<LOD		<LOD		0.1	(3.0)	<LOD		<LOD		<LOD	
Campesterol	1.2	(2.1)	2.1	(2.1)	3.7	(2.0)	1.3	(2.3)	5.6	(2.0)	0.8	(2.8)	5.5	(2.0)	9.2	(2.0)	59.9	(2.0)
Campestanol	0.1	(2.2)	0.1	(2.9)	0.1	(2.2)	0.1	(2.6)	0.1	(2.8)	0.1	(3.0)	0.1	(3.0)	0.3	(2.8)	1.4	(2.3)
Stigmasterol	0.3	(2.3)	0.1	(3.0)	1.2	(2.0)	2.9	(2.0)	0.7	(3.0)	1.5	(2.0)	9.9	(2.0)	1.0	(2.5)	123.1	(1.7)
Clerosterol	0.5	(2.3)	0.1	(3.0)	1.8	(2.0)	0.1	(2.9)	<LOD	(3.0)	<LOD	(3.1)	0.5	(2.4)	0.1	(2.8)	7.1	(2.1)
β-Sitosterol	64.6	(1.9)	6.7	(1.9)	195.3	(1.7)	108.8	(1.9)	19.4	(1.9)	45.8	(1.8)	360.7	(1.6)	35.0	(1.8)	3733.1	(1.6)
Sitostanol	0.3	(3.0)	0.1	(2.9)	0.7	(2.0)	0.2	(2.8)	0.1	(2.9)	0.2	(2.8)	<LOD		<LOD		<LOD	
Δ^{-5}−Avenasterol	0.2	(3.2)	0.3	(2.9)	0.2	(2.8)	0.9	(2.7)	1.6	(2.1)	0.3	(2.6)	3.4	(2.0)	5.9	(2.0)	94.7	(1.8)
Δ^{-7}−Avenasterol	0.2	(3.3)	0.3	(2.9)	1.5	(2.3)	1.1	(2.3)	1.9	(2.3)	0.3	(2.2)	1.0	(2.1)	1.3	(2.4)	21.8	(1.9)
Alcohols	44.0		7.9		44.1		21.0		16.2		26.1		131.8		21.0		465.1	
Sterols	67.3		9.6		204.4		115.2		29.5		48.8		381.9		52.8		4041.0	

Two trends were observed in the case of drupes (**Figure 1A**) for the variation of the concentration of fatty alcohols as a function of the ripeness state. In one of them —found in gordal and manzanilla varieties (used for production of table olives)—, the concentration of alcohols in drupes in states 4 and 5 is higher than in those in states 1–3. Furthermore, the concentration of fatty alcohols in the drupes of the ripeness state 1 is higher than in states 2 and 3. This behavior was also found by Ranalli *et al*. in 1998 *(21)* for other varieties. In that case, samples in state 1 had the same concentration of total fatty alcohols as samples in states 4 and 5.

The other trend was found in the picual drupes —a variety used for olive-oil production—, in which alcohols concentration decreases at higher maturation states. This is an explainable result taking into account that the amount of these compounds present in olive oil is limited by legislation and fatty alcohol concentrations found for drupes of all the varieties in ripeness states 4 and 5 are in agreement with those found by Ranalli *et al.* for frantoio and lechin varieties.

(A)



(B)

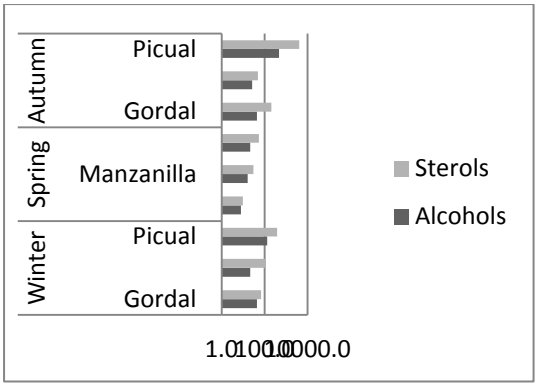


Figure 1. *Concentration of fatty alcohols and sterols in the three varieties under study. A and B, in drupes and leaves, respectively (all the results are expressed as µg/g of dry material).*

The concentration of total sterols in drupes (ranging between 48.9 and 6126.8 µg/g) is higher than that of fatty alcohols (**Table 1**). This is in agreement with the information found in the literature about the ratio between the alcohol and sterol fractions, which ranges between 1:3 and 1:10 *(19)*.

The concentrations of the sterol fraction were higher than the results provided for oil obtained from whole olive fruit but they were similar to the values provided by the oil obtained from the drupe seed *(19)*. This behavior can be easily explained taking into account that the whole drupe, seed included, was used in this study and the proposed extraction method was optimized for total extraction of these compounds (17).

A detailed study of the composition of this fraction reveals that sitosterol is by far the predominant sterol followed by campesterol and stigmasterol. These results are in agreement with the information found in the literature for whole drupe *(22)*. It should be emphasized that the

concentration of Δ^{-5}- avenasterol found in the samples under study is smaller than that found by other authors *(22)*.

Two trends were also observed in drupes for the variation of the concentration of sterols as a function of the ripeness state (**Figure 1A**). The first one was found for gordal and manzanilla drupes. The concentration of sterols increased with the ripeness state, with the exception of state 2, which showed the smallest concentration of these compounds. This result is similar to that proposed by Stiti *et al*. in 2007 *(22)*. In the case of picual drupes the behavior was similar to that in the other varieties, with the exception of state 4, which showed similar concentration to stage 2. This behavior was also found by *(21)*.

Leaves. The concentration of fatty acohols in leaves ranges between 7.9 and 465.1 µg/g. This concentration is five times smaller than the concentration present in drupes. A study of the individual composition of this fraction shows that hexacosanol is the alcohol present at the highest concentration. Furthermore, the concentration of tetracosanol and docosanol is ten times smaller than the concentration of hexacosanol. This is the main difference with the composition of drupes.

In leaves, the harvesting period has a clear influence (**Figure 1B**). The concentration of alcohols in autumn and winter is higher than in spring for all the varieties. It should be emphasized that the concentrations found for picual variety leaves were higher than those found for the other varieties. This could be related with the behavior of picual drupes, which present low fatty alcohol concentrations in ripeness states 4 and 5.

The concentration of sterols in leaves ranges between 9.6 and 4041.0 µg/g. This concentration is ten times smaller than that present in

drupes in the case of gordal and manzanilla varieties, but the concentration found for picual leaves is twice the concentration found in drupes. This fact can also be related with the presence of the smallest amount of sterols in picual drupes. Despite there is not information in the literature about the concentration of these compounds in olive leaves, there is information about the composition of the different fractions of the olive drupes and the highest concentration of sterols is present in the seed, which could be compared with leaves.

Two trends have also been observed in the modification of the sterols concentration with the season. In one of them, involving gordal and picual varieties, the concentration of sterols decreased from autumn to spring. The other trend was found in manzanilla leaves, in which sterols concentration increased from autumn to winter and decreased in spring.

Chemometric analysis. Statistical studies based on non-supervised pattern recognition techniques, such as Principal Component Analysis (PCA) and Hieratical Cluster Analysis (HCA), were developed in order to study the influence of the olive-tree variety and the harvest period on the concentration of sterols and fatty alcohols. The small number of samples used for development of the models hindered the use of supervised pattern recognition techniques, such as K-Nearest Neighbour (KNN) or Soft Independent Modelling of Class Analogy (SIMCA). Therefore, these results should be considered as a first approach to be completed in a future research, and a useful tool to visualize differences between samples.

In the case of olive leaves, normalization of the data was necessary. As can be seen in **Figure 2A**, the fatty alcohol and sterol composition in leaves harvested in winter and autumn is similar and different to that provided by leaves harvested in spring. Furthermore, identification of

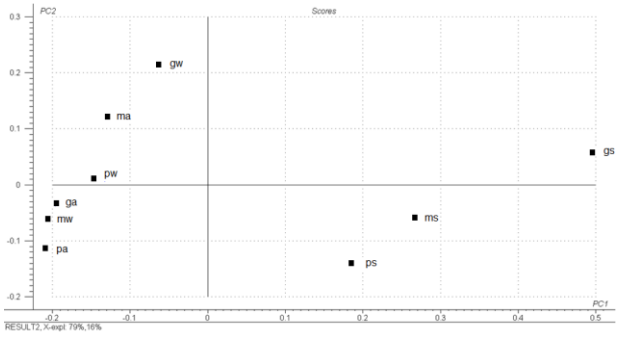
variety as a function of composition of these two groups of compounds is impossible. Target analytes which exert a higher influence in the development of the model are hexacosanol, docosanol, campesterol, octacosanol and Δ^{-5}- avenasterol. Three principal components were necessary to explain 96% of the data variability. This information was confirmed by means of the dendrogram obtained on the HCA analysis (**Figure 2B**). This dendrogram can divided into two main groups which are separated by an Euclidean squared distance 30. One group contains the samples harvested in spring and the other group contains the rest of the samples. Additional information can be obtained from this dendrogram for the samples harvested during autumn or winter. Gordal and picual leaves samples can be easily differentiated

(Euclidean squared distance 14). The composition of the manzanilla leaves harvested in autumn is similar to that provided by the picual leaves harvested in winter. The distance between these samples is smaller than 5. Therefore, it could be difficult find differences between these samples. Manzanilla leaves harvested during winter are totally different from the rest of the leaves harvested during autumn or winter but it is associated to that group of samples. This behavior can be explained taking into account that manzanilla is a variety which is harvested in advance.

Principal component model were developed using the raw data obtained after the analysis of the sterols and fatty alcohol composition of drupes from 5 different ripeness states. As can be seen in **Figure 3A**, drupes beloging to ripeness state 5 for all varieties and those of state 4 for manzanilla and gordal are different. Apart from this behaviour is that of states 1-to-3 for the three varieties. The joint use of these ripeness states allow differentiation between varieties as a function of sterols and fatty-

alcohols composition (see zoom in **Figure 3A**, ellipses have been used to join samples from the same variety). Three PC were necessary to explain 99% of the variability. The analytes with higher influence in the model were β-sitosterol, campesterol, estigmasterol, docosanol and pentacosanol.

(A)



(B)

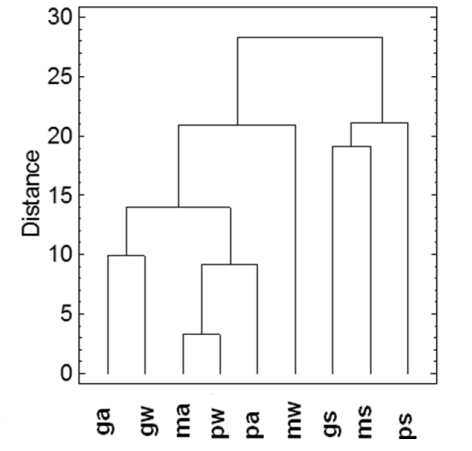
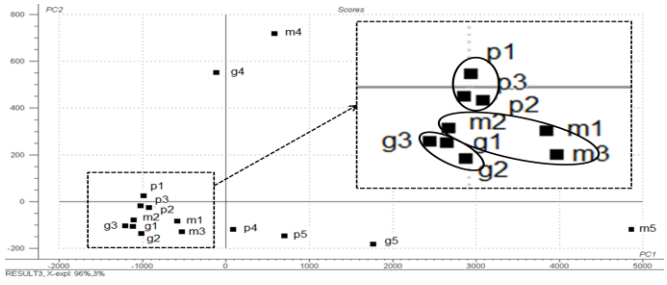


Figure 2. *Statistical analysis of main sterols and fatty alcohols families. A) PCA and B) HCA analysis of olive leaves (normalized data). Samples were identified using two letters. The first one is associated to the variety (g, gordal; m, manzanilla; p, picual) and the second one with the harvest period (a, autumn; s, spring; w, winter).*

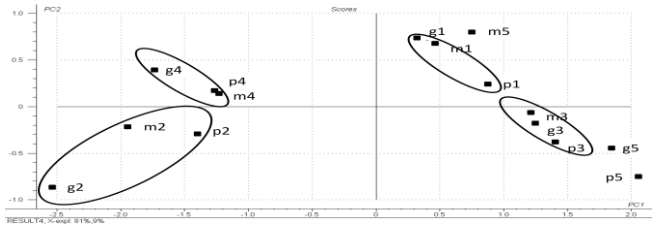
After data normalization and differentiation, it becomes clear that the samples are grouped as a function of the ripeness state (**Figure 3B**, ellipses have been used to join samples from the same variety). Manzanilla drupes in the ripeness state 5 have a different behaviour from the rest of the varieties with the same ripeness state. This behaviour can be associated with the high concentration of fatty alcohols present on the sample. The explained variability for 3 PC was 96%, and the most influential variables were campesterol, β-sytosterol, hexacosanol, tetracosanol, octacosanol and docosanol.

Hieratical cluster analysis was also developed in this case. As can be seen in **Figure 3C**, the obtained dendrogram can be divided into two groups, the first contains the samples in the ripeness state 2 and 4 and the second contains the rest of the ripeness states. The Euclidean squared distance for the samples from the same ripeness state is lower than 6 for ripeness states 1, 3 and 4. Therefore, these samples can be easily differenced. In the case of ripeness states number 2 and 5, one sample is not grouped correctly. Picual drupes in ripeness state 2 appear totally isolated from the rest of the samples of the same ripeness state. This can be explained taking into account Figure 3B. It is possible to see in this figure that the distance between the picual sample and the rest of the samples of the ripeness state 2 is the largest in the plot. Therefore, in HCA this sample cannot be grouped correctly. Something similar has been found for manzanilla drupes in ripeness state 5.

(A)



(B)



(C)

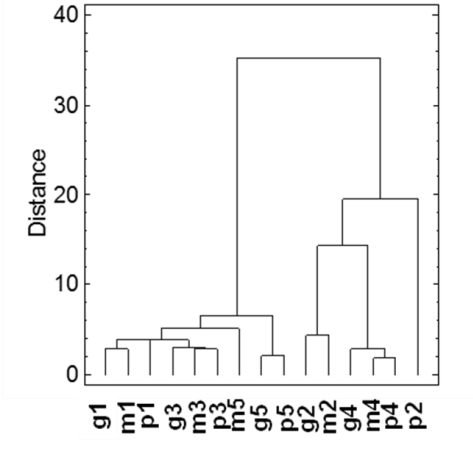


Figure 3. *Statistical analysis of analysis of main sterols and fatty alcohols families. A)) PCA of olive drupes (raw data),* ***B)*** *PCA of olive drupes (normalized and differentiated data); and, C) HCA analysis of olive drupes (normalized and differentiated data) . Samples were identified using a letter and a number. The letter is associated to the variety (g, gordal; m, manzanilla; p, picual) and the number from 1 to 5 to the ripeness state.*

Hieratical cluster analysis was also developed in this case. As can be seen in **Figure 3C**, the obtained dendrogram can be divided into two groups, the first contains the samples in the ripeness state 2 and 4 and the second contains the rest of the ripeness states. The Euclidean squared distance for the samples from the same ripeness state is lower than 6 for ripeness states 1, 3 and 4. Therefore, these samples can be easily differenced. In the case of ripeness states number 2 and 5, one sample is not grouped correctly. Picual drupes in ripeness state 2 appear totally isolated from the rest of the samples of the same ripeness state. This can be explained taking into account Figure 3B. It is possible to see in this figure that the distance between the picual sample and the rest of the samples of the ripeness state 2 is the largest in the plot. Therefore, in HCA this sample cannot be grouped correctly. Something similar has been found for manzanilla drupes in ripeness state 5.

Therefore, it is possible to say that drupe sterols and fatty alcohols composition can be used for the development of models based on supervised pattern recognition techniques —KNN and SIMCA— for empirical identification of the ripeness state of olive drupes. These models were not developed in this case owing to the small number of samples analyzed.

LITERATURE CITED

[1] Conde C.; Delrot S.; Gerós H. Physiological, biochemical and molecular changes occurring during olive development and ripening. *J. Plant Physiol.* **2008**, *165*, 1545–1562.

[2] Japón R.; Ruiz-Jiménez J.; Luque de Castro M. D. Discrimination and classification of olive tree varieties and cultivation zones by biophenol contents. *J. Agric. Food Chem.* **2006**, *54*, 9706–9712.

[3] Ercisle S.; Barut E.; Ipek A. Molecular characterization of olive cultivars using amplified fragment length polymorphism markers. *Genet. Mol. Res.* **2009**, *8*, 414–419.

[4] Ostlund R.; Lin X. Regulation of cholesterol absorption by phytosterols. *Curr. Atheroscler. Rep.* **2006**, *8*, 487–491.

[5] Berger A.; Jones P.; Abumweis S. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lipid Health Dis*. **2004**, *3*, 1–19.

[6] Angerosa F.; Camera L.; Cumitini S.; Gleixner G.; Reniero F. Carbon stable isotopes and olive oil adulteration with pomace oil. *J. Agric. Food Chem.* **1997**, *45*, 3044–3048.

[7] REGLAMENTO nº 702/2007 de la Comisión, de 21 de junio de 2007, por el que se modifica el Reglamento nº 2568/91 relativo a las características de los aceites de oliva y de los aceites de orujo de oliva y sobre sus métodos de análisis. *Diario Oficial de la Unión Europea L161 (22 de junio de 2007).*

[8] Segura A.; Carrasco A.; Cortacero S.; Gori A.; Cerretani L.; Fernández A. A simplified method for HPLC-MS analysis of sterols

in vegetable oil. *European J. Lipid Sci. Technol.* **2008**, *110*, 1142–1149.

[9] Lagarda M.J.; García G.; Farré R. Analysis of phytosterols in foods. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1486–1496.

[10] Giacomelli L.; Mattea M.; Ceballos C. Analysis and characterization of edible oils by chemometric methods. *J. Am. Oil Chem. Soc.* **2006,** *83*, 303–308.

[11] Salter A.M.; Tarling E.J. Regulation of gene transcription by fatty acids. *Animal,* **2007**, *1*, 1314-1320.

[12] Hilmarsson H.; Traustason B.; Kristmundsdottir T.; Thormar H. Virucidal activities of medium- and long-chain fatty alcohols and lipids against respiratory syncytial virus and parainfluenza virus type 2: comparison at different pH levels *Arch. Virol*. **2007,** *152*, 2225–2236.

[13] Volin P. Analysis of steroidal lipids by gas and liquid chromatography. *J. Chromatogr. A* **2001,** *935*, 125–140.

[14] Kim Y.; Dohnalek Z.; Kay B.; Rousseau R. Competitive oxidation and reduction of aliphatic alcohols over $(WO3)_3$ clusters. *J. Phys. Chem.* **2009,** *113*, 9721–9730.

[15] Paganuzzi, V. Distribution of the alcoholic components of the unsaponifiable matter within the olive drupe. I. Sterols. *Riv. Ital. Sostanze Gr*. **1979**, *56*, 431–437.

[16] Mijangos, O.; Ruiz-Jiménez, J.; Lagunez-Rivera, I; Luque de Castro, M.D. Fast ultrasound-assisted extraction of polar (phenols)

and nonpolar (lipids) fractions in *heterotheca inuloides. Sent to Food Chemistry for publication.*

[17] Orozco M.; Ruiz-Jiménez, J.; Luque de Castro, M.D. Ultrasound-assisted extraction and derivatization of sterols and fatty alcohols from olive leaves and drupes prior to determination by GC-tandem MS. *J. Chromatogr. A.* **2010,** *1217*, 1227–1235.

[18] Sánchez N.; Priego Capote F.; Luque de Castro M. D. Ultrasound-assisted extraction and silylation prior to gas chromatography–mass spectrometry for the characterization of the triterpenic fraction in olive leaves. *J. Chromatogr. A.* **2007,** *1165*, 158–165.

[19] Ranalli A.; Pollastri L.; Contento S.; Di Loreto G.; Lannucci E.; Lucera L., Russi F. Sterol and alcohol components of seed, pulp and whole olive fruit oils. Their use to characterise olive fruit variety by multivariates. *J. Sci Food Agri.* **2002,** *82*, 854–859.

[20] Rivera del Álamo R M.; Fregapane G.; Aranda F.; Gómez-Alonso S.; Salvador M. D. Sterol and alcohol composition of cornicabra virgin olive oil: the campesterol content exceeds the upper limit of 4% established by EU regulations. *Food Chem*. **2004,** 84,533–537.

[21] Ranalli A.; Tombesi A.; Ferrante M. and De Mattia G. Respiratory rate of olive drupes during their ripening cycle and quality of oil extracted. *J. Sci. Food Agric*. **1998**, *77*, 359–367

[22] Stiti N.; Triki S.; Hartmann M. Formation of triterpenoids throughout Olea europaea fruit ontogeny. *Lipids*. **2007,** *42*, 55–67.

PARTE II

Desarrollo y aplicación de plataformas orientadas:

Nutrimetabolómica

Esta Parte II de la Memoria es la más amplia, ya que abarca 5 capítulos con los siguientes contenidos:

Los Capítulos 4 y 5 constituyen una introducción a los estudios de nutrimetabolómica ya que se dedican a los estudios de caracterización de aceites sometidos a fritura simulada para la posterior preparación de piezas de bollería que ingerirían como desayudo los individuos obesos objeto del estudio. En los aceites (4 tipos con y sin adición de inhibidores de la oxidación de origen natural o sintético) y tras la etapa de calentamiento prolongado, se estudió la evolución de la concentración de la fracción insaponificable (esteroles, triterpenos y alcoholes de cadena larga), según se recoge en el Capítulo 4; y la fracción compuesta por inhibidores de la oxidación (fenoles y tocoferoles), que constituye el Capítulo 5. La enorme influencia del tipo de inhibidor añadido a los aceites en su comportamiento durante el calentamiento, puesta de manifiesto por estos estudios, condujo a su uso para la preparación de desayunos con los que comprobar su efecto en pacientes.

Los estudios de intervención utilizando muestras de suero de individuos obesos tras la ingesta de los aceites de fritura simulada se centraron en la evolución experimentada por esteroles, triterpenos y alcoholes de cadena larga en la sangre de los individuos en estudio (Capítulo 6) y a la de los ácidos grasos (Capítulo 7). En ambos casos se puso de manifiesto la repercusión del tipo de aceite en su metabolismo y el efecto beneficioso, protector, de los inhibidores de la oxidación naturales.

El último estudio que se recoge en esta Parte II de la Memoria, y que constituye el Capítulo 8, se engloba también en nutrimetabolómica, pero en este caso se desarrolló un método totalmente automatizado y basado en SPE–LC–MS/MS para la determinación de fenoles presentes en aceite de oliva virgen y sus metabolitos, que se aplicó a muestras de plasma de individuos sanos, extraídas después de la ingesta de un desayuno rico en aceite de oliva virgen extra.

CAPÍTULO 4

Influencia del calentamiento en la fracción insaponificable de los aceites comestibles vegetales enriquecidos con antioxidantes naturales

Influence of deep frying on the unsaponifiable fraction of vegetable edible oils with or without natural or artificial antioxidants

MARA ISABEL OROZCO, FELICIANO PRIEGO CAPOTE* AND MARÍA D. LUQUE DE CASTRO

[a]*Department of Analytical Chemistry,* Fine Chemistry Andalucía Institute, *Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain*

[b]*Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain*

Journal of Agricultural and Food Chemistry, 59(13) (2011) 7194-02

Influence of Deep Frying on the Unsaponifiable Fraction of Vegetable Edible Oils with or without Natural or Artificial Antioxidants

MARA ISABEL OROZCO, FELICIANO PRIEGO CAPOTE* AND
MARÍA D. LUQUE DE CASTRO

ABSTRACT

The influence of deep frying process mimicked by 20 heating cycles at 180 ºC on three representative fractions present in the unsaponifiable fraction of vegetable edible oils (*viz.* phytosterols, aliphatic alcohols and triterpenic compounds) has been studied. The target oils were extra-virgin olive oil (intrinsic content of phenolic antioxidants), refined sunflower oil enriched with phenolic compounds isolated from olive pomace, refined sunflower oil enriched with an artificial antioxidant (dimethylpolysiloxane) and refined sunflower oil without enrichment. The evolution of the target analytes as a function both of heating cycle and presence of natural or artificial antioxidants was also evaluated by comparison of the profiles after each heating cycle. Identification and quantitation of the target compounds were performed by gas cromatography–mass spectrometry (GC–MS) in single ion monitoring mode (SIM). The results showed that the addition of natural antioxidants to edible oils decrease the degradation from lipidic fraction produced during the simulated heating process. The addition of artificial antioxidant has similar behaviour for phytosterols and aliphatics alcohols. The employ of natural and artificial antioxidants improve the stability of the lipid fraction from tested vegetables oils used for frying without affecting its quality.

KEYWORDS: Olive oil, sunflower oil, fatty alcohols, sterols, triterpenic compounds, natural and artificial antioxidants, oil enrichment, deep frying, GC–MS.

INTRODUCTION

Deep fat frying is one of the most common processes used worldwide for preparation of cooked food. Fat or oil used for frying often determines the acceptability of food prepared with them. Although frying oil serves primarily as heat exchange medium, it often makes up a significant portion of the final food product. Both physical and chemical changes occurring in oil as a result of frying are mainly due to temperature, but also to interaction between oil, light, air, water, oxygen free radicals, enzymes action and food components *(1)*. During the frying process, the oil or fat is exposed to high temperature in the presence of air and moisture. Under these conditions, a cascade of chemical reactions is activated resulting in the loss of quality of the frying oil, and of the fried food, observed in the variation of the sensory and nutritional characteristics.

Although general modifications of the main fat constituents are well-known, it is not easy to foresee the rate of fat degradation due to the number of variables involved in the process. Dobarganes *et al.* classified these variables into those linked to the process itself, such as temperature, heating time, continuous or discontinuous heating, turnover rate, etc. *(2)*; those connected to the food subjected to frying such as lipid composition, main and minor constituents, etc., or those associated to the oil or fat used (*e.g.* unsaturation degree, initial quality and additives). The main chemical modifications affecting the quality of frying oil are caused by temperature, oxygen and moisture. It is worth noting that oxygen plays an important role in the deterioration of the oil during frying, but rarely a limiting factor. Under ambient conditions, autoxidation occurs by a free-radical mechanism where hydroperoxides play a primary role *(2)*. With temperature increase, oxidation leads to the formation of hydroperoxides and conjugated dienes, which do not accumulate due to their instability at frying temperature. These indermediates decompose spontaneously to form volatile organic compounds such as alcohols, ketones, aldehydes, epoxides and hydrocarbons, or generate dimers and trimers. At an advanced level, dimers and cyclic compounds can be polymerized. Oxidation plays a significant role in the development of rancid flavors that reduce the organoleptic characteristics, and in the formation of oxidized products that may cause a health hazard *(3)*. Hydrolysis alteration is caused by moisture content, even at trace level, resulting in free fatty acids, monoglycerides, diglycerides and glycerol *(4*). The previous chemical reactions affect massively to major fraction of vegetable oils, composed of triacylglycerols (95–98%), and have been widely studied *(5,6)*. However,

the effect on the minor fraction (2–5%), composed mainly by aliphatic alcohols, triterpenic compounds, sterols, hydrocarbons, volatile compounds and antioxidants, has been less studied. The minor fraction is of particular interest for the oil quality due to its contribution to organoleptic properties and product stability, but also from a nutraceutical point of view due to health benefits linked to many compounds in this fraction. Three representative groups present in the minor fraction are phytosterols, aliphatic alcohols and triterpene compounds.

Phytosterols are isoprenoid compounds with a sterol nucleus and an alkyl chain. They have nutritional interest because of their potential to lower both total serum cholesterol and LDL cholesterol in humans. This activity is caused by inhibition of the absorption of dietary cholesterol in the thin intestine *(7)* as well as by reabsorption of cholesterol excreted into the bile in the course of the enterohepatic cycle *(8)*. In the presence of oxygen and at frying temperature (150–190 ºC), sterols can lead to the formation of oxysterols, which are absorbed in the small intestine and incorporated into chylomicrons with toxic effects. Some examples of them are alteration of the structure and function of the cellular membranes or changes in the activity and expression of enzymes involved in cholesterol biosynthesis *(9)*. There is studies that report antioxidant activity of some phytosterols such as Δ5-avenasterol, its isomers fucosterol, as well other isomers, which protect oils subjected to frying temperatures from degradation oxidative. However, other studies report the low activity of phytosterols as ergosterol, stigmasterol, lanosterol and β-sitosterol. Their activity depend of the structure and number of double bonds.

Aliphatic alcohols are derived from natural fats and oils originated in plants, but also synthesized in animals and algae. Their historically overlooked significance in nutrition and health is at present recognized, as they are closely related to fatty acids, including the well-documented omega-3 fatty acids. Recently, it has been reported that sterols and aliphatic alcohols could be used to characterize virgin olive oil and, especially, to detect the adulteration of olive oil with hazelnut oil *(10)*. Aliphatic alcohols are clinically interesting as they are endowed with anticancer, antiviral, antifungal, and anti-HIV properties, and thus, with potential in medicine and as health supplements *(9)*.

A characteristic group of compounds present in seeds or drupes for extraction of edible oils is that formed by triterpenic compounds, which can be partially extracted in the oil as a part of the unsaponifiable fraction. The triterpenic fraction is especially interesting in olive oil and includes bioactive compounds such as oleanolic acid, ursolic acid, maslinic acid, uvaol, and erythrodiol. Several studies have shown that these compounds possess healthy properties *(11)*. However, they are used as a purity parameter to detect olive oil frauds with pomace olive oil *(12)*. Some papers have reported that high amounts of triterpenes deteriorate organoleptic oil quality *(13)*.

The presence of naturally existing or added antioxidants in oils exerts beneficial effects by avoiding their chemical alteration during heating. Natural antioxidants such as phenolic compounds have demonstrated an antioxidant activity superior to that of synthetic antioxidants. Therefore, there is an increased trend to replace the latter with natural antioxidants. The enrichment of edible oils with phenols protects them, for example, against oxidation that means better oil quality and prevention from the formation of toxic oxidation products such as cholesterol oxides *(14)*.

The aim of the present work was to evaluate the effect of adding natural or synthetic antioxidants to vegetable oils prior to be subjected to 20 cycles of simulated deep frying at 180 ºC (5-min heating each cycle). Three different fractions present in the unsaponifiable fraction were monitored to detect changes during the heating process. The alkaline saponification, a cleaning step, are required to remove interferents prior to final analysis by gas chromatography with flame-ionization or mass-spectrometry detection, where a derivatization step is required due to the low volatility and high molecular weight of the target compounds The isomeric character of many of these compounds justifies the frequent selection of high-resolution GC *versus* LC *(15–17)*, although UPLC or capillary LC could separate them efficiently *(18, 19)*.

MATERIALS AND METHODS

Reagents. Fatty alcohols —docosanol, tetracosanol, hexacosanol and octacosanol— and sterols —campesterol, stigmasterol, stigmastanol and β-sitosterol— were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as standards. The most representative and commercially available triterpenic compounds —*i.e.* erythrodiol from Extrasynthese (Genay, France), and uvaol, oleanolic acid and ursolic

acid from Sigma–Aldrich— were also used. Maslinic acid (>97% purity) isolated by a previously reported protocol *(20)* was a gift from A. García-Granados (University of Granada, Spain).

Betulinic acid from Sigma–Aldrich was used as internal standard (IS) for triterpenes quantitation, while eicosanol and cholestanol, also from Sigma–Aldrich, were used as IS for alcohols and sterols, respectively. The stock standard solutions of alcohols and sterols were prepared at 1000 μg/mL in chloroform, while campesterol was prepared at 100 μg/mL, also in chloroform. The standard solutions, which contained 4 alcohols, 4 sterols and 5 triterpenes, were prepared by appropriate dilution of the stock solutions. All solutions were stored at −20 ºC in glass until use.

n-Hexane LC gradient grade was provided by Panreac (Barcelona, Spain). Anhydrous sodium sulfate from Sigma–Aldrich was used as drying agent for the non-polar phase in the derivatization step. A 2 M KOH methanolic solution (Panreac) was used for oil saponification.

Aminopropyl-phase cartridges (500 mg) from Waters (Millipore, Milford MA, USA), and silica-phase cartridges from Supelco (Bellefonte, PA, USA) were used for solid-phase extraction. Bis-(trimethylsilyl)-fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich and pyridine from Merck (Darmstadt, Germany) were used in the derivatization step.

Apparatus and Instruments. Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium–alloy probe (12.70 mm diameter), which was immersed into a lab-made stainless steel container with eight compartments to place test tubes *(21)*. A Selecta Mixtasel centrifuge (Barcelona, Spain) was used to separate the immiscible phases after saponification. A mechanical electrical stirrer MS2 minishaker from Ika (Wilmington, USA) was used to assist the derivatization step.

A Varian CP-3900 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable-temperature injector and coupled to a Saturn 2100 ion-trap mass-spectrometer (Sunnyvalley, TX, USA) was used for analysis of fatty alcohols, sterols and triterpenic compounds. The chromatograph was equipped with a Varian CP 8400

autosampler and a Factor Four VF-5 ms fused silica capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness) provided by Varian.

Preparation of Edible Oils. Edible oils with a known content in antioxidants — extra-virgin olive oil as such (VOO), refined sunflower oil enriched with an extract of phenolic compounds from olive pomace (ASO), refined sunflower oil enriched with dimethylpolysiloxane as an artificial antioxidant (DSO), and refined sunflower oil without enrichment (SO)— were used. Koipesol (SOS Cuétara S.A, Madrid) provided the oils for subsequent enrichment in the laboratory.

Deep Heating Oil Procedure. Two litres of the selected oils was placed in a stainless deep fryer. The oil was subjected to 20 cycles heating at 180 ºC ± 5 ºC for 5 min/cycle. Three-mL aliquots from the target oil were removed after each heating period and stored at –20 ºC until use.

Sample Preparation. Two gram of the tested oil was placed in a test tube to which 2-mL 2 M KOH and 10-μL internal standards for sterols, fatty alcohols and triterpenic compounds (0.1% chloroform solutions of cholestanol and 1-eicosanol, and 0.1% ethanol solution of betulinic acid) were added. The tube was immersed in the water bath at 23 ºC, where ultrasonic irradiation —duty cycle 70%, output amplitude 30% of the converter— was applied for 15 min. After cooling at room temperature, the unsaponifiable fraction was extracted with 4-mL hexane, and the immiscible organic phase separated by centrifugation for 10 min at 1500 g. Finally, this phase was washed with distilled water until the aqueous phase resulted in neutral reaction. The unsaponifiable fraction was dried and the residue dissolved in 0.4-mL hexane. The resulting solution was passed through an aminopropyl column, in which the sterols, fatty alcohols and triterpenic dialcohols were bound to the sorbent while the no retained compounds were disposed off. The column was conditioned by two consecutive washing with 2-mL hexane. The analytical sample was then applied to the cartridge under vacuum suction. The interfering substances were removed by washing the cartridge with 4-mL hexane. Then, the fraction containing the target compounds was eluted with 6-mL 1:1 hexane–ethyl acetate.

Finally, conversion of sterols, fatty alcohols and triterpenic dialcohols into their more volatile derivatives is a necessary step prior to GC individual separation. With this aim, 200-μL clean extract was subjected to dryness by a nitrogen stream and the residue

reconstituted with 100-µL N-pyridine and homogeneized in a vial for 1 min; then, 98-µL N,O-bis(trimetilsylil)trifluore-acetamide was added and the mixture shaken vigorously in the vial for 1 min. Finally, 2-µL chloride trimethylsilylane was added, the mixture was shaken vigorously in the vial for 2 min more, then subjected to ultarasound (output amplitude 40% of the converter, duty cycle 50%) for 10 min to accelerate the derivatization reaction *(17, 22)*. Finally, the resulting solution was analysed by GC–MS.

Determination of Sterols, Fatty Alcohols and Triterpenic Compounds by GC–MS. The individual separation of sterols, fatty and triterpenes and determination by GC–MS was similar to that previously proposed by the authors *(22)*. Briefly, 1-µL analytical sample was injected into the chromatograph. The injector temperature was fixed at 250 ºC, and the injection was in the split–splitless mode. The splitter was opened (50:1) for 0.5 min, closed for 3.5 min and then opened at 100:1 split ratio for 10 min. The oven temperature program was as follows: initial temperature 70 ºC (held for 1.20 min), increased at 25 ºC/min to 320 ºC, followed by a second gradient at 2 ºC/min to 243 ºC; and finally, increased at 40 ºC/min to 270 ºC (held for 50 min). The total analysis time was 70.38 min, and 10 min extra time was necessary for re-establishing and equilibrating the initial conditions.

The ion-trap mass spectrometer was operated in single ion monitoring (SIM) mode after electron impact positive ionization (EI). The manifold, trap and transfer line temperatures were set at 220, 200 and 50 ºC, respectively. The analyses were performed with a filament–multiplier delay of 20 min and data acquisition was performed in the range *m/z* 50–650 in full scan mode, with a background mass of *m/z* 45. The emission current of the ionization filament was set at 80 µA. The scan time for data acquisition was set at 1.0 s in SIM mode while 3 microscans/s was selected in full scan mode. The values found for each monitored ion under SIM mode are listed in **Table 1**.

Statistical analysis. The variables potentially influencing the saponification procedure were studied by a multivariate approach. Statgraphics Centurion XV, Statpoint Technologies, Inc. (Warrenton, VA, USA) was used as statistical software with this purpose.

Table 1. Name, Formula, *m/z* Value and Retention Time for each Compound, and Number of Segments and Ions for Quantification in μSIS Detection Mode

	Compound name	Formula	*m/z*	Retention time	Segments	Ions for quantification
Aliphatic alcohols	1-Eicosanol (S.I)	$C_{20}H_{42}O$	298	26,247	1	355.3
	1-Docosanol	$C_{22}H_{46}O$	326	27,266	2	383.5
	1-Tetracosanol	$C_{24}H_{50}O$	354	29,471	3	411.3
	1-Hexacosanol	$C_{26}H_{54}O$	382	32,060	5	439.5
	1-Octasanol	$C_{28}H_{58}O$	410	34,987	6	370.2/460.2
Sterols	Cholestanol (S.I)	$C_{27}H_{48}O$	388	34,957	6	
	α-Cholestane	$C_{27}H_{48}$	388	30,962	4	357.0/413.3
	Campesterol	$C_{28}H_{48}O$	400	36,613	7	382.3/394.4
	Stigmasterol	$C_{29}H_{48}O$	412	36,997	7	
	ß-Sitosterol	$C_{29}H_{52}O$	416	37,938	8	215.3/383.5/396.0
	Stigmastanol	$C_{29}H_{50}O$	414	38,128	8	
Triterpenic compounds	Erythrodiol	$C_{30}H_{50}O$	426	40,365	9	496.5/216.1
	Uvaol	$C_{30}H_{50}O_2$	442	41,064	9	
	Oleanolic acid	$C_{30}H_{48}O_3$	456	42,335	10	
	Betulinic acid (IS)	$C_{30}H_{48}O_3$	456	41,794	10	203.3/320.3
	Ursolic acid	$C_{30}H_{48}O_3$	456	41,448	10	
	Maslinic acid	$C_{30}H_{48}O_3$	456	44,069	11	203.1/320.1

RESULTS AND DISCUSSION

Preparation of Vegetable Oils. Four different oils were selected to study the influence of antioxidants on the stability of the three target groups of compounds

present in the unsaponifiable fraction during simulated deep frying. Olive and sunflower oils were used as reference to assess the antioxidants effect, as the former oil is characterized by the natural presence of phenolic antioxidants, whereas sunflower oil loss them in the refining process. For normalization of the concentration of natural phenolic antioxidants in VOO, two different olive oils were mixed at a suited proportion for total phenol concentration of 400 μg/mL, expressed as caffeic acid according to the Folin-Ciocalteu test, while refined sunflower was used as such and also enriched as follows to compare the efficiency of artificial and natural antioxidants: (i) with a natural phenolic extract from olive pomace residue using the protocol described by Giron V. *et al*. *(23).* Enrichment was carried out up to a total phenols concentration of 400 μg/mL, also expressed as caffeic acid. (ii) with 400 μg/mL of synthetic antioxidant dimethylpolysiloxane. The speed and efficiency of the heating process depend on the temperature and quality of the oil. The heating temperature is usually between 150 and 190 °C and, for this experiment, it was set at 180 ºC.

Optimization of the Detection Step by GC–MS with an Ion-Trap. Mass spectrometry detection was performed in single ion monitoring by single ion monitoring mode. In this way, the ion-trap enriches those ions formed from the target compounds by electron impact ionization with respect to unwanted matrix ions by storing the former and ejecting the latter from the trap. This high-selectivity process is accomplished by definition of time segments for each compound in a chromatographic run according to elution times. Each segment should contain the most appropriate mass ranges to be stored. **Table 1** includes time segments and SIM ions mass selected for each monitored compound. The fragmentation schemes for representative compounds of each family (sterols, fatty alcohols and triterpenic compounds) are shown in **Figure. 1**.

Identification of analytes was ensured with standard solutions and spiked samples by comparison of mass spectra and retention times. **Figure 2** shows a chromatogram obtained from a multistandard solution. As can be seen, complete separation was achieved within 52 min. The retention times of sterols, fatty alcohols and triterpenic compounds depend on the length of their chains, double bonds number, position, geometry and branches. Also, the elution temperature program affects the elution order of sterols and triterpenic compounds with different number of double bonds.

The complexity of the sample and the trace levels at which the target analytes are present in vegetable oils demanded for a high sensitivity strategy to decrease quantification and detection limits. Large-volume injection was tested to improve sensitivity using injection in split–splitless mode to avoid solvent flow in excess through the column and prevent matrix residue deposition in it. Combination of split ratios from 50:1 to 100:1 and splitless mode was assayed as well as injector temperatures from 50 to 250 ºC with different ramps. The optimum split ratio (1:50) and the rest of the optimum conditions are described in the experimental section.

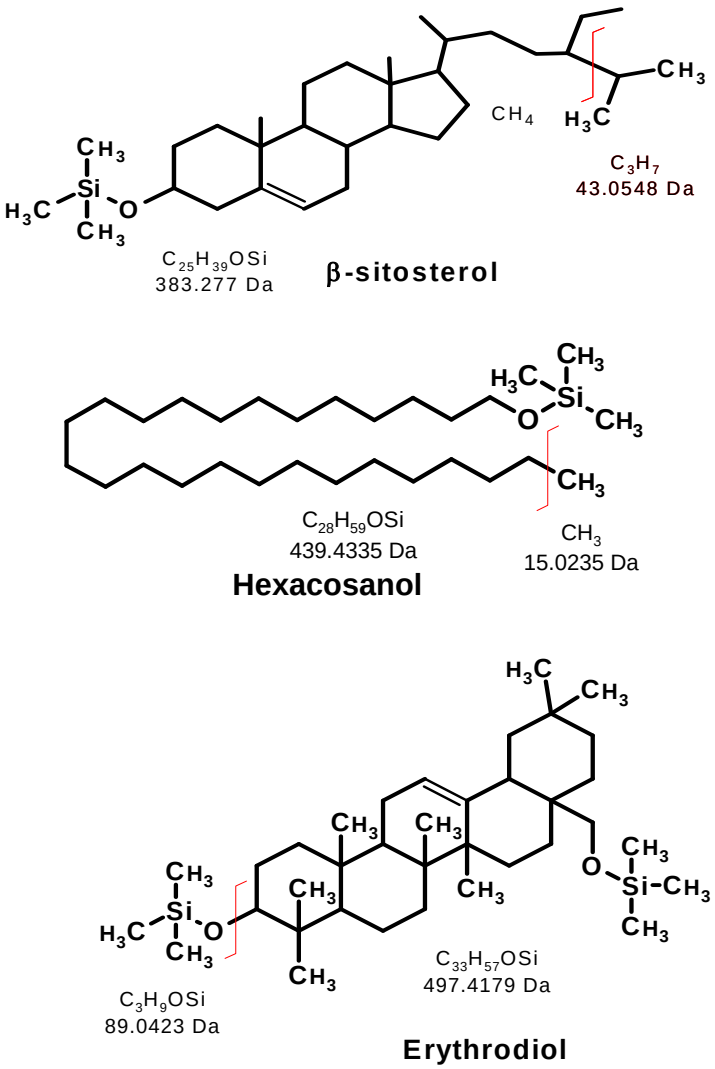


Figure 1. *Fragmentation of the derivatization products of the most representative target analytes of each fraction (β-sitosterol, hexacosanol and erythrodiol).*

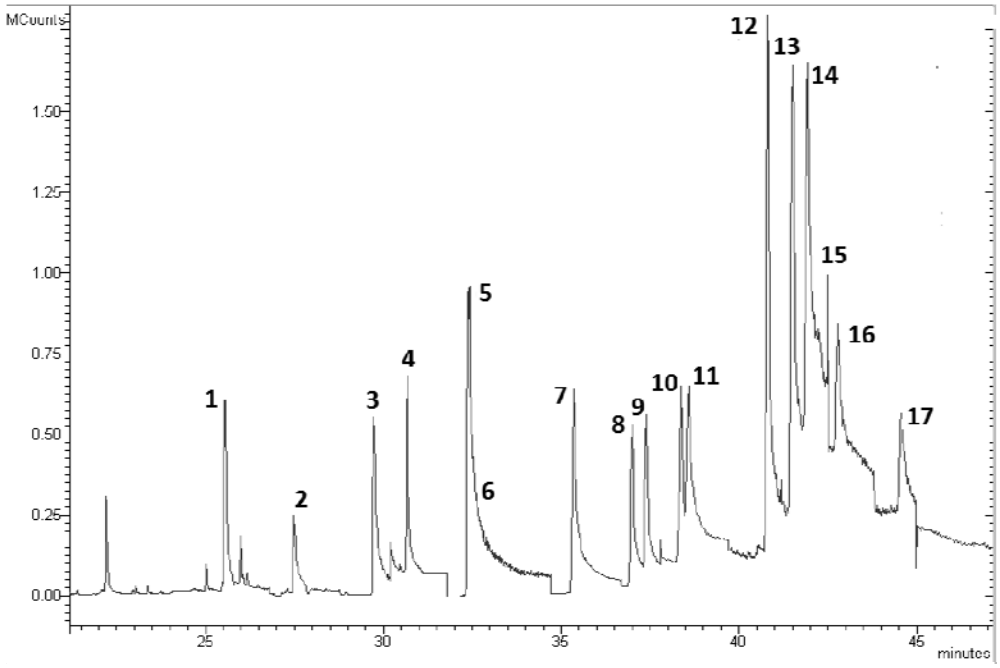


Figure 2. *Total ion chromatogram of a 15 µg/L standard solution of sterols, aliphatic alcohols and triterpenic compounds. 1, eicosanol (internal standard); 2, docosanol; 3, tetracosanol; 4, α-cholestane; 5,hexacosanol; 6, cholestanol (internal standard); 7, octacosanol; 8, campesterol; 9, stigmasterol; 10, β-sitosterol; 11,stigmastanol; 12, erythrodiol; 13, uvaol; 14, oleanolic acid; 15, betulinic acid (internal standard); 16, ursolic acid; and 17, maslinic acid.*

Characterization of the Individual Separation–Detection Method. Calibration plots were run for the thirteen analytes with commercial standards. Eicosanol, cholestanol and betunilic acid were used as IS for quantitation of sterols, fatty alcohols and triterpenic compounds, respectively. Calibration equations were set by using the ratio between the peak area of each compound and that of the IS of the given fraction as a function of concentration of each compound (see **Table 2**). The regression coefficients ranged between 0.9842 and 0.9988 for the linear dynamic range tested for each analyte, which was within its limit of quantification (LOQ) and 50 µg/mL. The characterization of the method was completed with the limits of detection (LODs) and those of quantification, which were calculated from the chromatograms obtained with standard solutions. The peak height-to-averaged background noise ratio was calculated, for which the background noise was estimated by the peak to peak baseline near the analyte peak. LODs and LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise (S/N) ratio of 3 and 10, respectively. The LODs for each analyte ranged between 0.21–1.09 µg/L for sterols, between 0.02–0.11 µg/L for fatty alcohols and 0.29–0.98 µg/L for triterpenic compounds. The LOQs

ranged from 0.70–3.64 µg/L; 0.06–0.36 µg/L and 0.97–3.27 µg/L for sterols, fatty alcohols and triterpenic compounds, respectively.

Table 2. Calibration Curves, Limits of Detection (LODs), Limits of Quantitation (LOQs) of Sterols, Aliphatic Alcohols and Triterpenic Compounds.

Compound	Calibration curve	R^2	Linear range	LOD (µg/L)	LOQ (µg/L)
Docosanol	$Y=3.0x\ 10^{-4}\ X\ +0.4559$	0.998	LOQ–0.50	0.06	0.19
Tetracosanol	$Y=3.0x\ 10^{-4}\ X\ +0.3302$	0,990	LOQ–0.50	0.04	0.09
Hexacosanol	$Y=1.5x\ 10^{-3}\ X\ +0.8455$	0.992	LOQ–0.50	0.02	0.06
Octacosanol	$Y=1.5x\ 10^{-3}\ X\ +0.3643$	0.999	LOQ–0.50	0.11	0.36
α-cholestane	$Y=3.0x\ 10^{-4}\ X\ +0.1654$	0.993	LOQ–0.50	0.29	0.98
Stigmasterol	$Y=8.0x\ 10^{-5}\ X\ +0.0448$	0-996	LOQ–0.50	0.43	1.43
β-Sitosterol	$Y=1.0x\ 10^{-4}\ X\ +0.1097$	0.997	LOQ–0.50	0.95	3.15
Stigmastanol	$Y=3.0x\ 10^{-4}\ X\ +0.1126$	0.999	LOQ–0.50	0.98	3.26
Campesterol	$Y=5.0x\ 10^{-5}\ X\ +0.0493$	0.991	LOQ–0.50	1.09	3.64
Erythrodiol	$Y=1.0x\ 10^{-3}\ X\ +1.630$	0.994	LOQ–0.50	0.29	0.97
Uvaol	$Y=6.0x\ 10^{-4}\ X\ +0.9327$	0.989	LOQ–0.50	0.55	1.86
Oleanolic acid	$Y=3.0x\ 10^{-4}\ X\ +0.177$	0.999	LOQ–0.50	0.98	3.27
Ursolic acid	$Y=4.0x\ 10^{-4}\ X\ +0.1004$	0.997	LOQ–0.50	0.37	1.23
Maslinic acid	$Y=9.0x\ 10^{-5}\ X\ +0.0359$	0.999	LOQ–0.50	0.88	2.02

Isolation of Sterols, Fatty Alcohols and Triterpenic Dialcohols from Oil Samples. Due to the low concentration of the minor compounds present in vegetable oils, saponification of triglycerides in alkaline media (2 M KOH) in methanol was required *(22)*. It is worth emphasizing that a direct analysis of oils without saponification is not possible with any chromatographic technique due to the complex

handling of triglycerides. Saponification is usually performed at 60–85 ºC or, less frequently, at room temperature, but always with long processing times *(3)*. In a previous research *(22)*, oil saponification was completed in 15 minutes using ultrasonic energy, which has proved a high efficiency to form emulsions and accelerate chemical reactions. The capability of this protocol, optimized for analysis of sterols and fatty alcohols in olive oil, was tested additionally for analysis of triterpenic compounds. The operational variables were duty cycle 70%, output amplitude 30% of the converter (135 W) at ambient temperature (23 ºC). The development of the process at ambient temperature is a significant benefit since degradation of labile compounds is avoided. After saponification and liquid–liquid extraction to a non-polar hexane phase, optimization of a clean-up step based on SPE was performed by testing different washing and elution solvents. Solvents such as ethyl ether, hexane and different hexane–ethyl acetate ratios were tested to cover a wide range of polarity. Washing was efficiently carried out using pure hexane (2 × 2 mL) to remove non-polar compounds present in the unsaponifiable fraction. On the other hand, elution of the target fractions with 50:50 (v/v) hexane-ethyl acetate (2 × 3 mL) was preferred. **Table 3** summarizes the variables studied in the optimization of the SPE step. The recovery of the SPE clean-up step was evaluated by spiking two olive-oil samples with the target compounds at two different concentrations (5 and 15 μg/L). The recoveries, calculated by a calibration curve for each analyte, were within 89–91%, which demonstrated an optimum efficiency of the SPE step. Evaluation of within-day and between-days variability was performed in a single experimental setup with replicates using natural samples (tested oils) subjected to the proposed method. Two analyses of these samples per day were carried out for 7 days.

Eq. (1) was used to determine the variance between days:

$$s_{between}^2 = (MS_{between} - MS_{within})/ n_j \qquad (1)$$

where MS is the mean square (residual sum of squares rated by the freedom degrees) and n_j is the number of replicates per day. The within-laboratory variability, s^2_{WR}, was calculated by Eq. (2)

$$s^2_{WR} = s_r^2 + s_{between}^2 \qquad (2)$$

where s_r^2 is the residual mean squares within-days, and $s_{between}^2$ is the variance due to the inter-day effect. The results obtained are listed in **Table 4**. Between-day precision, expressed as relative standard deviation (RSD), ranges between 2.8 and 5.1% and the within-day precision, also expressed as RSD, between 7.3 and 12.5%.

Table 3. Optimization of the Solid-Phase Extraction Step.

Variable	Tested range	Optimum value
SPE sorbent	Aminopropyl	Aminopropyl[a]
Sample volume(μL)	100–500	400
Volume of washing solvent (mL hexane)	2–12	4
Percentage of organic solvent in the elution phase (mL hexane)	0–50	50
Volume of eluant (mL 1:1 hexane–ethyl acetate)	5–20	6

[a] *Optimum sorbent in the method described by Orozco M., et al.*

Characterization and Quantification of Sterols, Fatty Alcohols and Triterpenic Compounds in the Tested Oils. The four oils selected for this study were preliminary characterized by analysis of the target compounds present in the unsaponifiable fraction. **Figure 3** shows the chromatographic profile of the unsaponifiable fraction of VOO, identified according to **Table 1.**

Table 5 summarizes the evolution of alcohols, sterols and triterpenic compounds in the four edible oils under study (note that the concentration of the target compounds is expressed as μg/mL) from the heating cycle 0 to cycle 20. As shows the table, SO and DSO provided considerably lower levels of aliphatic alcohols than VOO. These results could be ascribed to the refining process or, simply, to the raw seeds used for oil extraction. By contrast, ASO analysis reported levels of aliphatic alcohols similar to VOO, except for hexacosanol, which was found at 2.1 μg/mL in ASO as compared to 13 μg/mL present in VOO. This similarity between VOO and ASO in terms of aliphatic

alcohols concentration can be ascribed to the enrichment process with the extract from olive pomace using ethanol as extractant.

Table 4. Between-day Precision (S_r) and Within-day Precision (s_{wr}), Expressed as Relative Standard Deviation, of the Proposed Method.

Compound	**(s_r)**	**(s_{wr})**
Docosanol	3.0	7.5
Tetracosanol	3.1	7.5
Hexacosanol	2.7	8.6
Octacosanol	3.8	7.9
α-Cholestane	4.2	7.3
Stigmasterol	5.1	8.0
β-Sitosterol	2.8	7.6
Stigamastanol	3.7	7.9
Campesterol	3.9	7.4
Erythrodiol	4.0	12.5
Uvaol	3.2	7.2
Oleanolic acid	4.3	7.7
Ursolic acid	4.2	7.8
Maslinic acid	2.9	9.4

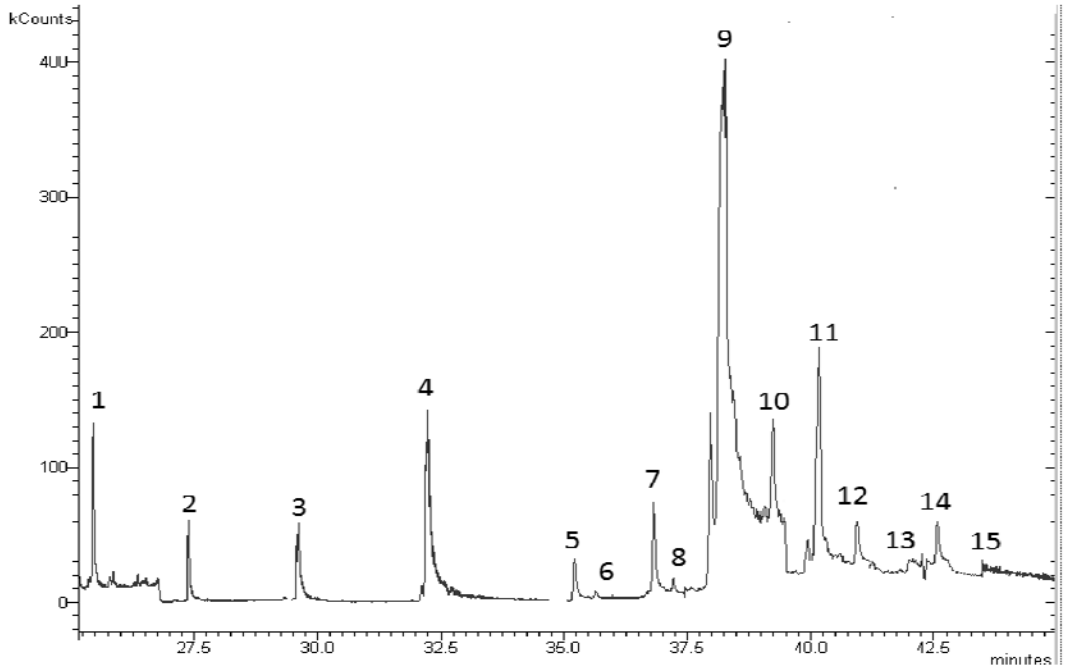
A different trend was found for phytosterols concentration. High levels of campesterol and stigmasterol were found in SO and DSO with concentrations of 133 and 230 μg/mL for campesterol and 101 and 64 μg/mL for stigmasterol, respectively. The concentrations of these sterols in VOO and ASO were below 65 μg/mL for campesterol and 43 μg/mL for stigmasterol. Concerning stigmastanol and sitosterol, they were found at higher concentrations in SO and VOO with concentrations around 18 and 506 μg/mL, respectively, as compared with their content in ASO and DSO. The concentration of sitosterol in sunflower oils was lower than in VOO.

Table 5. Evolution of Aliphatic Alcohols, Sterols and Triterpenic Compounds Contents in the Target Vegetable Oils During the Simulated Frying Process (all the results are expressed as µg/mL).

		Aliphatic Alcohols*				Sterols*				Triterpenic compounds*				
Oil	Cycle	DOC	TET	HEX	OCT	CAM	STE	STA	SIT	ERY	UVA	OLE	URS	MAS
VOO	**0**	4.75	11.93	13.07	<LOQ	42.02	43.31	8.23	506.19	18.67	14.40	5.05	5.26	28.10
	4	5.71	11.86	15.25	<LOQ	30.34	38.36	8.31	447.95	18.98	12.40	5.93	4.11	26.17
	8	5.20	2.93	1.22	<LOQ	24.75	35.40	6.75	222.42	14.14	6.57	<LOQ	<LOQ	<LOQ
	12	4.73	3.30	2.20	<LOQ	23.30	38.43	6.91	237.77	20.17	6.44	<LOQ	<LOQ	<LOQ
	16	3.53	3.23	1.55	<LOQ	20.07	36.07	7.84	269.72	18.52	5.32	<LOQ	<LOQ	<LOQ
	20	3.57	3.83	1.12	<LOQ	11.40	30.16	10.97	244.20	17.70	4.24	<LOQ	<LOQ	<LOQ
SO	**0**	0.40	0.01	0.22	<LOQ	133.63	101.53	18.12	308.09	7.01	5.77	<LOQ	<LOQ	<LOQ
	4	1.71	1.78	1.44	<LOQ	160.79	145.31	21.16	257.48	6.20	8.36	<LOQ	<LOQ	<LOQ
	8	1.54	3.13	1.64	<LOQ	153.65	139.82	25.61	246.69	7.21	11.45	<LOQ	<LOQ	<LOQ
	12	2.05	1.55	0.56	0.14	151.32	133.71	8.89	275.11	6.66	9.55	<LOQ	<LOQ	<LOQ
	16	1.61	2.01	0.82	0.68	123.18	125.25	8.91	268.47	6.46	8.22	<LOQ	<LOQ	<LOQ
	20	0.34	0.65	0.70	0.80	72.02	22.43	12.84	155.32	7.32	11.12	<LOQ	<LOQ	<LOQ
ASO	**0**	3.64	14.00	2.09	0.08	65.56	28.44	16.25	173.26	9.05	9.06	8.77	6.21	16.81
	4	5.92	7.24	4.13	0.28	202.73	168.19	30.54	258.72	7.48	7.61	7.41	6.61	19.45
	8	5.82	13.71	5.06	1.53	148.16	139.06	23.09	284.65	9.38	7.54	5.84	6.22	17.22
	12	5.77	18.37	6.87	2.11	141.70	90.80	22.88	241.71	9.88	11.08	4.18	<LOQ	<LOQ
	16	3.84	16.54	5.04	2.19	74.47	119.08	21.97	270.13	8.52	4.96	<LOQ	<LOQ	<LOQ
	20	<LOQ	4.21	0.11	0.40	51.49	44.05	6.12	121.76	0.14	0.75	<LOQ	<LOQ	<LOQ
DSO	**0**	1.13	4.78	2.82	<LOQ	230.46	64.05	2.24	286.52	6.37	2.08	2.27	2.09	6.62
	4	0.22	1.40	4.19	<LOQ	235.50	37.60	6.34	119.60	5.78	1.98	2.10	2.55	5.28
	8	1.14	1.01	5.75	<LOQ	185.85	49.71	17.64	55.21	5.44	1.57	2.30	2.73	5.36
	12	5.25	7.70	5.59	0.46	202.84	95.78	22.78	23.52	7.90	2.11	1.22	2.60	5.69
	16	5.75	7.19	4.17	0.59	169.27	85.30	25.72	28.58	8.34	1.60	1.28	2.77	5.80
	20	<LOQ	2.71	2.53	<LOQ	62.92	57.66	13.76	20.36	0.98	1.02	1.13	2.77	5.35

* *DOC, docosanol; TET, tetracosanol; HEX, hexacosanol; OCT, octacosanol; CAM, campesterol; STE, stigmasterol; SIT, β-sitosterol; STA,stigmastanol; ERY, erythrodiol;, UVA, uvaol; OLE, oleanolic acid; URS, ursolic acid and MAS, maslinic*

A)



B)

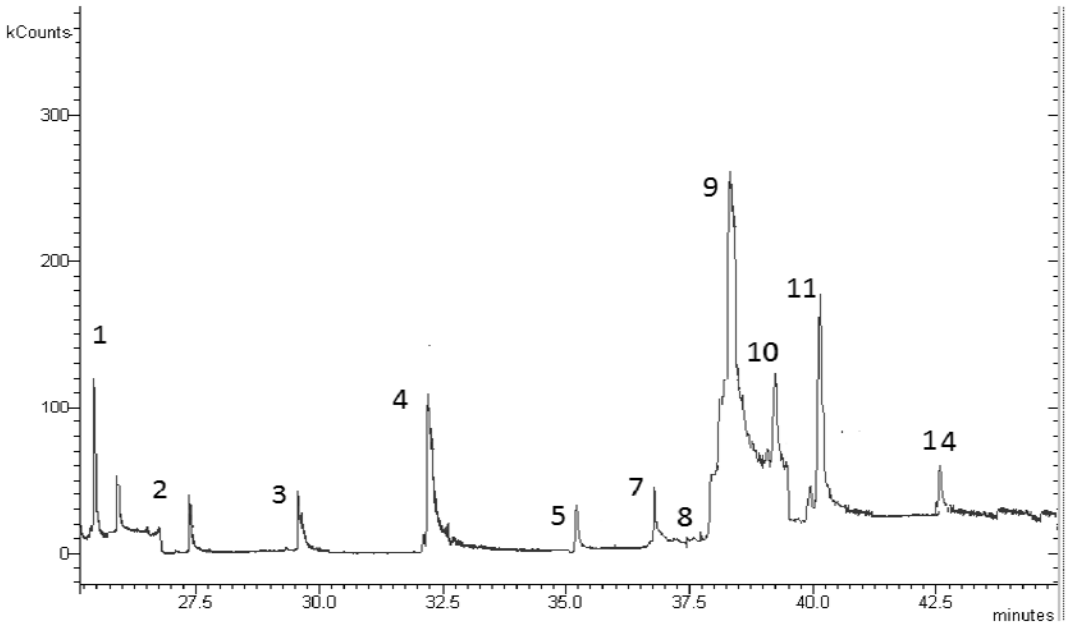


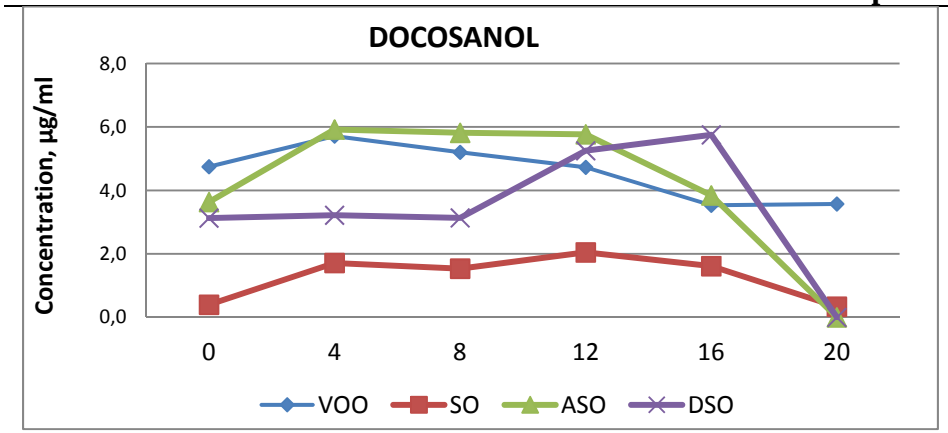
Figure 3. *Chromatograms from virgin olive oil in cycles zero (A) and twenty (B) of the heating process. Peak identification: 1, eicosanol (internal standard); 2, docosanol; 3, tetracosanol; 4, hexacosanol; 5, cholestanol (internal standard); 6, octacosanol; 7, campesterol; 8, stigmasterol; 9, β-sitosterol; 10, stigmastanol; 11, erythrodiol; 12, uvaol; 13; oleanolic acid; 14, betulinic acid (internal standard); and 15, ursolic acid.*

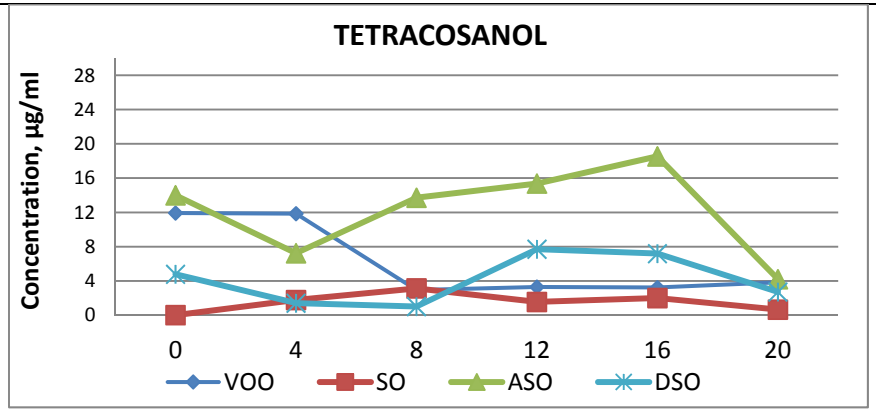
As suspected, the highest concentrations of triterpenic compounds were found in VOO, whereas low levels of them were detected in SO and DSO. The enrichment process caused an irregular increase in the concentration of triterpenic compounds in refined sunflower oil. The most concentrated triterpenes in VOO and ASO were maslinic acid and the two triterpenic dialcohols erythrodiol and uvaol.

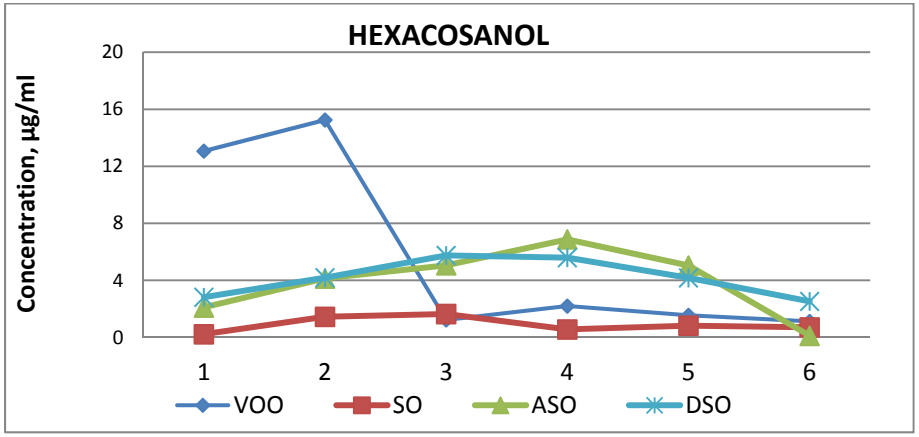
Characterization and Quantification of Sterols, Fatty Alcohols and Triterpenic Compounds in Fried Oils. The four target oils, (VOO, ASO, DSO and SO, the characteristics of which have been above exposed) were analysed with the proposed method to determine the concentration of the target analytes in them after each heating cycle. **Table 5** shows the concentrations of individual sterols, fatty alcohols and triterpenic dialcohols found in the oils after 4, 8, 12, 16 and 20 heating cycles. **Figure 3** compares chromatograms obtained from VOO before and after 20 heating cycles. The results obtained, illustrated in **Figure 4** for each monitored compound, are discussed for each target fraction.

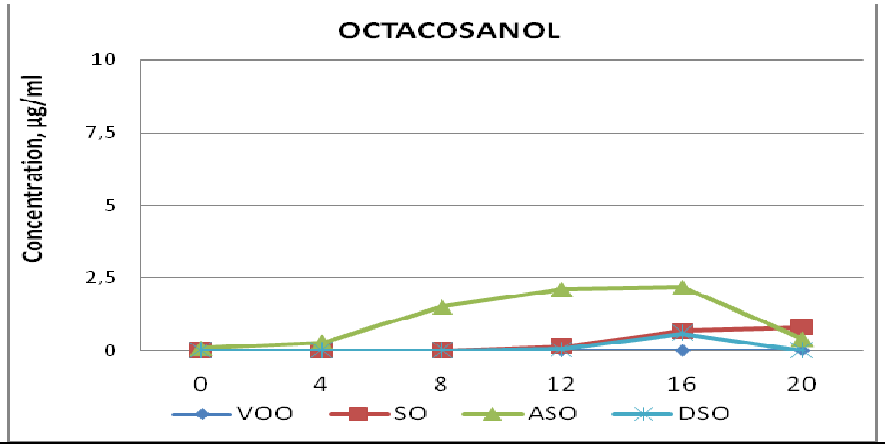
Triterpenic Compounds. Pentacyclic triterpenic acids as oleanolic, ursolic and maslinic acids were not detected in SO and DSO, while its detection in ASO was associated to the enrichment of the sunflower oil with extract from the solid residue generated in olive oil production. The main trend observed in VOO for triterpenic acids was a dramatic decrease of their concentration after 6 heating cycles (30-min heating), which led to non-detection of them after 8 frying cycles. As exposed above, similar levels of these compounds were transferred to ASO in the process for enrichment with the extracts. However, ursolic and maslinic acids were practically undetected after 10 heating cycles, and the same happened to oleanolic acid after 14 heating cycles. Therefore, it is clear that degradation of triterpenic acids was delayed for ASO as compared to VOO. Also, clear differences were observed for maslinic acid levels in VOO and ASO during the 7 first cycles. Thus, the concentration of maslinic acid increased during heating in ASO while the opposite trend was found in VOO. This behavior could be ascribed to the release of maslinic acid present in the extract from olive pomace, transferred to the oil and deconjugated during the first heating cycles.

Aliphatic alcohols

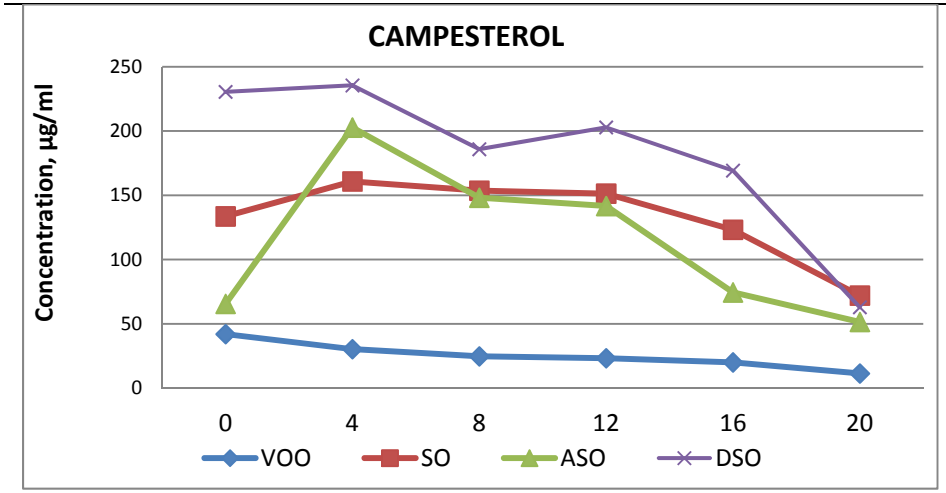


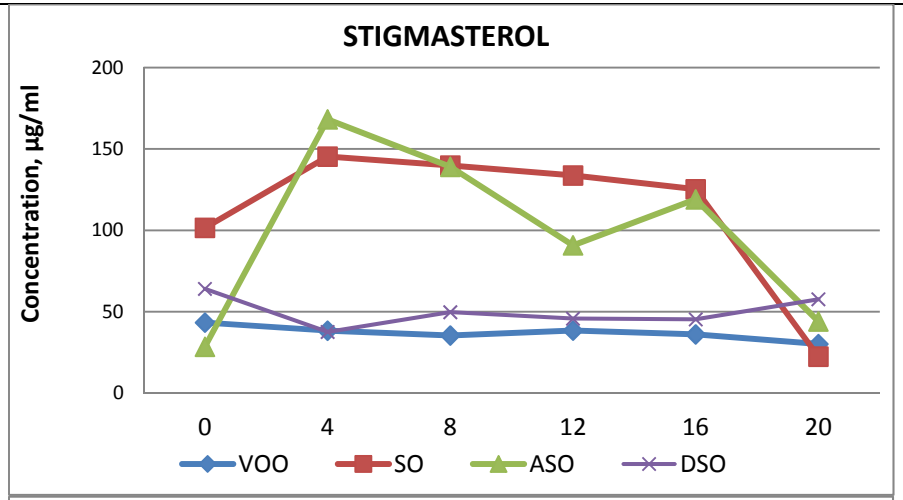


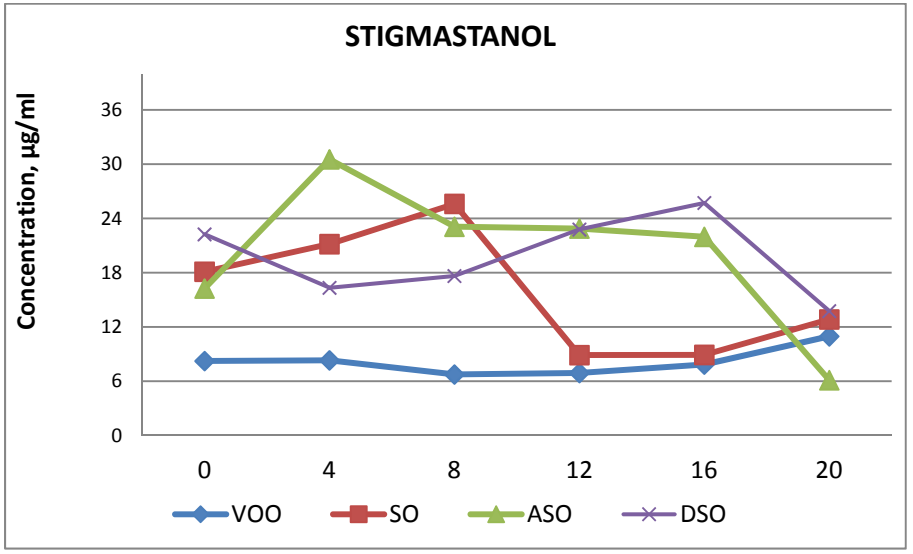


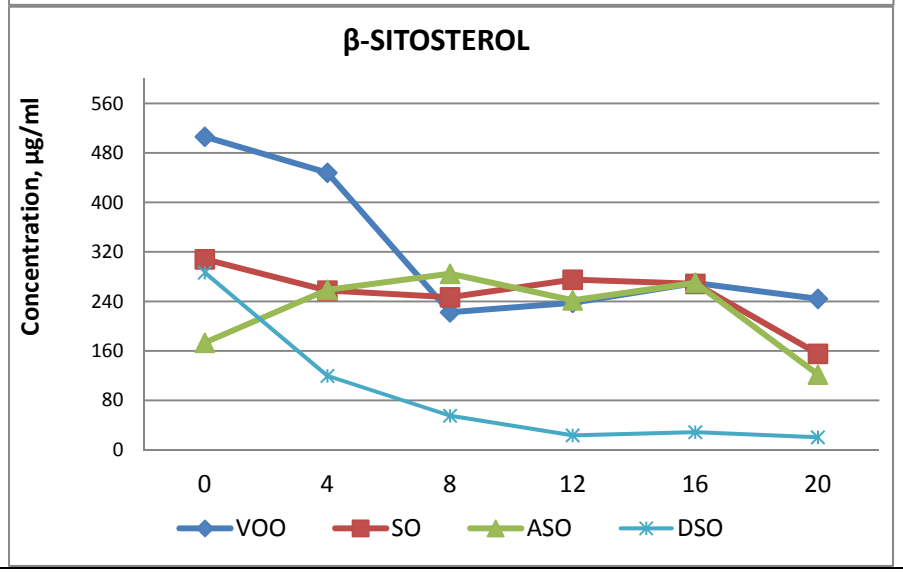


Sterols

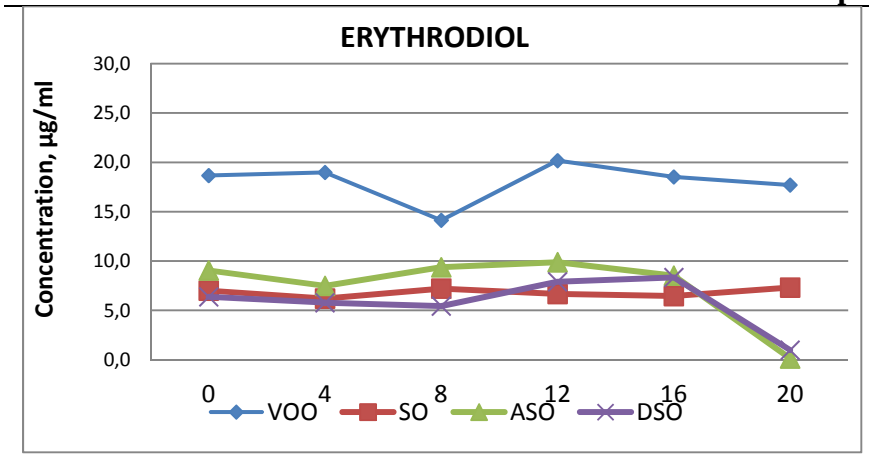

CAMPESTEROL
Concentration, µg/ml
250
200
150
100
50
0
0
4
8
12
16
20
VOO
SO
ASO
DSO

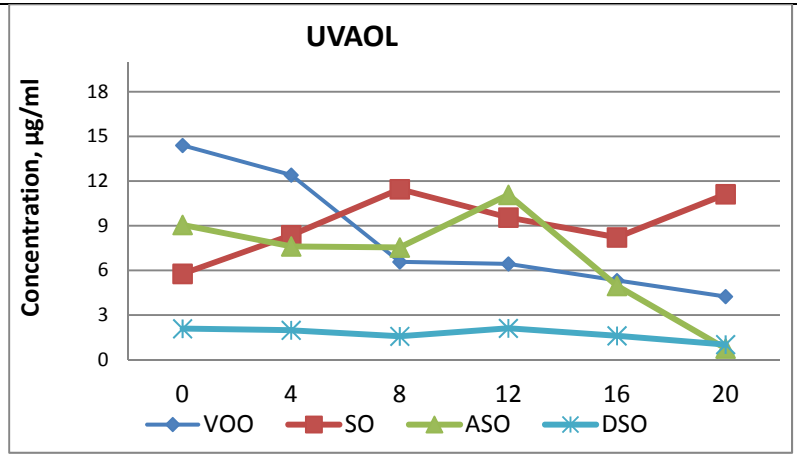

STIGMASTEROL
Concentration, µg/ml
200
150
100
50
0
0
4
8
12
16
20
VOO
SO
ASO
DSO

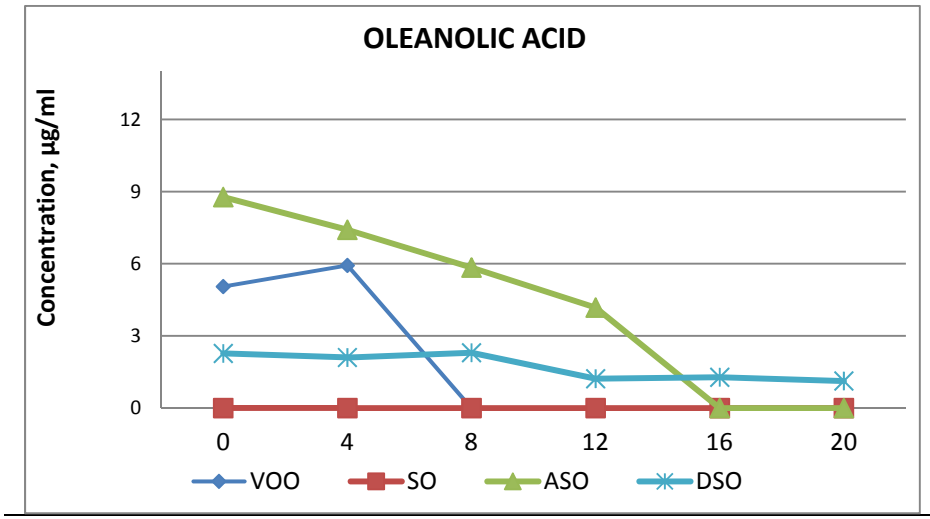

STIGMASTANOL
Concentration, µg/ml
36
30
24
18
12
6
0
0
4
8
12
16
20
VOO
SO
ASO
DSO

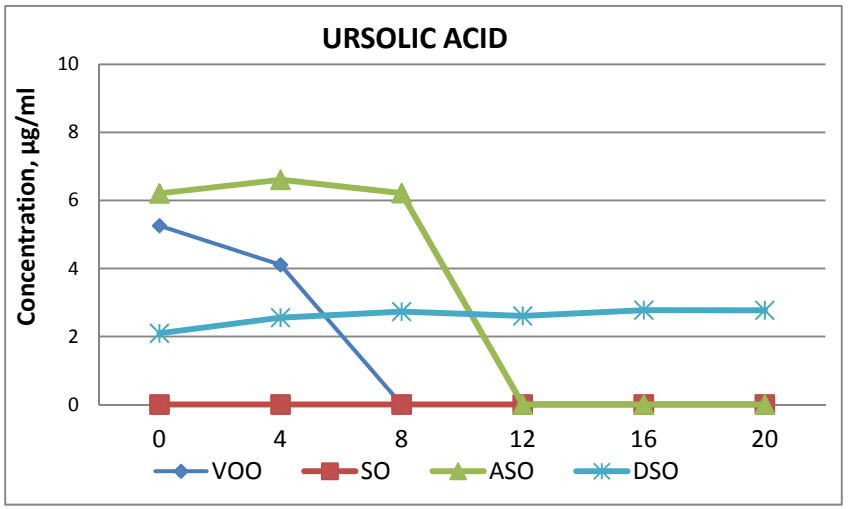

β-SITOSTEROL
Concentration, µg/ml
560
480
400
320
240
160
80
0
0
4
8
12
16
20
VOO
SO
ASO
DSO

Triterpenic dialcohols









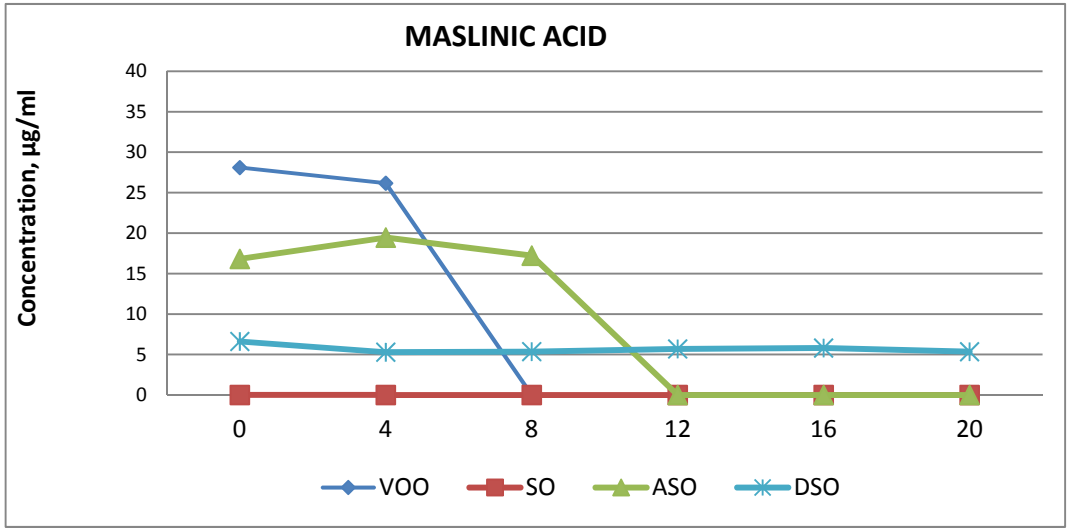


Figure 4. *Evolution of sterols, aliphatic alcohols and triterpenic compounds levels in the four tested oils, VOO, ASO, DSO and SO, during the twenty heating cycles.*

Triterpenic dialcohols were not detected in SO and DSO either. In VOO, the concentration of erythrodiol was practically not affected by heating. A different trend was observed for uvaol, the concentration of which decreased from 15 to 5 μg/mL during two hours, approximately. These two compounds were also detected in ASO because of the enrichment process. Uvaol showed a similar behavior in ASO and VOO but this triterpene was not detected in the aliquot sampled after 20 heating cycles. On the other hand, erythrodiol in ASO decreased its concentration after 17 heating cycles practically up to undetectable levels.

Phytosterols Fraction. Sterols were found in the unsaponifiable fraction of all oils under study. The concentrations of the monitored sterols changed during the heating process depending on the edible oil. Virgin-olive oil contained the lowest levels of campesterol and stigmasterol. On the other hand, DSO showed the highest concentration of campesterol (230 μg/mL) at cycle 0, which was decreased to 62 μg/mL after 20 cycles. Intermediate concentrations of campesterol were found in SO and ASO, which behave similarly: they underwent an increase of campesterol concentration during cycles 0-to-5/6, and a decrease in the level of this sterol for subsequent heating cycles. This trend was also found for stigmasterol in these two vegetable oils. β-sitosterol was the most concentrated phytosterol in all tested oils. The evolution of β-sitosterol in VOO consisted of a decrease during the eight cycles and a constant value during the rest of the heating process; on the contrary, this sterol experiences a total degradation in DSO after 12 heating cycles. On the contrary, SO and ASO underwent a slight decrease of β-sitosterol concentration by increasing the number of heating cycles. However, this alteration was not significant after the 20 heating cycles at 180 ºC. Oxidation mechanisms of phytosterols are believed to follow the same pathways as cholesterol oxidation to form oxysterols. Evaluation of the concentration of the different phytosterols during the heating process shows that autoxidation is favoured in VOO for β-sitosterol, which is the most concentrated of them. This is degraded very fast during the 6 first cycles (from 506 to 228 μg/mL); nevertheless, degradation of the rest of phytosterols is slower. Degradation of phytosterols is more uniform for SO, with a significant decrease in the concentration of campesterol (from 133 μg/mL to 72 μg/mL), stigmasterol (from 101 μg/mL to 22 μg/mL), stigmastanol (from 16 μg/mL to 9 μg/mL) and β-sitosterol (from 308 μg/mL to 155 μg/mL) during the twenty cycles. The heating

process of this oil affected principally to campesterol and β-sitosterol, which decreased their concentrations from 230 to 62 µg/mL for campesterol, and 286 µg/mL to 20 µg/mL in the case of β-sitosterol. A characteristic trend was observed in DSO for stigmasterol and stigmastanol, the concentrations of which increased during the initial heating cycles probably due to hydrolysis of conjugated forms. The concentration of target phytosterols also increased in ASO during the initial heating cycles. However, campesterol and stigmasterol decreased drastically their levels after 4 and 5 cycles, while stigmastanol and β-sitosterol concentrations were maintained up to cycle 16, when they were also degraded.

Aliphatic Alcohols Fraction. The fraction of aliphatic alcohols was detected in all edible oils under study. In VOO, this fraction followed a characteristic trend: the concentration of docosanol, tetracosanol and hexacosanol clearly decreased by increasing the number of heating cycles, while octacosanol was not detected at any time. Docosanol experienced a similar behaviour in sunflower oil enriched with olive pomace extract in comparison to VOO. In fact, this aliphatic alcohol was not detected in the last heating cycle. The concentration of the rest of alcohols increased after a given number of heating cycles. Thus, the concentration of tetracosanol increased after 10 cycles (although this was lowered after 13 cycles), octacosanol after 6 cycles (with a decrease after 18 cycles) and hexacosanol experienced a slight increase during the first 12 cycles with a subsequent fall in concentration for higher number of cycles. Aliphatic alcohols are frequently found forming conjugated structures in edible oils such as waxes, glycosides, fatty acids or sterols, which could justify this irregular behaviour. The same trend was observed in DSO for the different aliphatic alcohols, except for octacosanol, which was not detected along the heating test. The presence of natural or artificial antioxidants influence the chemical behaviour of oils during heating and the influence depends on the given compound. Anyway, the use of natural antioxidants could be considered as an excellent strategy to decrease the degradation from lipidic fraction produced during the heating, This improve the stability of oils used for frying without affecting its quality.

ACKNOWLEDGEMENTS:

The Spanish Ministerio de Ciencia e Innovación (MICINN) is thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

LITERATURE CITED

1. Lalas, S. Quality of Frying Oil. In *Advances in Deep-Fat Frying of Foods*, Edition 1.; S. Sahin and S. Glum, Eds., Publisher: Ankara, Turkey, 2008, CRC Press. pp. 57-80.
2. Dobarganes, M.C. and Velasco, J. Analysis of lipid hydroperoxides. *Eur. J. Lipid Sci. Tech*. **2002**, *104*(7), 420-428.
3. Erickson, M. D. Overview: Production and Composition of frying fats. Deep Frying Chemistry, Nutrition and Practical Applications, edition no 2.; Keys, AOAC press Eds.; Publisher: USA, 1996, pp.87-111.
4. Gillet, G.; Vitrac O. and Desobry S. Prediction of partition coefficients of plastic additives between packaging materials and food simulants. *Ind. Eng. Chem. Res.* **2010,**. *49*(16), 7263-7280.
5. Cert, A.; Moreda, W. and Pérez-Camino, M.C. Chromatographic analysis of minor constituents in vegetable oils. *J. Chromatogr. A.* **2000***, 881*(1-2), 131-148.
6. Andrikopoulos, N.K. Triglyceride species compositions of common edible vegetable oils and methods used for their identification and quantification. *Food Rev. Int.* **2002,** *18*(1), 71 – 102
7. X, O.R.a.L. Regulation of cholesterol absorption by phytosterols. *Curr. Atheroscler. Rep*. **2006**, 487-491.
8. Piironen, V.; Lindsay D.; Miettinen T.; Toivo J. And Lampi A.M. Plant sterols: biosynthesis, biological function and their importance to human nutrition. *J. Sci Food Agr*. **2000**, *80*(7), 939-966.
9. Lazzez, A., et al. Influence of Olive Maturity Stage and Geographical Origin on Some Minor Components in Virgin Olive Oil of the Chemlali Variety. *J. Agric. Food Chem.* **2008**, *56*(3), 982-988.
10. Cercaci L.; Rodriguez M.T. and Lercker G. Solid-phase extraction-thin-layer chromatography-gas chromatography method for the detection of hazelnut oil in olive oils by determination of esterified sterols. *J. Chromatogr. A*. **2003**, *985*, 211-220.
11. Somova, L.I.; Shode F.O.; Ramnanan P. and Nadar A.. Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from Olea europaea, subspecies africana leaves. *J.Ethnopharmacol.* **2003**, *84*(2-3), 299-305.
12. 1991, E.U.C., Regulation EEC/2568/91 on the characteristics of olive and olive pomace oils and their analytical methods. . *J. Eur. Commu*. **1991**. *L 248*,1-82.

13. Paganuzzi, V. Influence of origin and conservation state on the triterpenic dialcohols relative content of untreated olive oils III: pressed oils from Greece. *Fette Seifen Anstr.*. **2006**, *84*, 115-120.
14. Dobarganes, M.C. and Márquez-Ruiz, M. Physical Characteristics: Formation and Analysis of Oxidized Monomeric, Dimeric and Higher Oligomeric Triglycerides. Deep Frying Chemistry, Nutrition and Practical Applications, edition no 2.; Keys, AOAC press Eds.; Publisher: USA, 1996, pp.3-33.
15. Abidi, S.L., Chromatographic analysis of plant sterols in foods and vegetable oils. *J. Chromatogr. A*. **2001**, *935*(1-2), 173-201.
16. Dutta, P.C. and L. Normén, Capillary column gas-liquid chromatographic separation of [Delta]-5-unsaturated and saturated phytosterols. *J. Chromatogr. A.*. **1998**, *816*(2), 177-184.
17. Sánchez Ávila, N.; Priego Capote F., and. Luque de Castro M.D., Ultrasound-assisted extraction and silylation prior to gas chromatography-mass spectrometry for the characterization of the triterpenic fraction in olive leaves. *J Chromatogr. A*. **2007**,*1165*(1-2),158-165.
18. Toivo, J., et al. Determination of Sterols in Foods: Recovery of Free, Esterified, and Glycosidic Sterols. *J.Food Composition and Analysis*. **2001**, *14*(6),631-643.
19. Zarrouk, W.; Carrasco-Pancorbo A.; Zarrouk M.; Segura-Carretero A. and Fernández-Gutiérrez A. Multi-component analysis (sterols, tocopherols and triterpenic dialcohols) of the unsaponifiable fraction of vegetable oils by liquid chromatography-atmospheric pressure chemical ionization-ion trap mass spectrometry. *Talanta*. **2009**, *80*(2),924-934.
20. Jiang, B.; Kronenberg F.; Nuntanakorn P.; Qiu MH. and Kennelly E.J. Evaluation of the Botanical Authenticity and Phytochemical Profile of Black Cohosh Products by High-Performance Liquid Chromatography with Selected Ion Monitoring Liquid Chromatography−Mass Spectrometry. *J. Agric. Food Chem.* **2006**, *54*(9),3242-3253.
21. Mijangos Ricardez O.F.; Luque de.Castro, M.D.; Ruiz-Jiménez, J., Lagunez-Rivera L. Fast ultrasound-assisted extraction of polar (phenols) and nonpolar (lipids) fractions in heterotheca inuloides. *In press Phytochem. Analysis.*
22. Orozco-Solano, M.; Ruiz-Jiménez J. and Luque de Castro M.D. Ultrasound-assisted extraction and derivatization of sterols and fatty alcohols from olive leaves and drupes prior to determination by gas chromatography-tandem mass spectrometry. *J. Chromatogr. A*. **2010**, *1217*(8), 1227-1235.
23. Girón, M.V.; Ruiz-Jiménez J.; and Luque de Castro M.D. Dependence of Fatty-Acid Composition of Edible Oils on Their Enrichment in Olive Phenols. *J. Agric. Food Chem*. **2009**, *57*(7), 2797-2802.

CAPÍTULO 5

Influencia del proceso de fritura simulada en la fracción antioxidante de aceites vegetales tras el enriquecimiento con extractos de alperujo

Influence of simulated deep frying processes on the antioxidant fraction of vegetable oils with or without enrichment with natural or artificial antioxidants

M.I. Orozco-Solano, F. Priego-Capote, M.D. Luque de Castro*

Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain

Andalusian Institute of Fine Chemistry and Nanotechnology (IAQFN), University of Córdoba, E-14071, Córdoba, Spain

Journal of Agricultural and Food Chemistry, 59(18) (2011) 9806-14

Influence of simulated deep frying processes on the antioxidant fraction of vegetable oils with or without enrichment with natural or artificial antioxidants

M.I. Orozco-Solano, F. Priego-Capote*, M.D. Luque de Castro
Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain
Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain

Andalusian Institute of Fine Chemistry and Nanotechnology (IAQFN), University of Córdoba, E-14071, Córdoba, Spain

Abstract: The stability of the antioxidant fraction in edible vegetable oils has been evaluated during a simulated deep frying process at 180 ºC. Four edible oils (*i.e.* extra-virgin olive oil with a 400 μg/mL overall content in naturally existing phenols —oil *a*— , refined high-oleic sunflower oil without natural content of these compounds but enriched with hydrophilic antioxidants isolated from olive pomace after oil extraction —oil *b*—, refined high-oleic sunflower oil enriched with the artificial antioxidant dimethylsiloxane —oil *c*— and high-oleic sunflower oil without enrichment —oil *d*—) were subject to deep heating consisting of 20 cycles at 180 ºC for 5 min each to simulate a frying process. An oil aliquot was sampled after each heating cycle to study the influence of frying process on the antioxidant fraction composed of hydrophilic and lipophilic antioxidants such as phenols and tocopherols, respectively. The decomposition curves for each group of compounds caused by the influence of deep frying were studied to compare the resistance to oxidation of each prepared oil, taking oils *a* and *d* as reference. Different oxidation mechanisms of hydrophilic and lipophilic antioxidants were observed with a crucial role of dimethylsiloxane on the stability of the non-polar antioxidants.

***KEYWORDS*:** Edible oil enrichment, olive phenols, tocopherols, olive pomace, simulated deep frying, hydrophilic and lipophilic antioxidants.

INTRODUCTION

Physicochemical changes occurring during the frying process dramatically deteriorate the quality of oils and fats.[1] Physical changes mainly result in increased viscosity and foaming, colour changes and decreased smoke-point. Conjunctly, main chemical changes involve increased concentrations of free fatty acids and polar components as well as decreased levels of unsaturation, flavour quality and nutritive value by degradation of minor compounds affecting the organoleptic and nutraceutical properties.[2]

It is widely known that oils and fats deteriorate during storage in an oxidizing atmosphere by a process termed autoxidation. When fats or oils are heated in such atmosphere up to relatively high temperature, autoxidation is not only accelerated, but also followed by oxypolymerization and thermal-oxidative degradation. This overall process is referred to as thermal-oxidative decomposition,[3] which involves a set of physicochemical reactions such as thermoxidation, hydrolysis, polymerization, isomerization or cyclization. As a result of thermal-oxidative decomposition, monomeric, polymeric, primary and secondary oxidative compounds are formed, thereby affecting the quality of oil and fried product.[4] The reactions occurring during deep-fat frying depend on factors such as temperature, heating time, type of fryer, original quality of frying oil such as unsaturated fatty acid contents, food materials subjected to frying, oxygen concentration, humidity and, mainly, type and concentration of antioxidants. Antioxidants naturally present in oils encompass hydrophilic and lipophilic compounds. Thus, tocopherols are lipophilic compounds present in vegetable oils, including refined oils such as linseed and sunflower oils. On the contrary, hydrophilic antioxidants are exclusive of non-refined oils such as olive oil that is consumed as obtained. Among hydrophilic antioxidants, the most important are phenolic compounds, some of which (*e.g.* hydrixytyrosol, tyrosol, secoiridoids and their conjugated forms) are characteristic of olive oil. These compounds are primary antioxidants which either delay the oxidation step by reacting with free radicals or inhibit the propagation step by reacting with peroxy or alkoxy radicals avoiding the degradation of vegetable oils.[5]

Taking into account the relevance of antioxidants in oil stability, prevention or minimization of oil degradation during the frying process can be achieved by enrichment with either natural or artificial antioxidants. However, the use of synthetic oxidation inhibitors such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisol (BHA) can cause harmful effect on humans.[6] BHA has been shown to cause lesions in the rat forestomach. Other studies have shown that BHT may cause internal and external hemorrhages at high doses that is severe enough to cause death in some strains of mice and guinea pig.[7] According to the European Official Bulletin (Order of 24 August 2007), the maximum dose allowed of artificial additives such as BHT and BHA in frying oil and fats is 200 mg/kg of oil. With these premises, the use of these artificial additives has decreased because it is suspected that they may act as promoters of carcinogenesis. In this sense, enrichment of edible oils with natural antioxidants becomes of great interest.

Nowadays, the main source of natural antioxidants are vegetable plants.[8] In this sense, *Olea europaeae* has been studied as a promising source of powerful hydrophilic antioxidants, mainly taking advantage from agricultural residues such as leaves –after pruning- and also from the semisolid residue from olive oil production (*viz.* alperujo or olive pomace).[9] In fact, phenolic compounds have been found in olive pomace at concentrations 100 times higher than in olive oil, a fact explained by the polar nature of the semisolid residue versus the non-polar character of olive oil.[10]

The antioxidant profile of vegetable oils also includes lipophilic compounds such as tocopherols, which are not completely degraded in the refining procedure. Their high content in most vegetable oils[11] is a remarkable factor to be considered in the frying process. The aim of the present research was to evaluate the influence of a simulated frying process on the antioxidant profile of different edible oils with an intrinsic or added content of natural antioxidants or added artificial oxidation inhibitors. Both phenols and tocopherols were determined as representatives of hydrophilic and lipophilic antioxidants.

MATERIALS AND METHODS

Samples. Vegetable edible oils such as extra-virgin and refined olive oils, and high-oleic sunflower oil were provided by Koipesol (SOS Cuétara S.A, Madrid).

Reagents. HPLC grade ethanol, methanol and acetonitrile from Panreac (Barcelona, Spain) were used as solvents for extraction and chromatographic separation. Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system was used to prepare mobile chromatographic phases. Sodium carbonate, Folin Ciocalteu (F–C) reagent and orthophosphoric acid were also from Panreac.

HPLC grade n-hexane and heptane to dissolve oils prior to the F–C test and analysis of fatty acid methyl esters (FAMEs) were provided by Panreac. Anhydrous sodium sulphate from Sigma–Aldrich (Steinheim, Germany) was used in the derivatization step as drying agent for the non polar phase. Methanolic solution of 0.4 M KOH used in the derivatization of fatty acids, were purchased from Panreac.

Representative phenolic compounds present in olive oil and pomace (*i.e.* hydroxytyrosol, tyrosol, oleuropein, luteolin, apigenin, apigenin-7-glucoside, vanillic acid, *p*- and *o*-coumaric acids, ferulic acid, caffeic acid and syringic acid, this last used as internal standard, IS) were from Extrasynthese (Genay, France) and Sigma (St. Louis, USA). The stock standard solution of each phenol was prepared at 1000 μg/ml by dissolving 10 mg each phenol in 10 mL methanol. The α and δ-tocopherol were from Sigma. The stock standard solutions of tocopherols were prepared at 1000 μg/mL by dissolving 10 mg each tocopherol in 10 mL 2-propanol. Multistandard solutions were prepared by mixing the appropriate volume of each stock solution in methanol or 2-propanol. All solutions were stored in the dark at −20 ºC in glass vials until use. All fatty acid analytical-standards, supplied as FAMEs, to prepare the multistandard were from Sigma–Aldrich. Individual stock standard solutions were prepared by dilution of each compound in *n*-hexane (from C12 to C20) and heptane (C22:0). The stock standard solutions were stored –20 ºC.

Apparatus and Instruments. A mechanical electrical stirrer MS2 minishaker from Ika (Wilmington, USA) was used to favor the transference of hydrophilic phenols from the extract to sunflower oil.

An Agilent 1100 liquid chromatograph (Pittsburg, PA, USA), consisting of a quaternary pump, a vacuum degasser, a diode array detector (DAD) and a Reodyne

7725 high pressure injection valve (20 µL injection loop), was used for the analysis of the target compounds. The analytical column was an Inerstil ODS-2 (250×4.6 mm i.d. 5 µm) from GL Sciences Inc (Tokio, Japan). A Varian CP-3900 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable-temperature injector and coupled to a Saturn 2100 ion-trap mass-spectrometer (Sunnyvalley, TX, USA) was used to obtain the fatty-acid profiles. The chromatograph was furnished with a Varian CP 8400 autosampler and an SP^{TM} - 2380 fused silica capillary column (60 m×0.25 mm i.d., 0.2 µm film thickness) from Supelco (Bellefonte, PA, USA).

An Agilent 8453E UV–VIS spectrophotometer was used for determination of total phenols concentration by the F–C method.

A stainless deep fryer (Fagor F-206, Barcelona, Spain) was used for oils heating (simulation of the frying process) at 180 ºC.

Determination of the Fatty Acid Profile. The fatty acids in the oil were derivatized to more volatile compounds (FAMEs) for proper individual separation by gas chromatography. The IOC method for FAMEs preparation was used.[12]

The individual separation of FAMEs was carried out by GC, and then they were detected and quantified by MS using the GC–MS method developed by Sánchez-Avila *et al.*[13] modified in the injection step. Briefly, the injection volume was reduced to 1 µL, injected in splitless mode and the injector temperature was 250 ºC maintained during the whole run.

Preparation of Edible Oils Enriched with Antioxidants. Four edible oils were prepared for this study. Different extra-virgin olive oils with an intrinsic content of antioxidants were mixed up to a total concentration of phenolic antioxidants of 400 µg/mL, expressed as µg/mL of caffeic acid by the Folin-Ciocalteu test. High-oleic sunflower oil was enriched with two types of antioxidants: a synthetic oxidation inhibitor, dimethylpolysiloxane, at 400 µg/mL; and a natural extract from olive pomace in a protocol similar to that developed by Girón *et al.*.[14] For enrichment with natural antioxidants, 20 g of olive pomace was extracted with 100 mL ethanol. The resulting extract was evaporated to obtain a concentrated extract, which was put into contact with high-oleic sunflower oil and vigorously shaken. This oil was enriched up to a total

phenol concentration of 400 µg/mL, also expressed as caffeic acid. High-oleic sunflower oil without enrichment was used as reference.

Simulated Frying Procedure. Two liters of the target oil was placed in a stainless-steel deep fryer and subjected to 20 cycles at 180 ± 5 ºC for 5 min (total heating time: 100 minutes). Three-ml aliquots from the target oil were removed after each heating period and stored at –20 ºC until analysis.

Extraction of the Hydrophilic and Lipophilic Antioxidants from Oil. Aliquots of *ca*. 2-g of enriched or pure oils were dissolved with 2-ml hexane and shaken for 30 min with 20-mL methanol. The methanolic phase, which contained the antioxidants, was isolated by centrifugation, evaporated for preconcentration and stored at –20 ºC for subsequent analysis.

Determination of the Total Phenols Content. After extraction, the total concentration of phenols was estimated by the Folin–Ciocalteu method.[15] Briefly, 0.1-ml methanolic extract was, in this order, mixed with 2-mL water, 0.2-mL F–C reagent and 0.6-mL 20% (w/v) Na_2CO_3 aqueous solution. The resulting mixture was diluted with water to obtain a 5-mL final volume and then incubated for 30 min in a water bath at 50 ºC. The reaction product was monitored at 725 nm. Caffeic acid was used as standard for calibration.

LC–DAD Separation–Quantitation of Hydrophilic and Lipophilic Antioxidants. The applied method was that proposed by the International Oleic Council (IOC) for the individual determination of phenolic compounds in olive oil.[16] Additionally, tocopherols were eluted at the end of the chromatographic test with 100% methanol.

Statistical Analysis. Statgraphics Centurion XV, Statpoint Technologies, Inc. (Warrenton, VA, USA) was used as statistical software with this purpose. Additionally, statistical analysis to compare the levels of phenolic compounds for the different heating cycles was carried out by analysis of variance. In all cases, the confidence intervals were set at 95% and, thus, a *p*-value of 0.05.

RESULTS AND DISCUSSION

Characterization of the Method for the Determination of Antioxidants in Oils. The method proposed by the IOC is based on a chromatographic separation involving a solvent gradient from an acid aqueous phase to pure methanol for elution of less polar organic compounds. This method does not include the determination of tocopherols that, despite their non-polar character, can be eluted with pure methanol after flavonoids such as apigenin and luteolin. For this reason, tocopherols can be determined with phenols at 280 nm.

Identification of each phenol was based on comparison of the retention time and UV spectrum with those of the corresponding standards. Hydroxytyrosol acetate was identified by comparing its retention factor (retention time of each analyte/retention time of IS) and UV spectra with that reported by the IOC method. Secoiridoid derivatives were similarly identified. **Table 1** shows the maximum absorbance wavelength as well as the IOC and experimental retention factors for each analyte. Tocopherols were identified with individual standards of α and δ-tocopherol. **Figure 1.A and B,** shows chromatograms at quantification wavelength from the antioxidant fraction isolated from extra-virgin olive oil and sunflower oil enriched with extract from olive pomace prior to simulated frying.

Calibration plots were run by using the peak area of each analyte/peak area IS as a function of the concentration of each compound. The regression coefficients ranged between 0.995 and 0.998, as **Table 1** shows. Compounds with no calibration standards were quantified by the calibration curve of the phenol with a more similar structure. Thus, hydroxytyrosol acetate was quantified by the hydroxytyrosol calibration curve, while secoiridoid derivatives were overally quantified by the oleuropein calibration curve. Syringic acid, not present in any of the target oils, was used as internal standard.

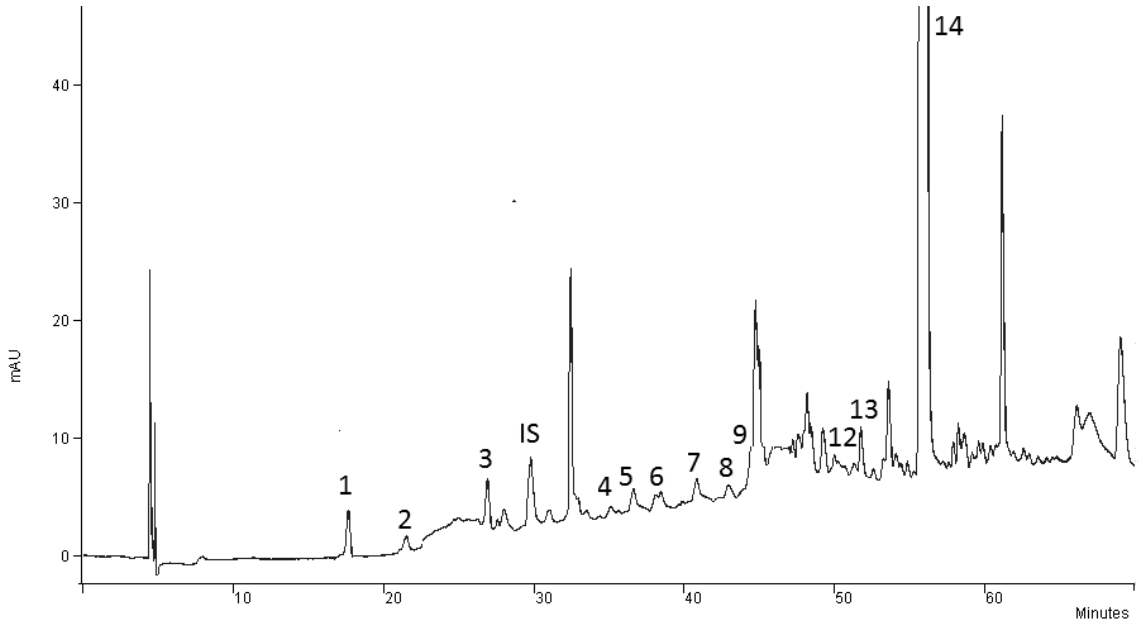
The characterization of the method was completed by calculation of detection (LOD) and quantification (LOQ) limits. The LOD for each analyte was expressed as the concentration of analyte which gives a signal 3σ above the mean blank signal (where σ is the standard deviation of the blank signal). The LODs ranged between 0.03 and 0.12 μg/mL for all the analytes. The limits of quantification (LOQs), expressed as the concentrations of analytes which gave a signal 10σ the mean blank signal, were from 0.10 to 0.40 μg/mL (**Table 1**).

Table 1. Limits of deection (LODs), limits of quantitation (LOQs), monitoring wavelength and experimental and theoretical response factors of the phenols and tocopherols found.

	Compound	Calibration curve*	R^2	Linear range	LOD (µg/ml)	LOQ (µg/ml)	Wavelength (nm)	Experimental factor	Theoretical factor
1.	Hydroxytyrosol	Y=0.046.(3.22)+0.0087.03(0.43)	0.996	LOQ–250	0.03	0.08	280	0.55	0.62
2.	Tyrosol	Y=0.0329(26..35)+0.0359(0.55)	0.996	LOQ–250	0.06	0.11	280	0.74	0.80
3.	Vanillic acid	Y=0.085(32.21.)+0.0902(0.86)	0.995	LOQ–250	0.03	0.21	260	0.94	0.96
5.	*p*-Coumaric acid	Y=0.2795(34.12)+0.2991(0.62)	0.995	LOQ–250	0.05	0.35	325	1.02	1.10
4.	Hydroxytyrosol acetate	As hydroxytyrosol			0.03	0.08	280	1.17	1.21
6.	Ferulic acid	Y=0.2971(48.23)+0.322(0.32)	0.995	LOQ–250	0.12	0.28	325	1.25	1.26
7.	*o*-Coumaric acid	Y=0.2526(43.55)+0.0316(0.39)	0.996	LOQ–250	0.09	0.20	325	1.42	1.31
8.	Secoridoid derivative (1)	As oleuropein			0.08	0.41	280	1.43	1.48
9.	Secoridoid derivative (2)	As oleuropein			0.08	0.41	280	1.46	1.74
11.	Oleuropein, secoridoid derivative (3)	Y=0.0603(28.42)+0.0337(0.9953)	0.998	LOQ–250	0.08	0.41	280	1.53	1.87
12.	Apigenin-7-glucoside	Y=0.0789(43.12)+0.0587(0.64)	0.998	LOQ–250	0.03	0.18	280	1.74	1.79
10.	Luteolin	Y=0.1322(32.55)+0.0099(0.75)	0.998	LOQ–250	0.11	0.12	325	1.83	1.88
14.	Apigenin	As apigenin-7-glucoside			0.03	0.18	280	1.96	1.95
15.	α-tocopherol	Y=0.0236.(1.94)+0.0188(0.71)	0.998	LOQ–500	0.05	0.12	280	2.03	1.98
16.	δ-tocopherol	Y=0.0721.(4.10)+0.0123(0.34)	0.998	LOQ–500	0.05	0.32	280	2.06	2.01

*Values in brackets

A)



B)

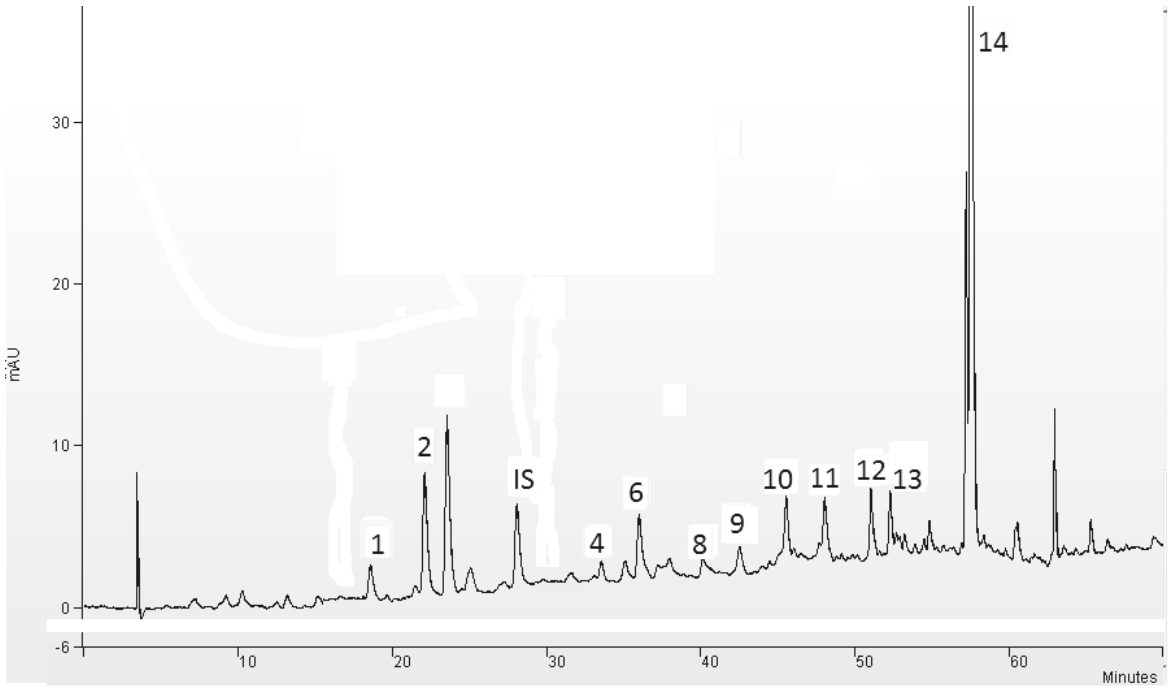


Figure 1. *Chromatograms provided by analysis of the antioxidant extracts from extra virgin olive oil (A) and sunflower oil enriched with extract from olive pomace (B) at λ= 280 nm. Peak identification: (A) -1, hydroxytyrosol; 2, tyrosol; IS (internal standard at 280 nm, syringic acid); 3, vanillic acid; 4, p-cumaric acid; 5, hydroxytyrosol acetate; 6, ferulic acid; 7, o-cumaric acid; 8, 9 and 10, secoiridoid derivatives; 11, apigenin-7-glucoside; 12, luteoline; 13, apigenin; 14, α-tocopherol.*

Evaluation of the Antioxidant Fraction in Edible Oils prior to Simulated Frying. The steps for extraction and individual separation–quantification of antioxidant compounds described under "Experimental" were applied to the target oils prior to and during the simulated heating process. The first step was the characterization of pure and enriched oils before simulated deep frying to compare their content in hydrophilic and lipophilic antioxidants. **Table 2** exposes the concentrations of the monitored antioxidants in the oils under study. As confirmed by **Figure 2.A** and **B**, hydrophilic antioxidants were not present in high-oleic sunflower oil and that enriched with dimethylsiloxane. This fact can be ascribed to the refining process by which the oils are exposed to high temperature (above 180 ºC) for short intervals for deodorization. These results were confirmed by the F–C test that provided null concentration of total phenols in both enriched and non-enriched sunflower oil.

Following with hydrophilic antioxidants, extra-virgin olive oil and sunflower oil enriched with extract from olive pomace, obviously, provided high concentrations of phenolic compounds (see **Figures 1.A** and **B**), which could be an indicator of a good antioxidant capacity. In previous studies, the transference of phenolic compounds from olive pomace extracts to vegetable edible oils with different composition of fatty acids was studied.[12,17,18] This research showed that the unsaturation level in the oil (content of mono- and polyunsaturated fatty acids) favoured its enrichment in phenolic antioxidants. Attending to the fatty-acid composition of sunflower oil, it seems to be suited for enrichment with olive phenols. In this research, high-oleic sunflower oil was selected for enrichment because of its fatty-acid profile, which is similar to that reported by extra-virgin olive oil. **Table 3** shows the fatty-acid composition of the target oils revealing this fact. As can be seen in **Tables 2 and 3**, olive oil and high-oleic sunflower oil enriched with hydrophilic phenols presented a similar fatty-acid profile, but also a similar composition in hydrophilic antioxidants as a result of the enrichment process. Compounds such as hydroxytyrosol, tyrosol, ferulic acid, vanillic acid, *o*- and *p*-coumaric acid, hydroxytyrosol acetate, luteolin and apigenin were found as simple phenols in both oils. Significant differences were found only in the concentration of secoiridoids in the enriched refined olive oil as compared to extra-virgin olive oil. Secoiridoid derivatives are formed by conjugation of hydroxytyrosol or tyrosol with eleanolic acid[19] and, therefore, the high concentration of them in olive oil could explain its resistance against thermal oxidation with respect to other vegetable oils such as

sunflower oil. Additionally, secoiridoid derivatives are direct responsible for the organoleptic properties of the oils.[20] As can be seen in **Table 2**, sunflower enriched oil presented a low concentration of secoiridoids as compared to extra-virgin olive oil (2.98 versus 22.85 µg/mL) before simulated frying. It is worth emphasizing the similar levels of hydroxytyrosol and tyrosol in olive oil and sunflower oil enriched with pomace extract. Taking into account these results, it would be foreseeable to predict the good stability of these two oils in the simulated frying process due to the presence of phenolic constituents with a reported high antioxidant activity.

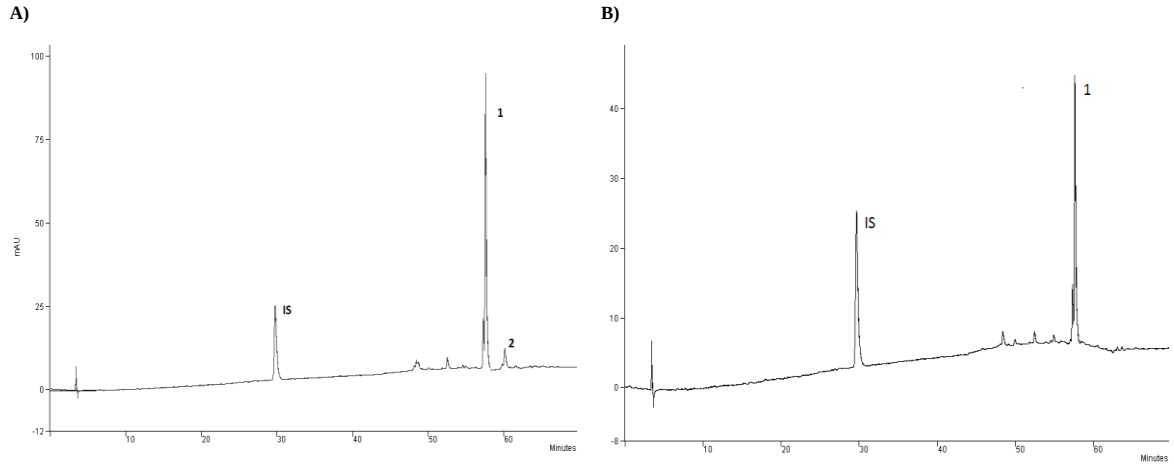


Figure 2. *Chromatograms provided by analysis of the antioxidant extracts from sunflower oil (A) and sunflower oil enriched with artificial antioxidant, DMSO (B) at λ= 280 nm. Peak identification: IS (internal standard, syringic acid; 1, α-tocopherol and 2, δ-tocopherol*

The initial content of tocopherols was determined by the method exposed in the experimental section. **Table 4** shows the concentration of α- and δ-tocopherol present in the four target edible oils before heating. The three types of high-oleic sunflower oil (namely, without enrichment and enriched with natural antioxidants and with the artificial oxidation inhibitor) presented higher concentration of α-tocopherol than extra-virgin olive oil (in the oils under study in the range 489.1–325.7 µg/mL vs 153.4 µg/mL of extra-virgin olive oil). δ-Tocopherol was only detected in high-oleic sunflower oil with a concentration of 15.22 µg/mL. Despite the refining process, the concentration of lipophilic antioxidants in sunflower oil is still above the levels found in olive oil.

Table 2. Concentrations, µg/ml, of phenols and tocopherols in the oils prior to heating

	Sunflower oil	High-oleic sunflower oil + artificial antioxidant	High-oleic sunflower oil + natural antioxidant	Olive oil
Hydroxytyrosol	<LOD	<LOD	13.51	12.38
Tyrosol	<LOD	<LOD	11.76	7.98
Vanillic acid	<LOD	<LOD	4.61	<LOD
Vanillin	<LOD	<LOD	<LOD	<LOD
p-Coumaric acid	<LOD	<LOD	1.96	1.28-
Ferulic acid	<LOD	<LOD	1.69	1.13
Hydroxytyrosol acetate	<LOD	<LOD	1.82	<LOD
o-Coumaric acid	<LOD	<LOD	1.12	<LOD
Secoridoids derivatives	<LOD	<LOD	2.98	22.85
Apigenin 7-glucoside	<LOD	<LOD	<LOD	1.17
Luteolin	<LOD	<LOD	2.43	3.01
Apigenin	<LOD	<LOD	2.17	1.64
α-tocopherol	489.12	325.76	439.32	153.43
δ-tocopherol	15.22	<LOD	<LOD	<LOD

Evaluation of the Influence of the Simulated Frying Process on the Phenolic Fraction of Target Vegetable Oils. Before analysis of the results from this study, it is important to review the hypothesis in the literature that compares the efficiency of hydrophilic and lipophilic antioxidants against thermal degradation. The presence of hydrophilic compounds in virgin olive oil and their high antioxidant activity can be explained by the so-called "polar paradox",[21] which establishes that "polar antioxidants are more effective in non-polar lipids whereas non-polar antioxidants are more active in polar-lipid emulsions". This means that hydrophilic antioxidants protect more effectively against oxidation than lipophilic antioxidants, because the latter remain dissolved in the oil while phenolic compounds are located at the air–oil interface.[22] The evolution of degradation caused for the simulated frying process in the antioxidant fraction was studied. With this purpose, the concentration of each compound was determined in the aliquots taken after each heating cycle. The thermal degradation of hydrophilic phenols in olive oil and sunflower oil enriched with olive pomace extract subjected to heating is shown in **Figure 3.A and B**. As can be seen, the degradation trends observed for the monitored phenols were quite similar for both oils with possibility to discriminate between two groups of compounds. The first group is that composed by tyrosol, hydroxytyrosol and secoiridoid derivatives because of their

antioxidant capacity. Thus, tyrosol was quite stable during the complete heating process with a concentration decrease, after 20 cycles, below 25% for both oils. This behaviour was in contrast with that observed for hydroxytyrosol, the concentration of which experienced a critical decrease of 80 and 100% for sunflower oil enriched with natural antioxidants and extra-virgin olive oil, respectively. Additionally, the decomposition curve for hydroxytyrosol was clearly more pronounced in extra-virgin olive oil, in which this phenol was below its limit after 8 heating cycles. A similar situation was observed for secoiridoid derivatives, which were rapidly decomposed in the first heating cycles being completely degraded after 16 heating cycles. In contraposition, secoiridoids were not statistically affected (95% confidence level) in enriched sunflower oil during the heating process. Therefore, it is clear that these conjugates are indicators of the frying process for olive oil as well as other vegetable oils enriched with phenols from olive-tree materials. Thus, in this case, the resistence to oxidation by a heating process is superior for sunflower oil enriched with natural phenolic antioxidants than for extra-virgin olive oil. In the latter, the effect of deep frying on the oil profile of hydrophilic antioxidants is particularly relevant with a direct incidence on the organoleptic properties of the resulting product and self-protective antioxidant capacity.

The other group, formed by the rest of monitored phenols, shared a behaviuor similar to tyrosol. In sunflower oil enriched with pomace extract, a reduction between 3.5 and 40% was observed for vanillic acid, *p*-coumaric acid, hydroxytyrosol acetate, ferulic acid, luteolin and apigenin. In the olive oil (see **Figure 3**.**A** and **B**), the final concentration of phenols such as *p*-coumaric acid, ferulic acid, apigenin-7-glucoside and apigenin as compared to their initial contents in oils was relatively constant with estimated degradation between 9.4 and 20%.

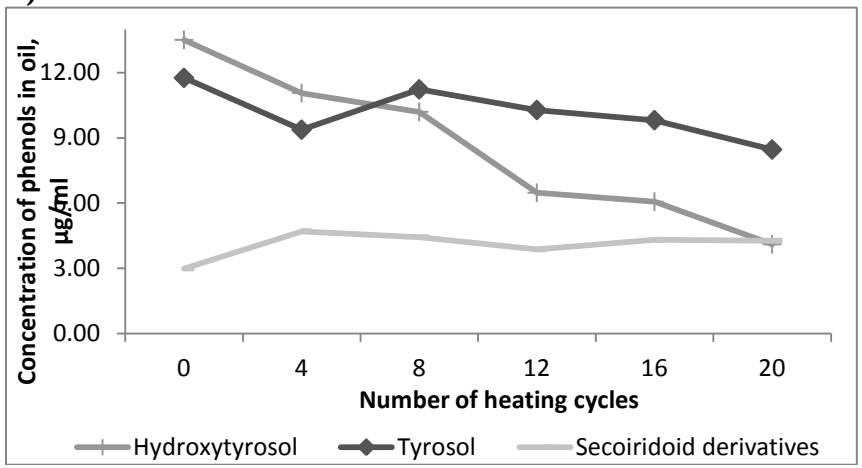
Evaluation of the Influence of the Simulated Frying Process on Tocopherols in Target Vegetable Oils. Tocopherols are important lipophilic antioxidants that are not completely decomposed during refining process of vegetable seed oils. For this reason, these compounds gain a special interest to compare the oxidative stability of oils and, particularly, taking into account their content in sunflower oil, which is above that in extra-virgin olive oil. The evolution of α-tocopherol in the four target oils during deep heating is shown in **Figure 3.C**. As shows, sunflower oil without antioxidants and that enriched with natural antioxidants presented an identical behaviour. A statistically significant degradation of α-tocopherol was observed just after 4 heating cycles. The

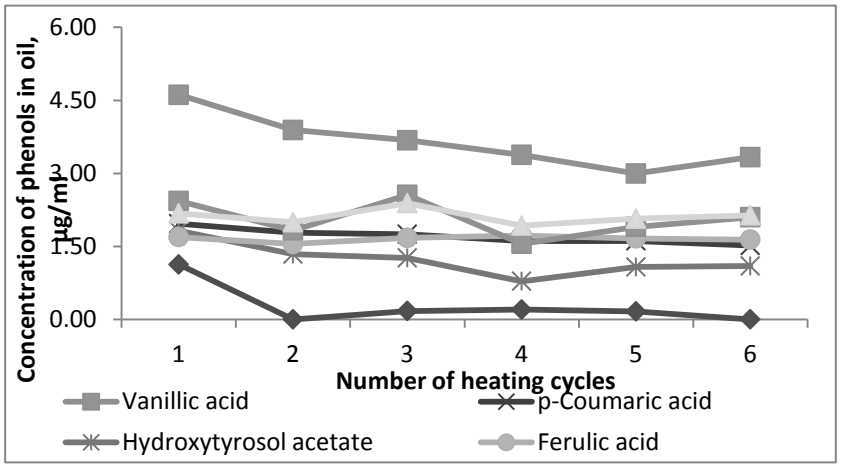
level of α-tocopherol felt down around 50% after 8 heating cycles and 80% (concentrations below 70 µg/mL) after 16 heating cycles, when its concentration levelled off for the following heating cycles.

Table 3. Esterified fatty acid profile in edible oils determined before the deep heating procedure. Concentrations are expressed as percentage (%).

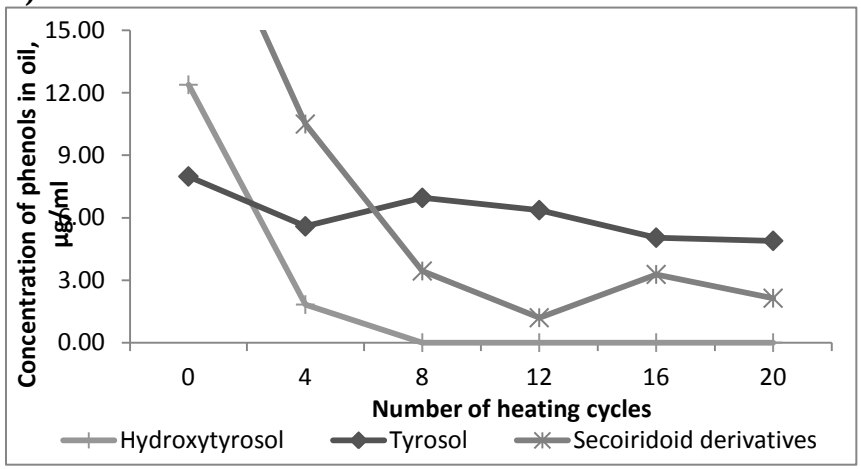
	Sunflower oil	**High-oleic sunflower oil +artificial antioxidant**	**High-oleic sunflower oil +natural antioxidant**	**Olive oil**
	EFA	**EFA**	**EFA**	**EFA**
c12	0.00	0.00	0.00	0.00
c14	0.07	0.07	0.10	0.03
c16:0	8.47	9.39	9.63	14.50
c16:1 n7 n9	0.06	0.16	0.15	0.71
c17	0.45	1.06	0.37	0.52
c17:1 n10	0.00	0.00	0.00	0.10
c18	6.49	6.22	5.86	7.23
c18:1n9 t	0.00	0.00	0.00	0.00
c18:1n9	0.00	0.00	0.00	0.80
c18:1n7	26.70	57.26	65.92	53.44
c18:2 t9,t12	0.00	0.00	0.00	0.00
c18:2 c, t	0.25	0.02	0.15	0.00
c18:2 t, c	0.00	0.02	0.00	0.00
c18:2 c, c	56.03	22.84	14.62	13.34
c19:1	0.00	1.61	1.72	0.46
c18:3 c, t, c	0.00	0.04	0.12	0.06
c18:3 c, c, c	0.13	0.20	0.06	0.38
c22:0	0.93	0.95	0.84	0.00
c20:4n3	0.03	0.05	0.00	7.62
c22:2 n6	0.00	0.00	0.36	0.00
c20:5 n3	0.11	0.11	0.10	0.10
c22:5 n3	0.27	0.00	0.00	0.69

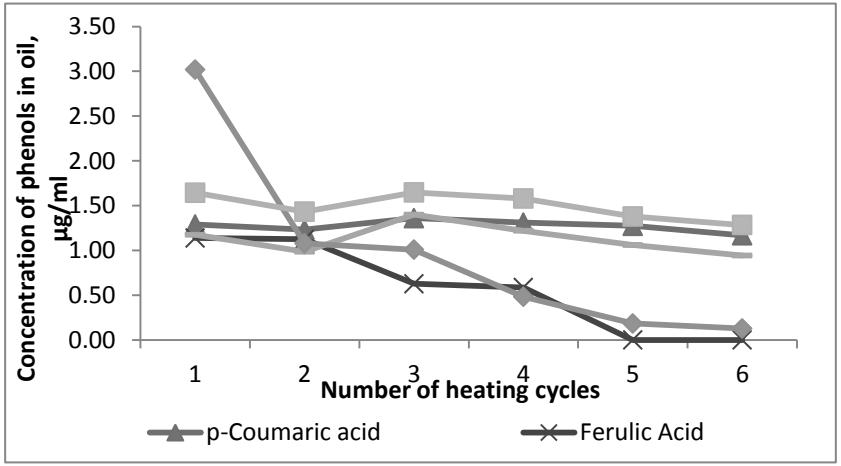
A)


Concentration of phenols in oil, µg/ml
12.00
9.00
6.00
3.00
0.00
0
4
8
12
16
20
Number of heating cycles
Hydroxytyrosol
Tyrosol
Secoiridoid derivatives


Concentration of phenols in oil, µg/ml
6.00
4.50
3.00
1.50
0.00
1
2
3
4
5
6
Number of heating cycles
Vanillic acid
p-Coumaric acid
Hydroxytyrosol acetate
Ferulic acid

B)


Concentration of phenols in oil, µg/ml
15.00
12.00
9.00
6.00
3.00
0.00
0
4
8
12
16
20
Number of heating cycles
Hydroxytyrosol
Tyrosol
Secoiridoid derivatives


Concentration of phenols in oil, µg/ml
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3
4
5
6
Number of heating cycles
p-Coumaric acid
Ferulic Acid

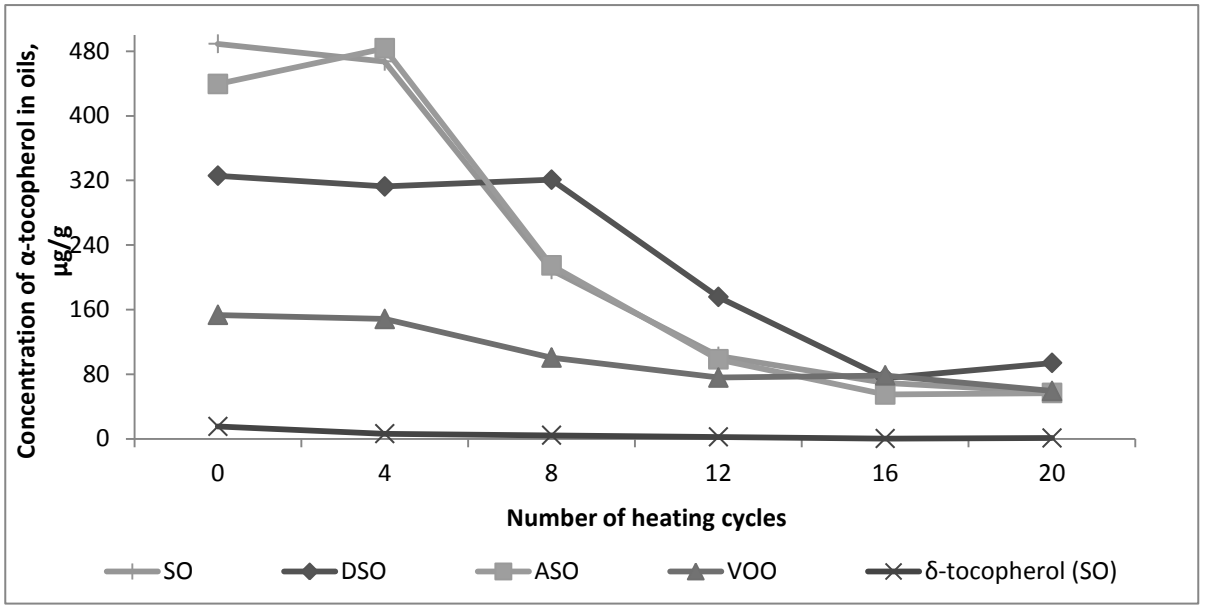


Figure 3. *Behaviour of biophenols and α- tocopherol during the twenty cycles in the simulated frying process at 180 ºC for: A) High-oleic sunflower oil enriched with alperujo extract, B) virgin olive oil and C) Four target oils*

Table 4. Evolution of tocopherols content during the heating procedure

	Sunflower oil		High-oleic sunflower oil +artificial antioxidant		High-oleic sunflower oil +natural antioxidant		Olive oil	
Cycles	**αT**	**δT**	**αT**	**δT**	**αT**	**δT**	**αT**	**δT**
0	489.12	15.22	325.76	nd	439.32	nd	153.43	nd
1	488.32	12.65	385.23	nd	423.87	nd	115.23	nd
2	473.12	7.93	351.67	nd	427.65	nd	146.32	nd
3	476.01	6.77	340.24	nd	421.21	nd	126.33	nd
4	467.33	6.23	312.43	nd	483.60	nd	148.33	nd
5	411.23	6.47	311.09	nd	306.12	nd	147.37	nd
6	223.40	5.58	267.65	nd	208.21	nd	173.45	nd
7	212.76	5.12	302.34	nd	215.65	nd	122.43	nd
8	209.12	4.32	320.76	nd	214.65	nd	100.53	nd
9	213.41	2.54	248.7	nd	223.45	nd	99.43	nd
10	183.23	2.11	210.36	nd	153.76	nd	98.46	nd
11	144.23	2.43	205.84	nd	76.43	nd	99.23	nd
12	102.36	2.31	175.63	nd	98.34	nd	75.76	nd
13	98.25	1.24	134.09	nd	120.43	nd	65.33	nd
14	77.54	1.19	107.23	nd	68.43	nd	78.87	nd
15	66.43	0.96	84.22	nd	85.23	nd	75.34	nd
16	69.23	0.23	75.34	nd	54.76	nd	78.45	nd
17	71.63	0.43	54.66	nd	52.43	nd	54.20	nd
18	68.23	0.78	68.85	nd	52.12	nd	62.12	nd
19	67.22	1.12	101.23	nd	50.76	nd	55.60	nd
20	58.23	0.87	93.96	nd	56.65	nd	59.12	nd

Concentrations are expressed as μg/mL
α T and δT: α- and δ-tocopherol
nd: no detected

In sunflower oil enriched with dimethylsiloxane, the degradation of α-tocopherol was delayed up to the 8th cycle, when its concentration was significantly decreased up to levels similar to those reported for the other two sunflower oils (*viz.* in the 16th cycle). Its concentration was also stabilized in heating cycles 16th to 20th. This could be justified if the non-polar character of dimethylsiloxane behaves its action as protective agent against decomposition of lipophilic antioxidants during deep heating. Furthermore, the comparison between the degradation curves of α-tocopherol for sunflower oil and sunflower oil prepared with olive pomace extract enables to conclude that the enrichment process with hydrophilic antioxidants does not exert any influence on the stability of lipophilic antioxidants. This is not the case with dimethylsiloxane, the retardant effect of which on oxidation and polymerization of frying oils is well-known;

the mechanism of action being based on the formation of a protective film at the oil–air interface that limits access to oxygen.[23]

Degradation of α-tocopherol was more attenuated in the case of extra-virgin olive oil despite the fact that its concentration was considerably lower than in sunflower oil. Thus, after 16 heating cycles the initial concentration of α-tocopherol was reduced to 50%, in contrast with the 75–85% decrease observed for sunflower oil versions for the same number of heating cycles. Apart from the predominant *α*- form, *δ*-tocopherol was also detected in pure sunflower oil but not in the other oils. *δ*-Tocopherol showed a decomposition curve similar to that of *α*-tocopherol but with a more pronounced degradation. Thus, the concentration of *δ*-tocopherol was reduced up to 85% of its initial concentration after 12 heating cycles.

ACKNOWLEDGMENTS

The Spanish Ministerio de Ciencia e Innovación (MICINN) and FEDER program are thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

LITERATURE CITED

1. Moreau, R.A.; Whitaker, B.D. and Hicks K.B. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Prog. Lipid. Res*. **2002**, *41*(6), 457–500.
2. Lalas, S. Quality of Frying Oil. In Advances in Deep-Fat Frying of Foods, Edition 1.; S. Sahin and S. Glum, Eds., Publisher: Ankara, Turkey, **2008**, CRC Press. pp. 57–80.
3. Kowalski B. Thermal-oxidative decomposition of edible oils and fats. DSC studies. *Thermochim. Acta*. **1991**, *184*(1), 49–57.
4. Andrikopoulos, N.K.; Kalogeropoulos, N.; Falirea A. and Barbagianni, M.N. Performance of virgin olive oil and vegetable shortening during domestic deep-frying and pan-frying of potatoes. *Int. J. Food Sci. Technol*. **2002,** *37*, 177–190.
5. Bera, D.; Lahiri, D. and Nag, A. Studies on a natural antioxidant for stabilization of edible oil and comparison with synthetic antioxidants. *J.Food Eng.* **2006,** *742* (4), 542–545.

6. Farag, R.S.; El-Baroty, G.S. and Amany, M.B. The influence of phenolic extracts obtained from the olive plants (cvs. Picual and Kronakii) on the stability of sunflower oil. *J. Food Sci. Technol*.. **2003,** *38*, 81–87.
7. Shahidi, F.; Janitha, PK and Wanasundara, PD. Phenolic antioxidants. Critical Reviews in *Food Sci. Nut.* **1992,** *32*, 67–103.
8. Moure, A.; Cruz, J.M.; Franco, D.; Domínguez, J.M.; Sineiro, J.; Domínguez, H.; Núñez, M.J.and Parajó, J.C. Natural antioxidants from residual sources. *Food Chem*. 2001, *72* (2), 145–17.
9. Benavente-García, O.; Castillo, J.; Lorente, J.; Ortuño, A., and Del Río, J.A. Antioxidant actvity of phenolics extracted from Olea Europaea. *J. Food Chem.* **2000,** *68*, 457–462.
10. Priego-Capote, F., Ruiz-Jiménez, J., and Luque de Castro, M.D. Fast separation and determination of phenolic compounds by capillary electrophoresis–diode array detection. Application to the characterisation of alperujo after ultrasound assisted extraction. *J. Chromatogr. A.* **2004**, *1045*, 239–246.
11. Desai, I.D.; Bhagavan, H.; Salkeld, R. and Dutra de Oliveira, J.E. Vitamin E content of crude and refined vegetable oils in Southern Brazil. *J. Food Compos. Anal*. **1998,** *1* (3), 231–238.
12. Identification and determination of fatty acids compounds in olive oil. International Olive Council.
13. Sánchez-Ávila, N.; Mata-Granados, J.M.; Ruiz-Jiménez, J. and Luque de Castro, M. Fast, sensitive and highly discriminant gas chromatography–mass spectrometry method for profiling analysis of fatty acids in serum. *J. Chromatogr. A.* **2009**, *1216* (40), 6864–6872.
14. Girón, M.V.; Ruiz-Jiménez J.; and Luque de Castro M.D. Dependence of Fatty-Acid Composition of Edible Oils on Their Enrichment in Olive Phenols. *J. Agric. Food Chem.* **2009**, *57*(7), 2797–2802.
15. Gutfinger, T. Polyphenols in olive oils. *J. Am. Oil Chem. Soc*. **1981**, *58*, 966–968.
16. Identification and determination of phenolic compounds in olive oil. International Olive Council. Resolution RES-4/94-/VO6.
17. Japón-Luján, R.; Luque de Castro, M. D. Liquid–liquid extraction for the enrichment of edible oils with biophenols from olive leaf extracts. *J. Agric. Food Chem*. **2008**, *56*, 2505–2511.

18. Bouaziz, M.; Feki, I.; Ayadi, M.; Jemai, H. and Sayadi, S. Stability of refined olive oil and olive-pomace oil added by phenolic compounds from olive leaves. *Eur. J. Lipid Sci. Technol*. **2010**, *112*, 894–905
19. Japón-Luján, R.; Luque de Castro, M. D. State-of-the-art and trends in the analysis of oleuropein and derivatives. *Trends Anal. Chem*. **2006,** *25* (5), 501-510.
20. Bendini, A.; Cerretani, L.; Carrasco-Pancorbo A.; Gómez, A.M.; Segura-Carretero A.; Fernández-Gutiérrez, A. and Lercker, G. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. an overview of the last decade.. *Molecules*. **2007**, *12*, 1679–1719.
21. Porter, W.L.; Black, E.D.; Gutfinger, T. Polyphenols in olive oils. *J. Am. Oil Chem. Soc*. **1981**, *58*(11), 966–968.
22. Frankel E**.**N. Antioxidants in lipid foods and their impact on food quality. *Food Chem*. **1996**, *57* (1), 51–55.
23. Jorge N.; Márquez-Ruiz, G.; Martín-Polvillo, M.; Ruiz-Mendez, M. V. and Dobarganes M. C. Influence of dimethylpolysiloxane addition to edible oils: dependence on the main variables of the frying process**.** *Grasas Aceites*. **1996**, *47*(1-2), 14-19.

CAPÍTULO 6

Análisis mediante CG–MS de esteroles, alcoholes alifáticos y triterpenos en muestras de suero de pacientes tras la ingesta de desayunos preparados con aceites comestibles sometidos a fritura simulada

GC–MS analysis of sterols, aliphatic alcohols and triterpenes in serum from individuals subjected to intervention breakfasts prepared with simulated frying edible oils

Mara Isabel Orozco-Solano, Feliciano Priego-Capote and

María Dolores Luque de Castro

[a]*Department of Analytical Chemistry, Fine Chemistry Andalusian Institute, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain*

[b]*Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain*

Enviado a Analytical and Bioanalytical Chemistry para su publicación

GC–MS Analysis of Sterols, Aliphatic Alcohols and Triterpenes in Serum from Individuals Subjected to Intervention Breakfasts Prepared with Simulated Frying Edible Oils

Mara Isabel Orozco-Solano, Feliciano Priego-Capote and
María Dolores Luque de Castro

Abstract Few analytical methods have been proposed to monitor minor compounds of the unsaponifiable fraction of vegetable edible oils in human biofluids. A method for targeted quantitative analysis of aliphatic alcohols, triterpenes and phytosterols in human serum is here proposed. Two endogenous sterols, cholesterol and α-cholestanol, were also monitored due to the influence of the three families of exogenous metabolites in the metabolism of cholesterol. The method has been applied to a cohort of obese individuals subjected to four intervention breakfasts prepared with edible oils after a simulated frying process. Edible oils used for this study were virgin olive oil, sunflower oil and high-oleic sunflower oil enriched with either an oxidation inhibitor (dimethylsiloxane) or an extract of phenolic compounds isolated from olive pomace. Serum saponification was accelerated by ultrasonic assistance and GC–MS in single ion monitoring (SIM) mode was used for determination of the target metabolites. Statistical analysis was based on multifactor ANOVA and on principal component analysis (PCA) to detect the contribution of both anthropometric features of the cohort selected and the type of intervention breakfast on the levels of the monitored compounds. The main discrimination according to the intervention breakfast was ascribed to endogenous

metabolites such as cholesterol and α-cholestanol but also to exogenous metabolites such as stigmastanol, octacosanol and erythrodiol.

KEYWORDS: Aliphatic alcohols · Phytosterols · Triterpenes · Vegetable oils · Natural antioxidants · Oxidation inhibitor · Simulated frying · GC–MS

Introduction

The intake of vegetable oils and dietary supplements is the main natural source of plant sterols, triterpenes and aliphatic alcohols in humans [1]. These compounds constitute a part of the non-polar unsaponifiable fraction present in vegetable edible oils and are endowed with protective effects against different pathologies such as metabolic disorders, certain types of cancer and cardiovascular diseases (anti-inflammatory [2], anti-bacterial [3], anti-oxidation [4,5] and anti-cancer properties [6].

Phytosterols, aliphatic alcohols and triterpenes can be detected in human tissues and fluids. The main constituents of the unsaponificable fraction in virgin olive oil (VOO) are sterols with a total content of 1.0–2.2 g Kg^{-1} of oil. Likewise, aliphatic alcohols, present in vegetable oils in lower amounts than sterols, are endowed with biological properties such as anticancer, antiviral, antifungal, and anti-HIV, and thus used in the treatment of various chronic diseases including diabetes and hypercholesterolemia [7-9]. Among other properties, short-chain compounds are used in cosmetics and food, and as industrial solvents. Also these compounds have been used as a purity parameter to detect olive oil frauds with pomace olive oil [10]; while some papers have reported that high amounts of triterpenes deteriorate organoleptic oil quality [11].

On the contrary, few studies have provided information on the distribution of triterpenic compounds in the plant in general and in VOO in particular, despite some of the biological properties of this oil (*viz.* anti-inflammatory [12], hepatoprotective , anti-tumour [13], anti-viral [14], anti-HIV [15], anti-microbial[15], anti-fungal[16], anti-diabetic[17], gastroprotective[18], and anti-hyperlipidemic [19] effects, antifeedant, and insecticidal activities [20]) are attributed to its unsaponifiable fraction. In addition, *in vivo* studies have demonstrated some benefits of this fraction in preventing hypertension [21]. Oleanolic acid, as well as uvaol and the maslinic acid derivative methyl maslinate have also shown to possess vasodepressor, cardiotonic, and antidysrhythmic properties

[22]; while oleanolic and maslinic acids are powerful pro-apoptotic agents in human colon cancer cells [23].

Bioavailability studies, of great interest in the clinical field, have shown that phytosterols are usually poorly absorbed in the intestine; therefore, only small amounts of them are detected in serum [24]. However, the intestinal absorption of plant sterols can be enhanced with marked effect on serum levels under phytosterolemia, an inborn error of sterol metabolism. Sitosterol (and also sitostanol) has been used therapeutically in diseases related with high intake of sterols in diet [25]. High intake of fat can cause variable effects on the level of serum cholesterol and on its metabolism as well as on the formation of other methyl sterols due to the function of the sterols as precursor [26]. Different studies have found that dietary phytosterols exert cardiovascular benefits due to their capability to regulate serum cholesterol levels in humans [27].

In the same way, long-chain aliphatic alcohols (LCFAs) are of clinical interest due to their prevention benefits against diabetes and hypercholesterolemia [28,29]. However, LCFAs metabolism is a matter of controversy as, due to the main structure of LCFAs, their hydrophobicity results in poor absorption, confirmed in both rats and monkeys using radiolabelled [^{14}C] octacosanol as well as by a study on octacosanol metabolism using LCFA supplements. Further research comparing the metabolism of LCFAs as supplements using therapeutic doses has helped to determine if mechanistic differences exist between the supplements under study [30].

Triterpenic dialcohols are also of great clinical interest thanks to their antioxidant properties [31,32,7]. The intake of these alcohols in VOO consumers is high, mainly of maslinic and oleanolic acids. It would be estimated that if the bioavailability of these compounds remains at 2.3%, as reported for triterpenoid 23-hydroxybethylinic acid, the intestinal epithelium is exposed to high concentrations of these compounds. Whether it is estimated that 30% of these compounds are not absorbed, remained unaltered in the intestinal tract and reach the colon, the concentration of maslinic and oleanolic acids in VOO consumers would be approximately 86 µM and 66 µM, respectively [33].

Taken into account the above non conclusive studies, it is of potential interest the development of analytical methods to be applied to clinical studies. Some of the global analytical methods in the literature for determination of sterols, aliphatic and triterpenic alcohols in biological samples (plasma, liver, adipose and small intestine)

require longer times for sample preparation [30, 37]. Steps such as sample homogenization and extraction use mixtures of polar and nonpolar solvents, being one of the most frequent 2:1 (v/v) chloroform–methanol mixture. Other steps involved in sample preparation are heating (for 10 min at 60 ºC), shaking and then centrifugation, followed by a saponification step using methanolic KOH before separation by liquid chromatography (LC), capillary zone electrophoresis (CZE), capillary electrochromatography (CEC) [34-36] or gas chromatography (GC) —previous derivatization to yield volatile trimethylsilyl derivatives— with subsequent quantification either by flame ionization (FID) or mass spectrometry (MS) detection [37,30].

The aim of the present research was to use a method previously developed by the authors and applied to the determination of these compounds in VOO [38] for their determination in serum. The final aim was to evaluate the influence of the intake of four independent breakfasts prepared with oils subjected to simulated deep frying on the profile of sterols, aliphatic alcohols and triterpenes in human serum. Gas chromatography with mass spectrometry detection was used to monitor the profile of target metabolites in human serum extracted from all individuals who consumed breakfasts prepared with these vegetable edible oils.

Materials and Methods

Chemicals

Fatty alcohols —docosanol, tetracosanol, hexacosanol and octacosanol—, phytosterols —campesterol, stigmasterol, stigmastanol and β-sitosterol— and endogenous sterols such as cholesterol and α-cholestanol were from Sigma–Aldrich (St. Louis, MO, USA) and used as standards. The most representative and commercially available triterpenic compounds —*i.e.* erythrodiol from Extrasynthese (Genay, France), and uvaol, oleanolic acid and ursolic acid from Sigma–Aldrich— were also used. Maslinic acid (>97% purity) isolated by a previously reported protocol [26] was a gift from A. García-Granados (University of Granada, Spain).

Betulinic acid from Sigma–Aldrich was used as internal standard (IS) for quantification of triterpenes and sterols, while eicosanol, also from Sigma–Aldrich, was used as IS for alcohols. The stock standard solutions of alcohols and sterols were prepared at 1000 μg mL^{-1} in chloroform, while the solutions of triterpenes were

prepared at 1000 μg mL^{-1} in ethanol. The standard solutions, which contained 4 alcohols, 6 sterols and 5 triterpenes, were prepared by appropriate dilution of the stock solutions and stored at −20 ºC in amber glass vials until use.

LC grade n-hexane was provided by Panreac (Barcelona, Spain). Anhydrous sodium sulfate from Sigma–Aldrich was used as drying agent for the nonpolar phase in the derivatization step. A 2 M KOH methanolic solution (Panreac) was used for oil saponification and a 0.01% 2,6-tert-butyl-4-methylphenol ethanolic solution (Panreac) was used as antioxidant prior to sample preparation.

Bis-(trimethylsilyl)-fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) from Sigma–Aldrich and pyridine from Merck (Darmstadt, Germany) were used in the derivatization step.

Oils and heating procedure

Koipesol (SOS Cuétara S.A, Madrid) provided the edible oils, virgin olive oil (VOO), high oleic sunflower oil and sunflower oil (SO), for this research. VOO and SO were used as such while high-oleic sunflower oil was enriched with either a phenolic extract with antioxidant properties or with an oxidation inhibitor. VOO, with a fatty acids (FA) composition of 70.5% monounsaturated FA (MUFA), 11.1% polyunsaturated FA (PUFA) and 18.4% saturated FA (SFA), was characterized by a total content in antioxidant phenols of 400 μg mL^{-1}, expressed as caffeic acid according to the Folin-Ciocalteu (F–C) test. On the other hand, SO (34.3% MUFA, 58.3% PUFA and 7.3% SFA) was characterized by the absence of phenolic antioxidants. High-oleic sunflower oil (71.8% MUFA, 18.0% PUFA and 10.2% SFA) was enriched either with an extract of phenolic compounds from olive pomace (ASO), up to a total phenol concentration of 400 μg mL^{-1} or with 400 μg mL^{-1} of dimethylpolysiloxane as an artificial oxidation inhibitor (DSO).

Two liters of each target oil was heated in a stainless deep fryer at 180±5 ºC for 20 cycles of 5 min (total heating time 100 min). Supplementary Table 1 lists the concentration of the target metabolites in oils after and before the deep frying process.

Supplementary Table 1. Concentration of aliphatic alcohols, sterols and triterpenic compounds in the target oils before and after the simulated frying process (all the results are expressed as μg mL^{-1}).

	Oil	VOO		SO		ASO		DSO	
	Simulated frying process	Before	After	Before	After	Before	After	Before	After
Aliphatic alcohols*	DOC	4.75	3.57	0.40	0.34	3.64	<LOQ	1.13	<LOQ
	TET	11.93	3.83	0.01	0.65	14.00	4.21	4.78	2.71
	HEX	13.07	1.12	0.22	0.70	2.09	0.11	2.82	2.53
	OCT	<LOQ	<LOQ	<LOQ	0.80	0.08	0.40	<LOQ	<LOQ
Sterols*	CAM	42.02	11.40	133.63	72.02	65.56	51.49	230.46	62.92
	STE	43.31	30.16	101.53	22.43	28.44	44.05	64.05	57.66
	STA	8.23	10.97	18.12	12.84	16.25	6.12	2.24	13.76
	SIT	506.19	244.20	308.09	155.32	173.26	121.76	286.52	20.36
Triterpenic compounds*	ERY	18.67	17.70	7.01	7.32	9.05	0.14	6.37	0.98
	UVA	14.40	4.24	5.77	11.12	9.06	0.75	2.08	1.02
	OLE	5.05	<LOQ	<LOQ	<LOQ	8.77	<LOQ	2.27	1.13
	URS	5.26	<LOQ	<LOQ	<LOQ	6.21	<LOQ	2.09	2.77
	MAS	28.10	<LOQ	<LOQ	<LOQ	16.81	<LOQ	6.62	5.35

***** *DOC, docosanol; TET, tetracosanol; HEX, hexacosanol; OCT, octacosanol; CAM, campesterol; STE, stigmasterol; SIT, β-sitosterol; STA,stigmastanol; ERY, erythrodiol; UVA, uvaol; OLE, oleanolic acid; URS, ursolic acid and MAS, maslinic acid; LOQ, limit of quantitation.*

Subjects

Twenty six obese individuals (body mass index above 29.4 Kg m^{-2}) were selected for this study. All of them gave their informed consent and underwent a comprehensive medical history, physical examination and clinical chemical analysis before enrolment. Participants with evidence of kidney, pancreas, lung, liver or thyroid disease were excluded. All subjects were non-diabetics, non-smokers, without clinical manifestations of cardiovascular disease and off treatment. The target cohort was composed by 17 post-menopausal women, age 48–70 years, and 9 men, age 39–70 years. None of the subjects was taking medication or supplementary vitamins with influential effect on serum lipidome. Anthropometric characteristics for the 26 subjects were as follows: age, 56.15±7.2 years; height, 159.3±6.1 cm; weight, 54.2±8.8 kg; BMI, 29.4–46.9 Kg m^{-2} and waist perimeter, 114.29±13.9 cm.

All volunteers received a randomized sequence of four breakfasts prepared in muffin format with each of the four different oils (0.45 mL of oil per kilogram of body weight per breakfast), previously subjected to the simulated frying process. The intake of each breakfast was programmed every two weeks (4 oils, 8 weeks) following a Latin square design.

Sampling protocol

Blood samples were collected into evacuated sterile tubes for whole blood hematology determination Vacutainer® (Becton Dickinson, Franklin Lakes, NJ, USA). The serum fraction was isolated after blood centrifugation at 1880 *g* (processing within 2 h after collection), placed in a plastic ware tube and stored at −80 °C up to analysis. All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki (2004) and supervised by the Ethical Review Board (ERB) of Reina Sofia Hospital (Córdoba, Spain), which approved the experiments.

Analytical method

The overall method for quantitative analysis of aliphatic alcohols, sterols and triterpenic compounds in serum samples started by a step for protein precipitation by adding 1200 µL chloroform and 600 µL methanol to 200 µL of human serum placed in a test tube with 10 µL of each internal standard (0.1% chloroform solution of eicosanol and 0.1% ethanol solution of betulinic acid). The tube was centrifuged for 15 min at 1880 *g*. The

chloroform fraction was separated and mixed with 300 µL of 2,6-tert-butyl-4-methylphenol methanolic solution to inhibit oxidation of the target compounds. Then, 1200 µL of methanol 2 M in KOH was added and the reaction mixture was immersed into a water bath at 20 ºC, where ultrasound —duty cycle 45%, output amplitude 50% of the converter— was applied for 10 min. After cooling at room temperature, the unsaponifiable fraction was extracted with 2.4 mL of hexane, and the immiscible organic phase separated by centrifugation for 10 min at 1500 *g*. This phase was washed with distilled water until neutral reaction of this. The unsaponifiable fraction was dried in a speed-vac apparatus and the residue reconstituted with 100 µL pyridine and homogeneized in a vial for 1 min. Then, 98 µL of BSTFA was added and the mixture vigorously shaken in the vial for 1 min. Finally, 2 µL of TMCS was added, the mixture was vigorously shaken in the vial for 2 min more, then subjected to ultrasound (output amplitude 40% of the converter, duty cycle 50%) for 10 min to accelerate the derivatization reaction [39,40]. Finally, the resulting solution was analyzed by GC–MS.
The individual separation of sterols, fatty and triterpenes and the separation–determination by GC–MS was similar to that previously proposed by the authors for the analysis of the target compounds in oils sujected to a simulated frying process [38]. Briefly, 1 µL of the analytical sample was injected into the chromatograph. The injector temperature was fixed at 250 ºC and the injection was in the split–splitless mode. The splitter was opened (50:1) for 0.5 min, closed for 3.5 min and then opened at 100:1 split ratio for 10 min. The oven temperature program was as follows: initial temperature 70 ºC (held for 1.20 min), increased at 25 ºC min^{-1} to 120 ºC, followed by a second gradient at 2 ºC min^{-1} to 243 ºC; and finally, increased at 4 ºC min^{-1} to 270 ºC (held for 5 min). The total analysis time was 71 min, and 10 min extra time was necessary for re-establishing and equilibrating the initial conditions.

Mass spectrometry detection was performed in single ion monitoring (SIM) mode after electron impact positive ionization (EI). The parameters were set as follows: 220 ºC the manifold, 200 ºC trap and 50 ºC transfer line. The analyses were performed with a filament–multiplier delay of 20 min and data acquisition was performed in the range *m/z* 50–650 in full scan mode, with a background of *m/z* 45. The emission current of the ionization filament was set at 80 µA. The scan time for data acquisition was set at 1.0 s in SIM mode, while 3 microscans s^{-1} was selected in full scan mode. The values found for each monitored ion under SIM mode are listed in Table 1.

Statistical analysis

A total of 312 samples were obtained from the twenty-six obese volunteers who received the four breakfasts. Sampling was carried out before the intake of each breakfast (26 individuals × 4 breakfasts = 104 samples) and 2 and 4 h after intake (26 individuals × 4 breakfasts × 2 times = 208 samples).

Table 1. GC–MS parameters for quantitation of the target compounds

	Compound name	Formula	Retention time	Segment	Ions for quantification
Aliphatic alcohols	1-Eicosanol (S.I)	$C_{20}H_{42}O$	27.121	1	355.3
	1-Docosanol	$C_{22}H_{46}O$	28.310	2	383.5
	1-Tetracosanol	$C_{24}H_{50}O$	29,710	3	411.3
	1-Hexacosanol	$C_{26}H_{54}O$	31.121	5	439.5
	1-Octasanol	$C_{28}H_{58}O$	32.285	6	370.2/467.2
Sterols	**α**-Cholestanol	$C_{27}H_{48}O$	34.837	6	445.5/460.5
	Cholesterol	$C_{27}H_{46}O$	32.971	6	458.5/443.5
	Campesterol	$C_{28}H_{48}O$	36.728	7	382.3/394.4
	Stigmasterol	$C_{29}H_{48}O$	37.519	7	
	ß-Sitosterol	$C_{29}H_{50}O$	38.521	8	215.3/383.5/396.0
	Stigmastanol	$C_{29}H_{52}O$	38.975	8	
Triterpenes	Erythrodiol	$C_{30}H_{50}O_2$	40.653	9	216.1/496.5
	Uvaol	$C_{30}H_{50}O_2$	41.586	9	
	Oleanolic acid	$C_{30}H_{48}O_3$	42.601	10	
	Betulinic acid (IS)	$C_{30}H_{48}O_3$	41.730	10	203.3/320.3
	Ursolic acid	$C_{30}H_{48}O_3$	41.915	10	
	Maslinic acid	$C_{30}H_{48}O_3$	44.575	11	203.1/320.1

Evaluation of data normalization was carried out by one variable analysis for each target analyte with estimation of standardized skewness and kurtosis coefficients. Distribution of the data were log-transformed, when appropriate, to meet the assumption of the statistical tests. Descriptive statistics were presented as means ± SD or as percentages. The Pearson test was used to evaluate correlations between each pair of variables at the 99.0% confidence level for the linear relationship between them.

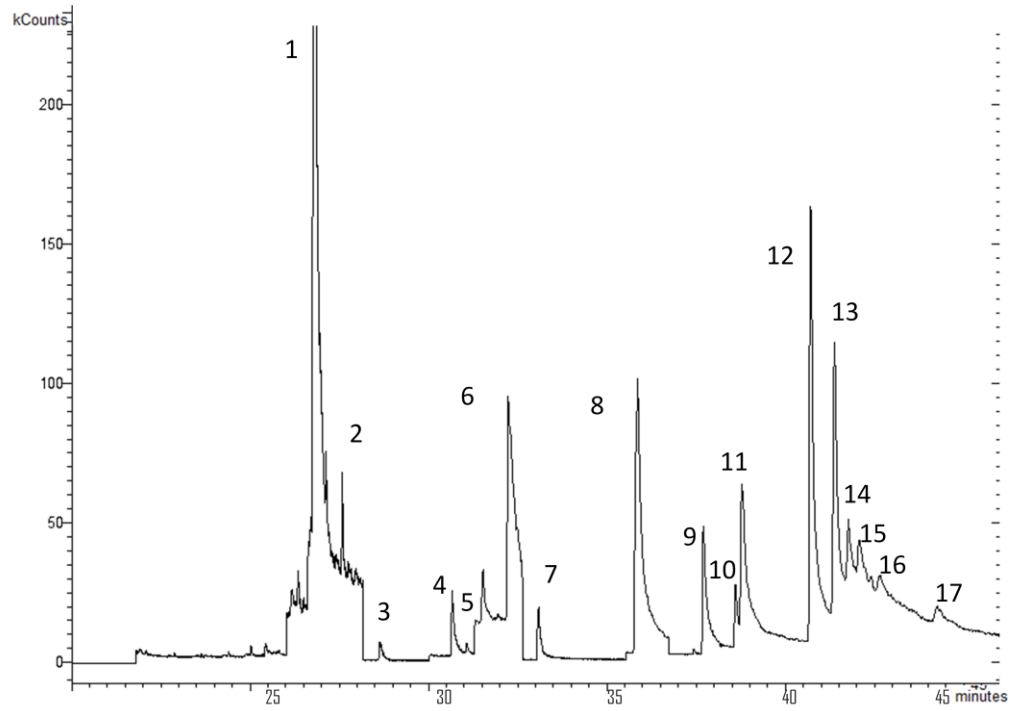
The influence of anthropometric characteristics (*viz.* age, BMI and gender) and variables of the intervention study (*viz.* type of breakfast ingested and time after intake) on the concentration of the target metabolites in serum was evaluated by multifactor ANOVA. The type of breakfast was categorized according to the oil used for breakfast preparation (VOO, ASO, DSO, SO) and control samples were taken prior to the breakfast intake. The time of serum sampling was categorized as prior to intake, 2 and 4 h after intake. Fisher's least significant difference (lsd) algorithm was used to discriminate among the means of concentration of the target analytes in serum from the different diets by multifactor ANOVA studies for 95% of confidence level. Unsupervised analysis by principal component analysis (PCA) was used to evaluate the similarity to the concentration of analytes for obese individuals after intake of different breakfasts.

Results and discussion

Validation of the method selected for analysis of target metabolites

Identification of the analytes was ensured with standard solutions and spiked samples by comparison of mass spectra and retention times. Supplementary Figure 1 shows a chromatogram obtained from a serum sample spiked with a multistandard solution. As can be seen, complete separation was achieved within 52 min. The retention times of sterols, fatty alcohols, and triterpenic compounds depended on the length of their chains, double bond number, position, geometry, and branches. Also, the temperature program affected the elution order of sterols and triterpenic compounds with different numbers of double bonds. The absence in the samples of the selected internal standards was checked in preliminary tests by targeted analysis. The fragmentation schemes for representative compounds of each family (sterols, fatty alcohols, and triterpenes) are shown in Supplementary Figure 2.

A serum pool was used to validate the proposed protocol. Calibration plots were run for the target analytes using the peak area as a function of the standard concentration of each compound by applying the standard addition method using sterols, aliphatic alcohols and triterpenes stock standard solutions. Analytical replicates (n=2) of serum aliquots spiked with the target metabolites within the linear dynamic range were injected. Table 2 summarizes the main parameters estimated in the characterization of the method. As can be seen, two calibration ranges were defined for all metabolites without effect on the linearity of the regression models. The limits of detection (LOD) and quantification (LOQ) were determined by analysis of serum aliquots spiked with dilution series of all analytes to obtain the concentrations that provided signals equivalent to three and ten times the background noise, respectively. LODs ranged from 0.02 to 1.09 ng mL^{-1}, while LOQs were from 0.06 to 3.64 ng mL^{-1}. Table 2 reveals a better sensitivity of the method for aliphatic alcohols than for sterols and terpenoids.

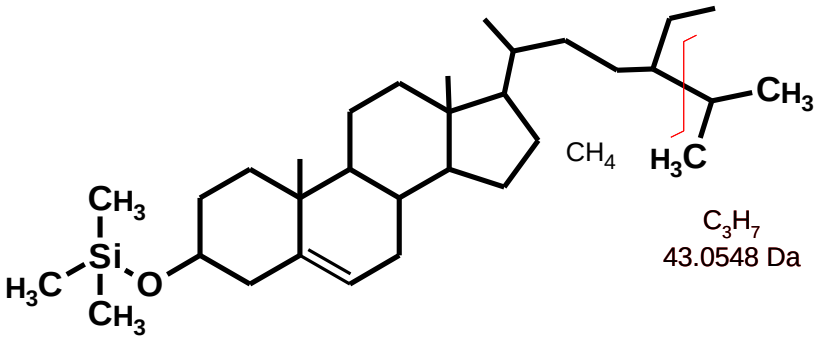


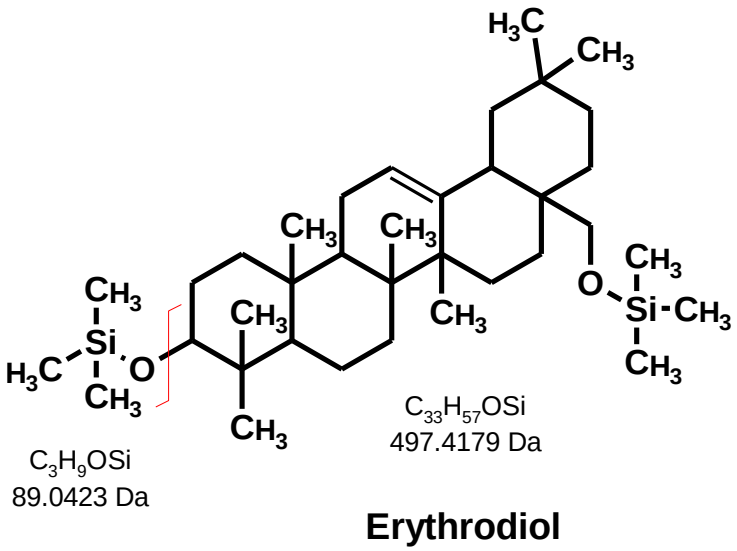
Supplementary Figure 1. *Total ion chromatogram obtained by GC–MS analysis of a serum sample spiked with a multistandard solution: Peaks: 1, eicosanol (internal standard); 2, docosanol; 3, tetracosanol; 4, hexacosanol; 5, octacosanol; 6, α-cholestanol; 7, cholesterol; 8 campesterol; 9, stigmasterol; 10, β-sitosterol; 11, stigmastanol; 12, erythrodiol; 13, uvaol; 14; oleanolic acid; 15, betulinic acid (internal standard); 16, ursolic acid; 17, maslinic acid.*

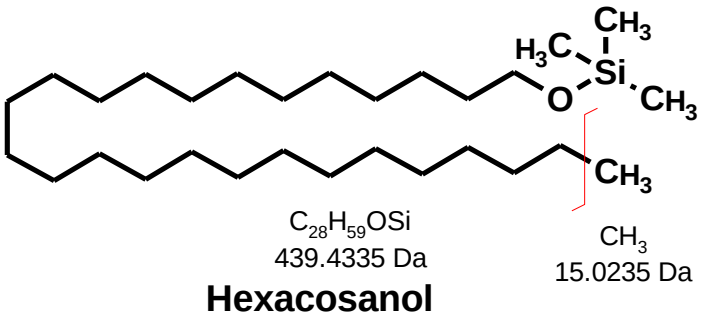
Table 2. Characterization of the GC–MS method for determination of the target compounds

Compounds	Calibration curve	R^2	Linear range (µg L^{-1})	LOD (µg L^{-1})	LOQ (µg L^{-1})
Docosanol	$Y=8.2x\ 10^{-3}\ X\ +0.0629$	0.991	LOQ–50	0.06	0.19
	$Y=3.0x\ 10^{-4}\ X$	0.996	50–25000		
Tetracosanol	$Y=2.8x\ 10^{-3}\ X\ +0.1609$	0,996	LOQ–100	0.04	0.09
	$Y=3.0x\ 10^{-4}\ X$	0.990	100–25000		
Hexacosanol	$Y=8.2x\ 10^{-3}\ X\ +0.0709$	0.995	LOQ–100	0.02	0.06
	$Y=1.7x\ 10^{-3}\ X + 1.0430$	0.995	100–25000		
Octacosanol	$Y=7.5x\ 10^{-3}\ X\ +0.0004$	0.998	LOQ–50	0.11	0.36
	$Y=1.5x\ 10^{-3}\ X\ +0.7749$	0.999	100–25000		
Cholesterol	$Y=1.2x\ 10^{-3}\ X\ +0.0004$	0.993	LOQ–200	0.08	1.58
	$Y=3.0x\ 10^{-4}\ X\ +0.0557$	0.996	200–25000		
α-Cholestanol	$Y=4.0x\ 10^{-3}\ X\ +0.0021$	0.993	LOQ–50	0.41	1.02
	$Y=7.2x\ 10^{-3}\ X\ +0.1533$	0.994	100–25000		
Campesterol	$Y=4.0x\ 10^{-4}\ X\ +0.0150$	0.995	LOQ–100	1.09	3.64
	$Y=6.0x\ 10^{-5}\ X\ +0.0009$	0.991	100–25000		
Stigmasterol	$Y=4.0x\ 10^{-4}\ X\ +0.0082$	0.992	LOQ–50	0.43	1.43
	$Y=8.0x\ 10^{-5}\ X\ +0.1064$	0.995	50–25000		
β-Sitosterol	$Y=1.6x\ 10^{-2}\ X\ +0.208$	0.991	LOQ–25	0.95	3.15
	$Y=1.0x\ 10^{-4}\ X\ +0.0485$	0.998	1500–25000		
Stigmastanol	$Y=1.5x\ 10^{-3}\ X\ +0.0340$	0.990	LOQ–100	0.98	3.26
	$Y=3.0x\ 10^{-4}\ X\ +0.2354$	0.993	100–25000		
Erythrodiol	$Y=1.2x\ 10^{-2}\ X\ +0.1011$	0.994	LOQ–50	0.29	0.97
	$Y=1.1x\ 10^{-3}\ X\ +1.0815$	0.999	50–25000		
Uvaol	$Y=1.3x\ 10^{-2}\ X\ +0.0685$	0.996	LOQ–50	0.55	1.86
	$Y=6.0x\ 10^{-4}\ X\ +1.8051$	0.995	50–25000		
Oleanolic acid	$Y=1.0x\ 10^{-3}\ X\ +0.0250$	0.995	LOQ–200	0.98	3.27
	$Y=4.0x\ 10^{-2}\ X\ +0.5819$	0.989	200–25000		
Ursolic acid	$Y=6.0x\ 10^{-4}\ X\ +0.0064$	0.999	LOQ–200	0.37	1.23
	$Y=3.0x\ 10^{-4}\ X\ +0.0701$	0.998	200–15000		
Maslinic acid	$Y=2.0x\ 10^{-4}\ X\ +0.0103$	0.990	LOQ–1500	0.88	2.02
	$Y=1.0x\ 10^{-4}\ X\ +0.1760$	0.998	1500–15000		

Intra-day and inter-day variability were evaluated in a single experimental setup with duplicates by analyses carried out on seven days [41]. Serum aliquots spiked with a multistandard solution at an intermediate concentration (500 ng mL^{-1}) according to the linear dynamic ranges were selected for this precision study. Table 3 shows the results obtained for both variability parameters expressed as percentage of relative standard deviation. As can be seen, variability was always below 10.7%. The accuracy of the method was estimated by comparative analysis of spiked and non-spiked samples to evaluate potential matrix effects. The accuracy was calculated as a function of the recovery factor by comparison of both groups of samples. This parameter was above 78% for all metabolites, except for two triterpene derivatives: acids oleanolic and maslinic, which reported values of 71.2 and 72%, respectively.







Supplementary Figure 2*. Fragmentation of the derivatization products of the most representative target metabolites of each fraction (β-sitosterol, hexacosanol, and erythrodiol).*

Profiling of the target metabolites in the cohort selected for the study

Preliminary tests were focused on the analysis of the target metabolites in serum samples from 26 individuals in basal state, which means before the intake of the prepared breakfasts according to the experimental plan. The serum profiles for the target metabolites in the selected cohort as a function of the individuals' gender are shown in Table 4, as well as the anthropometric characteristics, which show that the selected cohort was formed by obese individuals with BMI ranging from 29.4 to 46.9 Kg m^{-2}. Among the aliphatic alcohols included in the method only octacosanol was detected in the target cohort. Concerning sterols, endogenous cholesterol and α-cholestanol together with exogenous campesterol, β-sitosterol and stigmastanol were above their LOQs. Among triterpenes, uvaol and erythrodiol were the only compounds found in serum from obese individuals, while maslinic acid, ursolic acid and oleanolic acid were not detected. Serum concentrations of the target metabolites in the cohort under study were normalized by logarithmic transformation to operate with parametric statistical tests (standardized skewness and kurtosis coefficients below ±2.0). The *t*-Student test revealed no differences in the concentration of the target metabolites panel for male and female individuals, which allowed the joint analysis of data without taking into account the gender as variability source.

Supplementary Table 2. Correlation parameters obtained multiple variable analysis test.

	α-Cholestanol	Campesterol	Erytrodhiol	Sitosterol	Uvaol	Stigmastanol	Cholesterol	Octacosanol	Age	Weight	Waist perimeter	Body mass index	Height
α-Cholestanol		0.5033	0.4311	-0.2401	-0.2847	0.0140	0.6385	-0.1888	-0.0200	-0.0983	-0.0753	-0.1562	0.0120
		(267)	(121)	(127)	(88)	(186)	(267)	(253)	(267)	(267)	(267)	(267)	(267)
		0.0000	0.0000	0.0065	0.0072	0.8501	0.0000	0.0026	0.7451	0.1092	0.2203	0.0106	0.8457
Campesterol	0.5033		-0.2392	-0.1842	-0.3409	-0.1295	0.3504	0.1166	-0.0541	-0.0007	-0.0048	-0.0206	0.0242
	(267)		(145)	(140)	(94)	(219)	(312)	(291)	(312)	(312)	(312)	(312)	(312)
	0.0000		0.0038	0.0293	0.0008	0.0557	0.0000	0.0470	0.3405	0.9908	0.9328	0.7167	0.6709
Erytrodhiol	0.4311	-0.2392		0.0771	0.5975	0.4681	0.4682	-0.2441	-0.1256	0.1277	0.2367	0.0083	0.1750
	(121)	(145)		(76)	(88)	(101)	(145)	(132)	(145)	(145)	(145)	(145)	(145)
	0.0000	0.0038		0.5079	0.0000	0.0000	0.0000	0.0048	0.1321	0.1259	0.0041	0.9207	0.0353
Sitosterol	-0.2401	-0.1842	0.0771		0.0449	0.0357	-0.1424	0.2431	-0.0342	-0.1026	-0.0991	0.0820	-0.2087
	(127)	(140)	(76)		(70)	(108)	(140)	(140)	(140)	(140)	(140)	(140)	(140)
	0.0065	0.0293	0.5079		0.7119	0.7139	0.0932	0.0038	0.6881	0.2276	0.2440	0.3357	0.0133
Uvaol	-0.2847	-0.3409	0.5975	0.0449		0.3363	-0.2764	0.1198	0.3441	0.2729	0.2617	0.1226	0.2873
	(88)	(94)	(88)	(70)		(73)	(94)	(94)	(94)	(94)	(94)	(94)	(94)
	0.0072	0.0008	0.0000	0.7119		0.0036	0.0070	0.2501	0.0007	0.0078	0.0108	0.2392	0.0050
Stigmastanol	0.0140	-0.1295	0.4681	0.0357	0.3363		0.4287	0.0840	-0.0724	0.0926	-0.0167	0.0511	0.0790
	(186)	(219)	(101)	(108)	(73)		(219)	(206)	(219)	(219)	(219)	(219)	(219)
	0.8501	0.0557	0.0000	0.7139	0.0036		0.0000	0.2297	0.2858	0.1722	0.8058	0.4516	0.2446
Cholesterol	0.6385	0.3504	0.4682	-0.1424	-0.2764	0.4287		-0.2132	0.0689	-0.1067	-0.0598	-0.0328	-0.1152
	(267)	(312)	(145)	(140)	(94)	(219)		(291)	(312)	(312)	(312)	(312)	(312)
	0.0000	0.0000	0.0000	0.0932	0.0070	0.0000		0.0002	0.2247	0.0598	0.2922	0.5642	0.0421

Octacosanol	-0.1888	0.1166	-0.2441	0.2431	0.1198	0.0840	-0.2132		-0.0300	0.1352	0.1235	0.1191	0.0507
	(253)	(291)	(132)	(140)	(94)	(206)	(291)		(291)	(291)	(291)	(291)	(291)
	0.0026	0.0470	0.0048	0.0038	0.2501	0.2297	0.0002		0.6105	0.0210	0.0352	0.0424	0.3891
Age	-0.0200	-0.0541	-0.1256	-0.0342	0.3441	-0.0724	0.0689	-0.0300		-0.4963	-0.2034	-0.3062	-0.3931
	(267)	(312)	(145)	(140)	(94)	(219)	(312)	(291)		(312)	(312)	(312)	(312)
	0.7451	0.3405	0.1321	0.6881	0.0007	0.2858	0.2247	0.6105		0.0000	0.0003	0.0000	0.0000
Weight	-0.0983	-0.0007	0.1277	-0.1026	0.2729	0.0926	-0.1067	0.1352	-0.4963		0.6913	0.7648	0.6824
	(267)	(312)	(145)	(140)	(94)	(219)	(312)	(291)	(312)		(312)	(312)	(312)
	0.1092	0.9908	0.1259	0.2276	0.0078	0.1722	0.0598	0.0210	0.0000		0.0000	0.0000	0.0000
Waist perimeter	-0.0753	-0.0048	0.2367	-0.0991	0.2617	-0.0167	-0.0598	0.1235	-0.2034	0.6913		0.6948	0.2915
	(267)	(312)	(145)	(140)	(94)	(219)	(312)	(291)	(312)	(312)		(312)	(312)
	0.2203	0.9328	0.0041	0.2440	0.0108	0.8058	0.2922	0.0352	0.0003	0.0000		0.0000	0.0000
Body mass index	-0.1562	-0.0206	0.0083	0.0820	0.1226	0.0511	-0.0328	0.1191	-0.3062	0.7648	0.6948		0.0554
	(267)	(312)	(145)	(140)	(94)	(219)	(312)	(291)	(312)	(312)	(312)		(312)
	0.0106	0.7167	0.9207	0.3357	0.2392	0.4516	0.5642	0.0424	0.0000	0.0000	0.0000		0.3293
Height	0.0120	0.0242	0.1750	-0.2087	0.2873	0.0790	-0.1152	0.0507	-0.3931	0.6824	0.2915	0.0554	
	(267)	(312)	(145)	(140)	(94)	(219)	(312)	(291)	(312)	(312)	(312)	(312)	
	0.8457	0.6709	0.0353	0.0133	0.0050	0.2446	0.0421	0.3891	0.0000	0.0000	0.0000	0.3293	

For each analyte or anthropometric parameter the data in the first line correpond to correlation, the second to sample size and the third to p-value

Table 3. Precision and accuracy study by estimation of intra-day and inter-day variability and recovery factor, respectively. Precision parameters are expressed as percentage of relative standard deviation

Compound	Intra-day variability (%)	Inter-day variability (%)	Recovery(%)
Docosanol	2.9	4.1	88.7
Tetracosanol	2.9	5.8	84.3
Hexacosanol	3.1	6.9	92.1
Octacosanol	3.3	7.3	95.5
Cholesterol	4.4	10.7	100
α-Cholestanol	3.8	10.0	80.2
Campesterol	3.5	7.3	89.8
Stigmasterol	3.0	7.3	78.6
β-Sitosterol	3.9	6.9	82.3
Stigmastanol	4.1	8.0	80.2
Erythrodiol	4.2	8.2	80.2
Uvaol	2.9	8.3	87.4
Oleanolic acid	3.7	10.2	71.2
Ursolic acid	3.7	9.1	78.8
Maslinic acid	3.2	9.4	72.0

Statistical analysis of the influence of the intervention breakfast on the concentration of the target metabolites

The statistical analysis started by a test focused on finding univariate correlations between pairs of normalized concentration of metabolites. The univariate Pearson test by setting confidence level at 99% was selected taking into account the biological variability of the cohort under study. The results revealed high correlations between monitored metabolites such as those of endogenous cholesterol and phytosterols, aliphatic alcohols and triterpenoids, which could be a link to correlate the metabolism of cholesterol and that of exogenous metabolites. Supplementary Table 2 shows the correlation parameters obtained by this statistical test.

Table 4. Anthropometric features and average concentrations, as μg mL^{-1}. of the target metabolites before intake of the intervention breakfasts. Concentrations are expressed as mean ± standard deviation.

Parameter	Female		Male	
	Mean ± SD	Range	Mean ± SD	Range
Age (years)		39–63		48–70

Weight (kg)		79–125		69–120
Height (cm)		164–176		146–164
Waist perimeter (cm)		104–145		88–140
Body mass index (Kg m^{-2})		29.4–41.3		30.7–46.9
Docosanol	N.D.	—	N.D.	—
Tetracosanol	N.D.	—	N.D.	—
Hexacosanol	N.D.	—	N.D.	—
Octacosanol	0.42 ± 0.9		0.51 ± 0.7	
Cholesterol	3512.64 ± 821.4	2589.32–4214.75	3.327.30±774	2512.75–4098.32
α-Cholestanol	4.12± 0.8	3.30-4.85	4.18± 0.6	3.38–4.79
Campesterol	7.78± 1.1	6.68–8.54	7.63± 1.6	6.55–8.34
Stigmasterol	N.D.		N.D.	—
β-Sitosterol	9.27± 1.5	7.82–10.54	9.35 ± 0.9	7.80–10.34
Stigmastanol	1.93± 0.4	1.58–2.30	1.95± 1.2	0.78–3.03
Erythrodiol	0.31± 0.1	0.23–0.40	0.35 ± 0.2	0.20-0.41
Uvaol	0.65± 0.2	0.44-0.78	0.68± 0.5	0.47-0.76
Oleanolic acid	N.D.	—	N.D.	
Ursolic acid	N.D.	—	N.D.	—
Maslinic acid	N.D.	—	N.D.	—

Concentrations nondetected. (N.D.).

A multifactor ANOVA was carried out to compare the contribution of anthropometric features such as age, gender and BMI together with the intervention breakfast in the levels of the monitored compounds. The significance was stratified into three levels as follows: $0.01 < P < 0.05$, $0.001 < P < 0.01$ and $P < 0.001$. The multifactor ANOVA report is included in Table 5. As can be seen, the BMI and the individuals' age were the two anthropometric properties contributing to explain the levels of monitored metabolites in the studied cohort, while the gender did not explain serum levels in them. Thus, the BMI was highly significant to explain the levels of cholesterol and α-cholestanol as well as those of campesterol, octacosanol and stigmastanol. The age of individuals was other critical variable in this ANOVA study since it was highly significant to explain the levels of all the target metabolites. Finally, the intervention diet did also contribute to explain the levels of all metabolites, except for β-sitosterol and uvaol.

The incidence of anthropometric features such as gender, age and BMI was assessed by principal component analysis (PCA), as shows Figure 1. No discrimination between individuals in terms of metabolites concentration by these anthropometric features was observed. Similarly, the influence of the intervention breakfast on the levels of monitored metabolites was studied by multivariate analysis with unsupervised PCA. Figure 2.A shows partial discrimination into two groups of samples according to the concentration of the target compounds in serum from individuals after intake of

breakfasts prepared with the edible oils sujected to simulated frying. The two differentiated groups were formed by individuals after intake of breakfasts prepared with SO and VOO on the one side, and by individuals after intake of breakfasts prepared with ASO and DSO. Therefore, there is an effect in the concentration of monitored compounds caused by the intervention breakfasts. Three principal components were necessary to explain 84% of the data variability, although the commented discrimination was observed along PC1 in *x* axis. Taking into account the number of samples involved in the development of the models, these results are considered as a first approach of the research. For this analysis, no distinction was made between sampling times after breakfast consumption. The main compounds responsible for this discrimination were examined in the loadings plot associated to this PCA test. Figure 2.B shows the contribution of erythrodiol and α-cholestanol to explain the variability associated to PC1, although the contribution of the other monitored metabolites should also be considered. An additional task was to find discrimination patterns for each intervention breakfast according to the serum sampling time in post-basal state. As previously mentioned, serum samples were extracted at 2 and 4 h after the breakfast intake to detect post-basal metabolic differences. Supplementary Figure 3 illustrates that no differences were found for any intervention breakfast using the panel of monitored metabolites.

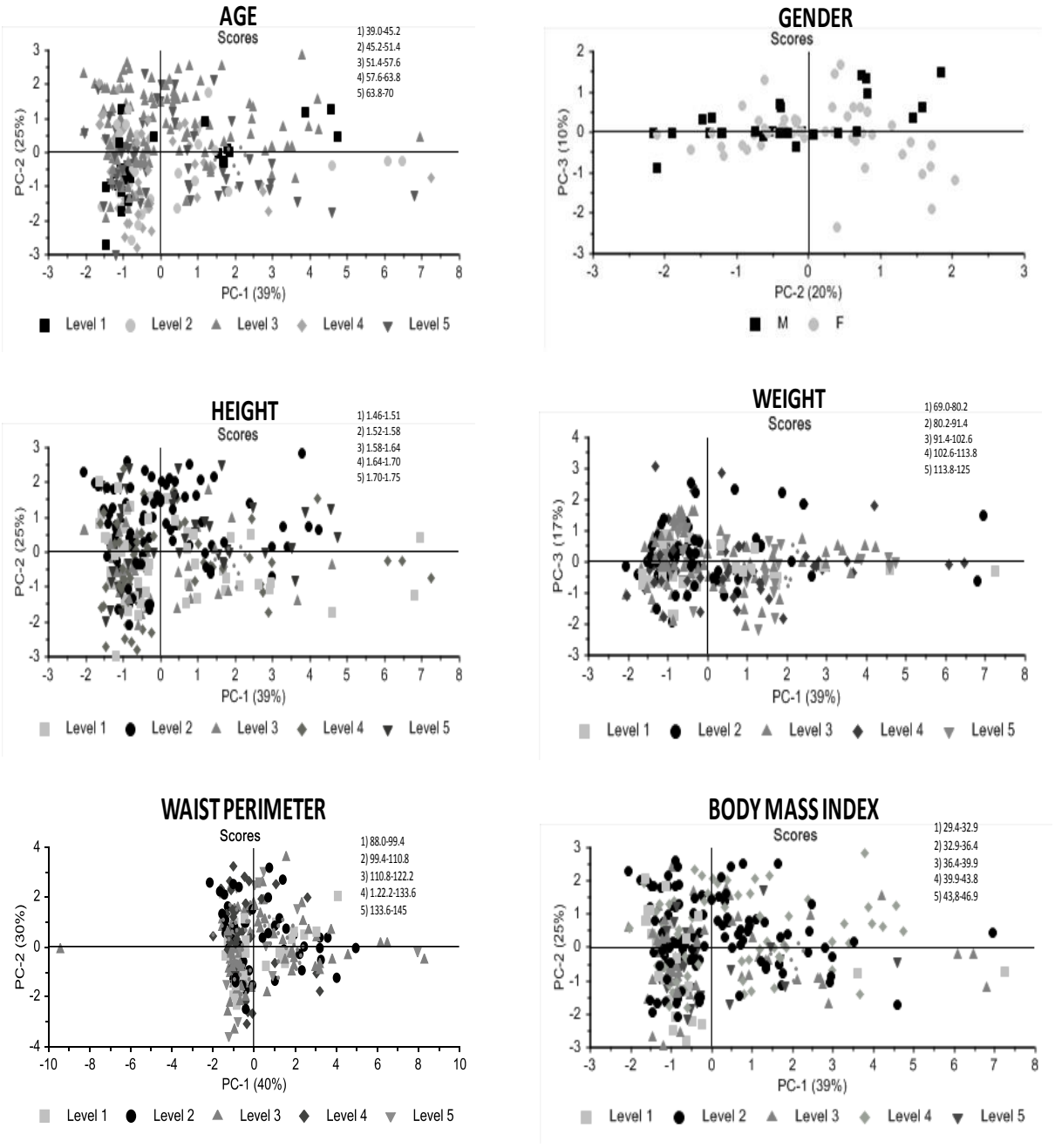
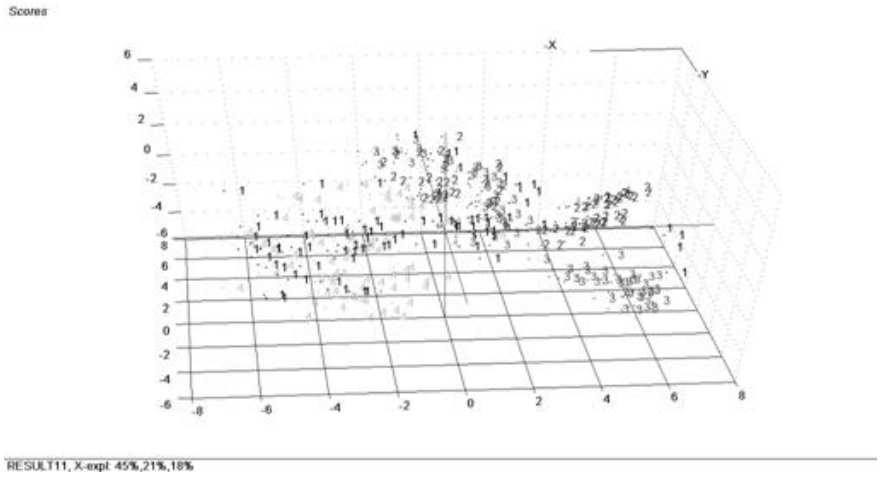


Figure 1. *Principal component analyses of control individuals (prior to breakfast intake) for anthropometric characteristics attending to serum levels of monitored target analytes*

A)



B)

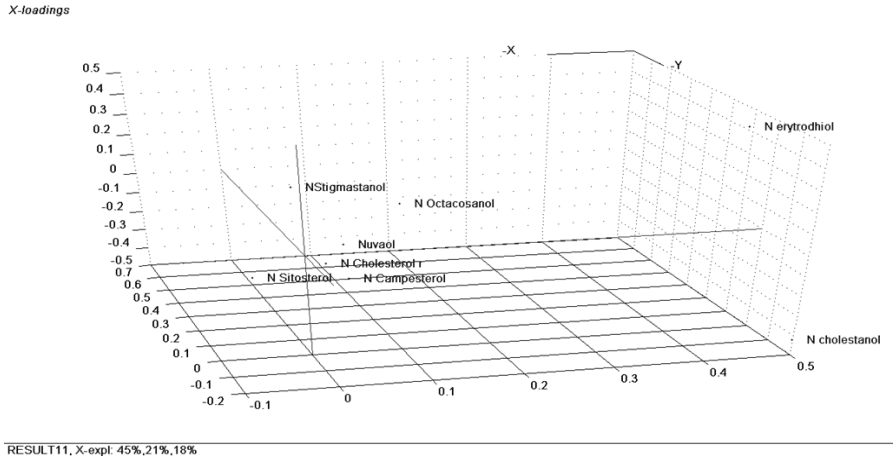


***Figure 2**. Principal component analysis of serum samples from individuals after intake of prepared breakfasts attending to the levels of the monitored target analytes. Scores graph (A). Number code is as follows: breakfasts prepared with pure sunflower oil (1), sunflower oil enriched with dimethylsiloxane (2), sunflower oil enriched with antioxidants from olive pomace (3), and, virgin olive oil (4). Loadings graph (B).*

Levels of the target metabolites in the individuals of the cohort after intake of intervention breakfasts

After discrimination of the individuals as a function of the intervention breakfasts the average levels of the monitored metabolites found in serum were compared in individuals after intake of each breakfast. The values detected in basal state were taken as a reference in this comparison intended to detect metabolic changes associated to the intake of a particular breakfast. Figure 3 shows the average means plot considering 95%

of least significance difference (LSD) intervals for each compound. Campesterol and β-sitosterol did not report discrimination capability despite they were the two most concentrated phytosterols in the oils. A different behavior was observed for stigmastanol which was detected at higher levels in individuals who consumed ASO- and DSO-based breakfasts, while individuals who consumed VOO and SO-based breakfasts reported ever lower levels than the cohort in the basal state. As Supplementary Table 1 shows stigmastanol was the most concentrated phytosterol in ASO and DSO oils after the simulated deep frying.

Table 5. Multifactor ANOVA revealing the contribution of the different factors to explain serum levels of sterols, aliphatic alcohols and triterpenic compounds. The results are expressed as sum of squares and the percent each one represents in the total observed variability.

*0.01 <P < 0.05; ** 0.001 < P< 0.01; *** P < 0.001

	A:Diet	B:Gender	C:BMI	D:Age
Cholesterol	203,55(74,66)***	0,02(0,01)	0,66(0,24)***	400,27(146,82)***
Campesterol	40,82(16,45)***	224,59(90,49)	132,55(53,41)***	191,85(77,3)***
Sitosterol	170,08(129,16))	0,01(0))	0,62(0,47)	487,63(370,32))***
Stigmastanol	333,64(156,81)***	479,03(225,14)	793,45(372,91)*	100,66(47,31)***
α-Cholestanol	682,22(62,84)***	266,31(24,53)	202,86(18,69)***	763,42(70,32)**
Erythrodiol	0,4(0,11)***	963,59(263,58)	129,6(35,45)	132,71(36,3)***
Uvaol	0(0)	0,06(0,04)	961,19(706,61)	139,18(102,31***)
Octacosanol	147,75(30,77)***	154,28(32,13)	123,48(25,72)***	0,83(0,17)***

Endogenous cholesterol was characterized by a similar trend to that observed in stigmastanol. Thus, cholesterol levels were higher in individuals who consumed DSO- and ASO-based breakfast, while individuals after intake of breakfasts prepared with VOO and SO presented lower cholesterol levels than those in basal state. α-cholestanol is a sterol detected in human biofluids under certain physiological situations. Serum levels of this cholesterol-related metabolite allowed individuals to be clearly discriminated after intake of breakfast prepared with ASO.

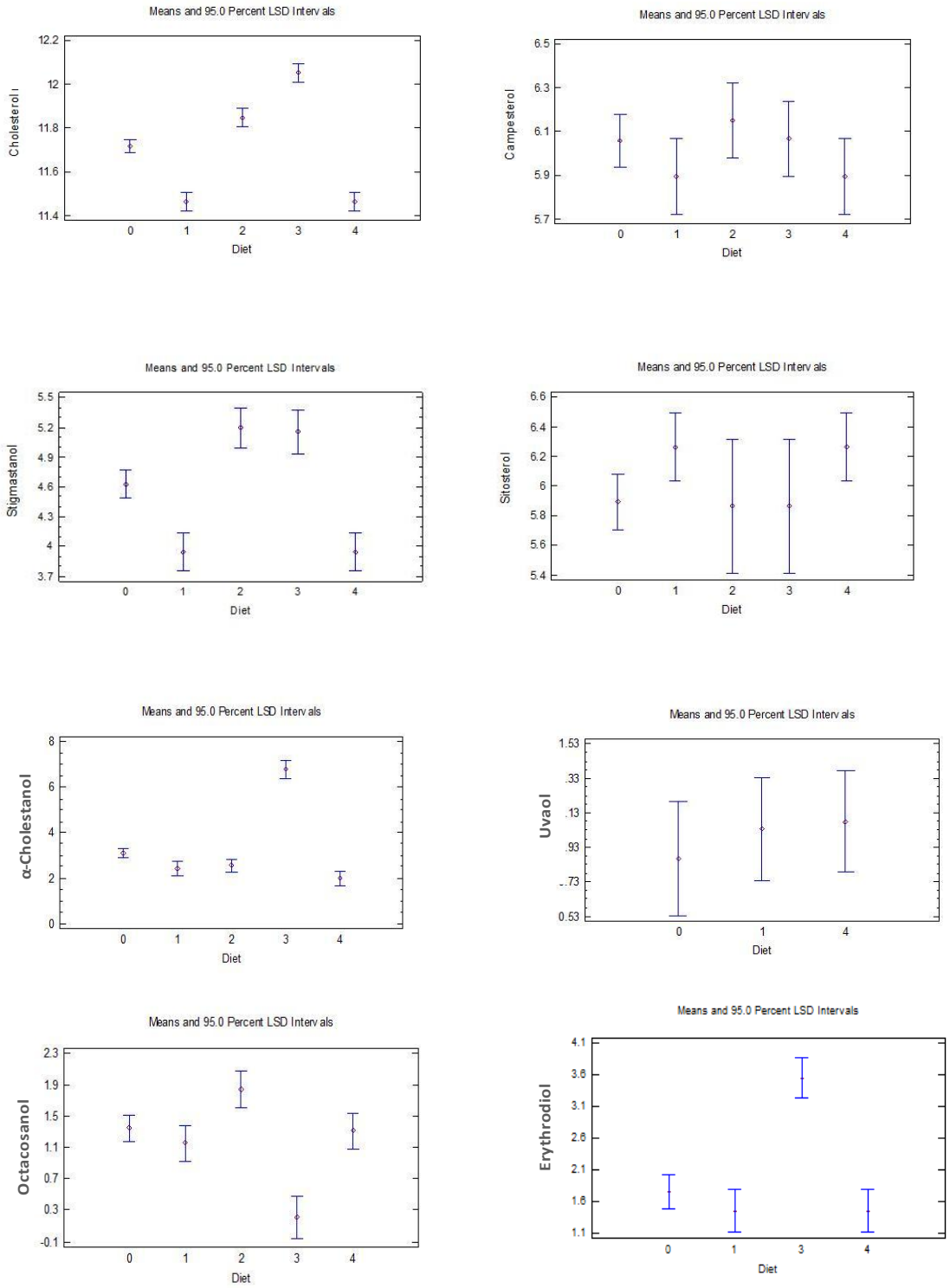
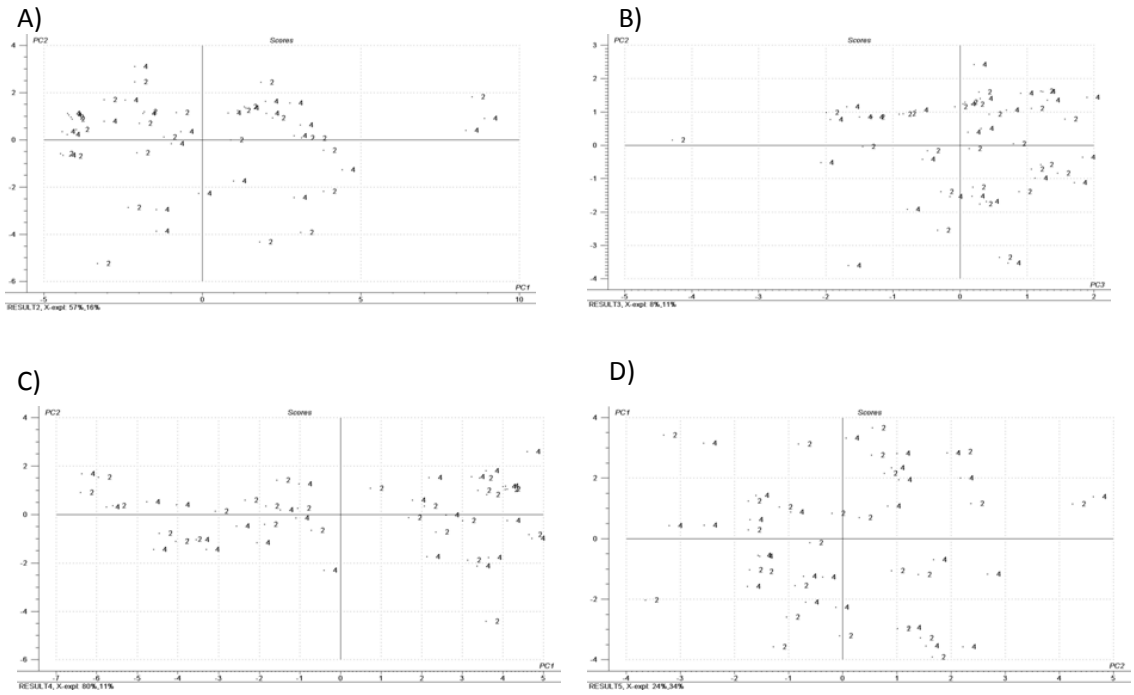


Figure 3*. Average levels plots for cholesterol, campesterol, stigmastanol, β-sitosterol, α-cholestanol, octacosanol, uvaol and erythrodiol detected in serum obtained from individuals at: (0) basal state; (1) VOO-based breakfast; (2) ASO-based breakfast; (3) DSO-based breakfast; SO-based breakfast.*



Supplementary Figure 3*. PCA scores graphs from the analysis of serum samples from individuals after intake of each prepared breakfast attending to the levels of the target analytes. Distinction was made between the two sampling times after breakfast intake. Number code is as follows: sampling at 2 h after breakfast intake (2), sampling at 4 h after breakfast intake (4). Name code is as follows: intake of breakfasts prepared with pure sunflower oil (A), sunflower oil enriched with dimethylsiloxane (B), sunflower oil enriched with antioxidants from olive pomace (C), and, virgin olive oil (D).*

Octacosanol also enabled to differentiate individuals after intake of intervention breakfasts prepared with ASO and DSO, despite this aliphatic alcohol was scarcely detected in the studied oils. Serum levels of this aliphatic alcohol in individuals after intake of DSO-breakfast were above the levels found in basal state and after intake of the other two breakfasts (VOO and SO). The opposite situation was observed in individuals after consumption of the ASO-breakfast since serum from them reported the lowest levels of octacosanol.

Concerning triterpenes, uvaol was only detected in individuals in basal state and in those who ingested breakfasts prepared with VOO and SO. In the case of erythrodiol, the average means plot demonstrated its discrimination capability to differentiate individuals after intake of ASO-based breakfast. This situation could be quite logical since this oil was enriched with a hydroalcoholic extract from olive pomace, which contained this triterpene at high levels in comparison to other edible oils. However, the

content of erythrodiol in VOO was higher than that measured in the other three edible oils although its concentration in individuals after VOO-breakfast did not provide discrimination capability.

Concluding remarks

A method for determination of sterols, aliphatic alcohols and triterpenes has been applied to detect discrimination patterns associated to the single intake of four intervention breakfasts prepared with different edible oils subjected to a simulated frying process for quantitative comparison of the profile of the monitored compounds. Metabolic differences have been detected both in endogenous metabolites such as cholesterol and α-cholestanol but also in exogenous compounds such as stigmastanol, octacosanol and erythrodiol. The level of the target compounds in the oils before and after the simulated frying process has been considered for interpretation of the results obtained in this study.

Acknowledgements The Spanish Ministerio de Ciencia e Innovación (MICINN) is thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

References

1. Cert A, Moreda W, Pérez-Camino MC (2000) Chromatographic analysis of minor constituents in vegetable oils. J. Chromatogr. A. 881 (1–2):131-148

2. Bouic PJD (2002) Sterols and sterolins: new drugs for the immune system. Drug Discov. Today 7 (14):775-778

3. Zhao C-C, Shao J-H, Li X, Xu J, Zhang P (2005) Antimicrobial constituents from fruits of Ailanthus altissima swingle. Archives of Pharmacal Research 28 (10):1147-1151

4. Seppanen C, Song Q, Saari Csallany A (2010) The Antioxidant Functions of Tocopherol and Tocotrienol Homologues in Oils, Fats, and Food Systems. J Am Oil Chem Soc 87 (5):469-481

5. Farhoosh R, Tavassoli-Kafrani MH, Sharif A (2011) Antioxidant activity of the fractions separated from the unsaponifiable matter of bene hull oil. Food Chem 126 (2):583-589

6. Awad AB, Fink CS (2000) Phytosterols as Anticancer Dietary Components: Evidence and Mechanism of Action. J Nut 130 (9):2127-2130

7. Cercaci L, Rodriguez-Estrada MT, Lercker G (2003) Solid-phase extraction–thin-layer chromatography–gas chromatography method for the detection of hazelnut oil in olive oils by determination of esterified sterols. J. Chromatogr. A. 985 (1–2):211-220

8. Volin P (2001) Analysis of steroidal lipids by gas and liquid chromatography. J. Chromatogr. A. 935 (1–2):125-140

9. Kim YK, Dohnálek Z, Kay BD, Rousseau R (2009) Competitive Oxidation and Reduction of Aliphatic Alcohols over (WO3) 3 Clusters. J Phys Chem C. 113 (22):9721-9730

10. Lerma-García MJ, Ramis-Ramos G, Herrero-Martínez JM, Gimeno-Adelantado JV, Simó-Alfonso EF (2009) Characterization of the alcoholic fraction of vegetable oils by derivatization with diphenic anhydride followed by high-performance liquid chromatography with spectrophotometric and mass spectrometric detection. J. Chromatogr. A. 1216 (2):230-236

11. Caponio F, Gomes T, Summo C (2003) Assessment of the Oxidative and Hydrolytic Degradation of Oils Used as Liquid Medium of In-oil Preserved Vegetables. J. Food Sci 68 (1):147-151

12. Siqueira Jr JM, Peters RR, Gazola AC, Krepsky PB, Farias MR, Rae GA, de Brum-Fernandes AJ, Ribeiro-do-Valle RM (2007) Anti-inflammatory effects of a triterpenoid isolated from Wilbrandia ebracteata Cogn. Life Sci 80 (15):1382-1387

13. Akihisa T, Nakamura Y, Tagata M, Tokuda H, Yasukawa K, Uchiyama E, Suzuki T, Kimura Y (2007) Anti-Inflammatory and Anti-Tumor-Promoting Effects of Triterpene Acids and Sterols from the Fungus Ganoderma lucidum. Chem Biodivers 4 (2):224-231

14. Wu Z-J, Ouyang M-A, Wang C-Z, Zhang Z-K (2007) Six new triterpenoid saponins from the leaves of oblonga and their inhibitory activities against TMV replication. Chem Pharmac Bull 55 (3):422-427

15. Kalogeropoulos N, Chiou A, Ioannou M, Karathanos VT, Hassapidou M, Andrikopoulos NK (2010) Nutritional evaluation and bioactive microconstituents (phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the Mediterranean countries. Food Chem. 121 (3):682-690

16. Rocha AD, de Oliveira AB, de Souza Filho JD, Lombardi JA, Braga FC (2004) Antifungal constituents of Clytostoma ramentaceum and Mansoa hirsuta. Phytotherapy Research 18 (6):463-467

17. Villaseñor IM, Canlas AP, Faustino KM, Plana KG (2004) Evaluation of the bioactivity of triterpene mixture isolated from Carmona retusa (Vahl.) Masam leaves. J Ethnopharmac 92 (1):53-56

18. Sánchez M, Theoduloz C, Schmeda-Hirschmann G, Razmilic I, Yáñez T, Rodríguez JA (2006) Gastroprotective and ulcer-healing activity of oleanolic acid derivatives: In vitro–in vivo relationships. Life Scis79 (14):1349-1356

19. Zhao P, Gao DF, Xu M, Shi ZG, Wang D, Yang CR, Zhang YJ (2011) Triterpenoid Saponins from the Genus Camellia. Chem Biodivers 8 (11):1931-1942

20. Garcia-Granados A, Martinez A, Moliz J, Parra A, Rivas F (1998) 3-b-Hydroxyolean-12-en-28-oic Acid (Oleanolic Acid). Molecules 3 (7):M87

21. Somova LO, Nadar A, Rammanan P, Shode FO (2003) Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. Phytomedicine 10 (2–3):115-121

22. Somova LI, Shode FO, Mipando M (2004) Cardiotonic and antidysrhythmic effects of oleanolic and ursolic acids, methyl maslinate and uvaol. Phytomedicine 11 (2–3):121-129

23. Rodriguez-Rodriguez R, Perona JS, Herrera MD, Ruiz-Gutierrez V (2006) Triterpenic Compounds from "Orujo" Olive Oil Elicit Vasorelaxation in Aorta from Spontaneously Hypertensive Rats. J Agr Food Chem 54 (6):2096-2102

24. Rozner S, Garti N (2006) The activity and absorption relationship of cholesterol and phytosterols. Colloids and Surfaces A: Physicochemical and Engineering Aspects 282–283 (0):435-456

25. Gould RG, Jones RJ, LeRoy GV, Wissler RW, Taylor CB (1969) Absorbability of β-sitosterol in humans. Metabolism 18 (8):652-662

26. Miettinen TA, Vanhanen H (1994) Serum concentration and metabolism of cholesterol during rapeseed oil and squalene feeding. Am. J. Clin. Nutr. 59 (2):356-363

27. Ellegård LH, Andersson SW, Normén AL, Andersson HA (2007) Dietary Plant Sterols and Cholesterol Metabolism. Nut Reviews 65 (1):39-45

28. Borg J, Toazara J, Hietter H, Henry M, Schmit G, Luu B (1987) Neurotrophic effect of naturally occurring long-chain fatty alcohols on cultured CNS neurons. FEBS Letters 213 (2):406-410

29. Borg J, Kesslak PJ, Cotman CW (1990) Peripheral administration of a long-chain fatty alcohol promotes septal cholinergic neurons survival after fimbria-fornix transection. Brain Res 518 (1–2):295-298
30. Marinangeli CP, Kassis AN, Jain D, Ebine N, Cunnane SC, Jones PJ (2007) Comparison of composition and absorption of sugarcane policosanols. British J Nut 97 (2):381-388
31. Somova LI, Shode FO, Ramnanan P, Nadar A (2003) Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from Olea europaea, subspecies africana leaves. Journal of Ethnopharmacology 84 (2–3):299-305
32. Lazzez A, Perri E, Caravita MA, Khlif M, Cossentini M (2008) Influence of Olive Maturity Stage and Geographical Origin on Some Minor Components in Virgin Olive Oil of the Chemlali Variety. J Agr Food Chem 56 (3):982-988
33. Preedy VR (2010) Bioactive Foods and Extracts: Cancer Treatment and Prevention.
34. Morin P, Daguet D, Coïc JP, Dreux M (1999) Usefulness of methylated-β-cyclodextrin-based buffers for the separation of highly hydrophobic solutes in non-aqueous capillary electrophoresis: Application to the separation of derivatized phytosterols. J Chromatogr A 837 (1–2):281-287
35. Abidi SL (2004) Capillary electrochromatography of sterols and related steryl esters derived from vegetable oils. J Chromatogr A 1059 (1–2):199-208
36. Lerma-García MJ, Simó-Alfonso EF, Ramis-Ramos G, Herrero-Martínez JM (2008) Rapid determination of sterols in vegetable oils by CEC using methacrylate ester-based monolithic columns. Electrophoresis 29 (22):4603-4611
37. Fernández-Arche A, Marquez-Martín A, Vazquez RdlP, Perona JS, Terencio C, Perez-Camino C, Ruiz-Gutierrez V (2009) Long-chain fatty alcohols from pomace olive oil modulate the release of proinflammatory mediators. J Nut Biochem 20 (3):155-162
38. Orozco-Solano MI, Priego Capote F, Luque De Castro MD (2011) Influence of deep frying on the unsaponifiable fraction of vegetable edible oils enriched with natural antioxidants. J Agric Food Chem 59 (13):7194-7202
39. Sánchez-Ávila N, Mata-Granados JM, Ruiz-Jiménez J, Luque de Castro MD (2009) Fast, sensitive and highly discriminant gas chromatography–mass spectrometry method for profiling analysis of fatty acids in serum. J. Chromatogr. A. 1216 (40):6864-6872
40. Orozco-Solano M, Ruiz-Jiménez J, Luque de Castro MD (2010) Ultrasound-assisted extraction and derivatization of sterols and fatty alcohols from olive leaves and drupes

prior to determination by gas chromatography–tandem mass spectrometry. J. Chromatogr. A. 1217 (8):1227-1235

41. Massart DL, Vandeginste BGM, Buydens LMC (1997) Handbook of Chemometrics and Qualimetrics. v. 20, parte 1. Elsevier

CAPÍTULO 7

Análisis de ácidos grados esterificados y no esterificados en suero de individuos obesos después de la ingesta de desayunos preparados con aceites sometidos a fritura simulada

Analysis of esterified and non-esterified fatty acids in serum from obese individuals after intake of breakfasts prepared with fried edible oils

Mara Isabel Orozco-Solano, Feliciano Priego-Capote and

María Dolores Luque de Castro

[a]*Department of Analytical Chemistry, Fine Chemistry Andalusian Institute, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain*

[b]*Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain*

Enviado a Analytical and Bioanalytical Chemistry para su publicación

Analysis of esterified and non-esterified fatty acids in serum from obese individuals after intake of breakfasts prepared with oils heated at frying temperature

M.I. Orozco-Solano, F. Priego-Capote, M.D. Luque de Castro[a]

Abstract In this study, levels of esterified and non-esterified fatty acids (EFAs and NEFAs, respectively) were compared in obese individuals (body mass index between 30–47 Kg m^{-2}) in basal state and after intake of four different breakfasts prepared with oils heated at frying temperature. The target oils were three sunflower oils —pure, enriched with dimethylsiloxane (400 μg mL^{-1}) as lipophilic oxidation inhibitor, and enriched with phenolic compounds (400 μg mL^{-1}) as hydrophilic oxidation inhibitors—, and virgin olive oil with a natural content of phenolic compounds of 400 μg mL^{-1}. The intake of breakfasts was randomized to avoid trends associated to this variability source. EFAs and NEFAs were subjected to a sequential derivatization step for independent GC–MS analysis of both fractions of metabolites in human serum. Derivatization was assisted by ultrasonic energy to accelerate the reaction kinetics, as demanded for high-throughput analysis. Statistical analysis supported on univariate (multifactor ANOVA) and multivariate approaches (PCA and PLS-DA) allowed identifying the main variability sources and also discriminating individuals after intake of each breakfasts. Individuals samples after intake of breakfasts prepared with virgin olive oil were clearly separated from those who ingested the resting breakfasts. The main compounds contributing to discrimination were omega-3 and omega-6 EFAs with special emphasis on arachidonic acid and eicosapentaenoic acid (EPA). These two polyunsaturated fatty acids (PUFAs) are the precursors of eicosanoid metabolites which are of vital importance as they play important roles in inflammation and in the pathogenesis of vascular and malignant diseases as cancer.

Keywords: Deep frying · Dietary intervention · Serum · Esterified fatty acids · Non-esterified fatty acids · Targeted nutrimetabolomics Principal components analysis

Introduction

Dietary fat plays a main nutritional role as a key source of energy despite the concerns about fat intake in developed countries, in which dietary fat has been documented as a relevant risk factor to suffer coronary heart diseases [1,2]. A study including seven countries suggested that the Mediterranean diet, which is rich in monounsaturated fatty acids (MUFA) with olive oil as main fat, is associated with low cardiovascular disease rates [3]. Likewise, epidemiological studies have shown an inverse correlation between the intake of long chain n-3 polyunsaturated fatty acids (PUFA) and the incidence of cardiovascular diseases such as atherosclerosis [4]. In fact, eicosapentaenoic acid (EPA) is currently recommended by the American Heart Association to reduce the risk for coronary heart disease (CHD) [5]. The presence of saturated fatty acids (SFA) has also been a matter of study [3]. Nutritional tests have shown that replacing SFA (C12:0–C16:0) with PUFA decreases LDL cholesterol concentration and the total/HDL cholesterol ratio. A similar but less significant effect has been observed by replacing SFA by MUFA [5].

Deep fat frying has become a worldwide way of cooking food. Fried foods are consumed with sustainable popularity despite their considerable fat content and the consumer's awareness of the relationship between food, nutrition and health. Deep frying involves negative aspects that can be harmful for health because the quality of oils and prepared food is affected by this process. The quality of oils used for frying is a function of degradation mechanisms taking place under heating. Three degradation processes occur during frying: hydrolysis, oxidation and isomerization. Hydrolysis of fat produces diacyl glycerides and free fatty acids; the former can further be hydrolyzed into monoglycerides and ultimately into glycerol and fatty

acids [6-8]. Complex oxidation reactions take place during frying with formation of products which may include oxidized monomeric, dimeric, and oligomeric triglycerides as well as volatile components such as aldehydes, ketones and hydrocarbons. The last compounds are volatilized and removed from the oil by steam generated during deep frying. Oils exposed to high temperature in the presence of air give rise to thermal alteration products such as cyclic fatty-acid monomers, geometrical fatty-acid isomers, non polar dimeric and oligomeric triglycerides [8,9].

Fat degradation results in loss of nutritional value of food as well as in changes of physiological properties [10], which cause rejection from consumers mainly owing to the effect on human health of the products formed under frying conditions. The relationship between the long-term consumption of lipid oxidation products and human health is not clear, but it is generally recognized that over-used and abused oils undoubtedly contain oxidized material, which if chronically consumed in large amounts could pose human health risk. Among the negative effects, it is worth mentioning cytotoxicity, atherogenicity, mutagenicity, carcinogenicity and metabolic interferences such as obesity [11]. There are few studies on the relationship between fried food and obesity [12,9].

Diet quality and physical exercise are the two main stays for treatment of obesity. There is a direct relationship between obesity and serum levels of fatty acids [13]. Therefore, it is expected that monitoring fatty acids can provide evidences about the influence of diet and other factors such as the lack of physical activity, genetic susceptibility, and endocrine disorders on obesity. Concerning the mechanisms involved in the occurrence of obesity, inflammation has been found as one of the most relevant processes, which plays a key role in the metabolic consequences of obesity as well as in other chronic degenerative conditions [14]. The inflammation is primarily driven by the production of pro-inflammatory fatty acids derived from arachidonic acid, the levels of which are entirely controlled by the diet [15,16]. Obese

individuals have prolonged elevations in postprandial lipemia and an exacerbated inflammatory response to high fat meals, which can increase the risk for cardiovascular diseases [17]. This last group and type 2 diabetes are associated with obesity and are also linked to inflammation and oxidative stress [18].

The determination of fatty acids is commonly carried out by gas chromatography (GC) separation as methyl ester derivatives (fatty-acid methyl esters, FAMEs) with possibility of individual separation of *cis/trans* isomers [9,18,19]. Concerning sample preparation, derivatization of fatty acids is mandatory for appropriate chromatographic separation. FAMEs prepared at room temperature by alkali-catalyzed transesterification are currently used for fatty-acid profiling by GC–FID [9]. Direct transmethylation, butylation, silylation, reaction with hydroxides such as tetramethylammonium hydroxide and trimethylphenylamine hydroxide have been employed as derivatization agents for profiling FAs [20,10,21,22]. Fatty acids also can be derivatized with 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide, forming 3-acyloxymethyl-1-methylpyridinium iodide [21]. Apart from that, the method can be completed by discrimination between esterified and non-sterified fatty acids (EFAs and NEFAs, respectively), which involves a sequential derivatization step for dual analysis of both groups of metabolites.

The research here presented is focused on determining the effect of the intake of vegetable oils heated at frying temperature with a natural or added content of oxidation inhibitors on the fatty acids profile of obese individuals. For this purpose, a simple and fast method based on sequential methylation of EFAs and NEFAs by using ultrasonic energy to enhance the kinetics of the derivatization step prior to CG–MS analysis was planned. Statistical analysis by univariante and multivariate approaches was also planned to be used to find potential differences in the fatty acids profiles as a consequence of the intervention breakfasts.

Material and methods

Chemicals and reagents

Chromatographic grade methanol, *n*-hexane and heptane were provided by Panreac (Barcelona, Spain) for the different steps of the analytical protocol. Anhydrous sodium sulfate from Sigma–Aldrich (Steinheim, Germany) was used as drying agent for the non-polar phase in the derivatization process. Potassium hydroxide and sulfuric acid, also from Sigma–Aldrich, were used in the derivatization process.

Fatty acid methyl esters (FAMEs) were purchased from Sigma–Aldrich to be used as analytical standards in the determination of fatty acids. Each individual stock standard solution was prepared as follows: high-purity fatty acid standards ranging from C12 to C20 compounds were diluted in *n*-hexane, and those from C22 to C28 compounds were diluted in heptane. Stock standard solutions were stored at −20 °C. A mixture of the standard solutions in either *n*-hexane or heptane containing all fatty acids in the range 0.05–50 μg/mL was daily prepared by mixing aliquots of each individual solution. The samples were spiked with an internal standard (IS, C19:1 methyl ester) at 5 μg mL^{-1}.

Apparatus and instruments

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz and 450 W) equipped with a cylindrical titanium alloy probe (12.70 mm diameter). A thermostated water bath and a vortex from Selecta (Barcelona, Spain) were used in the reference derivatization step. An Eppendorf Concentrator 5301 (Hamburg, Germany) was used for solvent evaporation.

A Varian CP 3800 gas chromatograph coupled to a Saturn 2200 ion-trap spectrometer (Varian, Sugar Land, TX, USA) was used to carry out GC–MS analyses for determination of FAMEs. A SP™-2380 fused silica capillary column (60 m × 0.25 mm I.D., 0.2 μm film thickness) provided by Supelco (Bellefonte, PA, USA) was used as analytical column.

Instrument control and mass spectrometry data were managed by a personal computer running the Saturn GC–MS WorkStation software (6.30 version).

Oils and heating procedure

The four edible oils used for this study were prepared as follows:

(1) Extra virgin olive oil (VOO). Different virgin olive oils were mixed up to a final concentration of phenolic compounds of 400 μg mL^{-1} equivalent to caffeic acid by application of the Folin−Ciocalteu (F–C) test. The final composition in fatty acids was: 70.5% monounsaturated fatty acids (MUFAs), 11.1% PUFAs, 18.4% saturated fatty acids (SFAs).

(2) Refined high-oleic sunflower oil enriched with an extract of phenols isolated from olive pomace by the protocol described by Girón *et al.* [23] (PSO). The enrichment was carried out up to a final concentration of 400 μg mL^{-1} total phenols, expressed as caffeic acid. The fatty acids composition of this oil was: 67.7% MUFAs, 17.6% PUFAs and 5.8% SFAs.

(3) Refined high-oleic sunflower oil spiked with dimethylsiloxane as synthetic oxidation inhibitor (DSO) at 400 μg mL^{-1}. The fatty acids composition was: 71.8% MUFAs, 18.0% PUFAs and 10.2% SFAs.

(4) Pure refined high-oleic sunflower oil with nil content in phenolic compounds (SO), which reported the following fatty acids profile: 34.3% MUFAs, 58.3% PUFAs and 7.3% SFAs.

A volume of 2 L of each oil was placed in a stainless-steel deep fryer, and heated at 180ºC ± 5 ºC for 20 cycles to mimic a conventional frying process. The enrichment step of the refined edible oils had the aim of enhancing oils stability and improving their healthy properties.

Cohort selected for the study

A total of 26 obese individuals (body mass index in the range 29.37–46.88 Kg m^{-2}) were recruited for this study. The cohort was formed by 19 female and 7 male individuals with average age of 56±7.1. They received four breakfasts consisting of skimmed milk and muffins prepared with each of the four oils heated at frying temperature (0.45 mL of oil per kilogram of body weight). The administration of each breakfast was held at randomization and following a cross Latin square design. The volunteers ate one of the breakfasts every two weeks (4 oils, 8 weeks). Sampling was programmed just before intake of each breakfast (basal state) and 2 and 4 h after intake (post-basal states). During the sampling period (4 h after intake) the volunteers did not consume any other food.

Sample collection

Programmed sample collection was performed by the Blood Donors Department of Reina Sofia Hospital (Córdoba, Spain). All steps from blood extraction to storage were developed according to the guidelines dictated by the World Medical Association Declaration of Helsinki (2004). The study protocol was approved by the Reina Sofia University Hospital's Human Research Review Committee, and complied with both institutional and "Good Clinical Practice" guidelines. Individuals selected for this study were informed to obtain consent prior to sample extraction. Venous blood was collected into a plastic Vacutainer® tube from Becton Dickinson (Franklin Lakes, NJ, USA) without additives. Blood samples were processed within 1 h after collection and centrifuged at 4000 × g for 10 min to isolate serum, which was then placed in plastic tubes and stored at –80 ºC until analysis.

Extraction and derivatization procedure

The method reported by Sánchez-Ávila *et al*. [24] for analysis of EFAs and NEFAs was used in this study. It consists of a two-step methylation for sequential analysis of EFAs and NEFAs in serum without necessity of a step for protein removal. For this purpose, 50 μL serum was

spiked with 5 μg mL^{-1} IS in reaction tubes and vortexed with 0.5 mL of 0.4 M KOH–CH_3OH for 30 s. Then, the tube was placed in a water bath in which the ultrasound probe was also plunged, and the reaction mixture was ultrasonically irradiated (duty cycle 0.3 s, 30% output of the converter amplitude) for 5 min. After derivatization, extraction was carried out twice using 0.5 mL *n*-hexane each time for removal of EFA methyl esters; then, the organic extractant was evaporated to dryness and the solid residue from the extract reconstituted in 500 μL *n*-hexane. The remaining polar phase was put into contact with anhydrous sodium sulfate to remove traces of water and then, 0.5 mL of 1 M H_2SO_4–CH_3OH was added for the second derivatization step. The reaction tube and the ultrasonic probe were introduced again in the water bath and the reaction mixture was ultrasonically irradiated (duty cycle 0.8 s, output 80% of the converter amplitude) for 15 min. The mixture was extracted twice with 1 mL *n*-hexane using a vortex for 30 s to obtain the NEFA methyl esters. The resulting solution was evaporated to dryness and reconstituted with 250 μL *n*-hexane prior to determination by GC–MS.

Gas chromatography–mass spectrometry analysis

High-purity helium (99.9%) at 1 mL min^{-1} was used as carrier gas. Sample injection was carried out by programmed temperature vaporization (PTV) as follows: initial temperature 70 °C, held for 0.2 min, increased at 100 °C min^{-1} to 250 °C, and then kept for 70 min. The injection was in the split–splitless mode. The splitter was opened (100:1) for 0.5 min, closed for 3.5 min and then opened at 100:1 split ratio for 10 min.

The samples were analyzed using the following oven temperature program: initial temperature 70 °C (held for 1 min), increased by 25 °C min^{-1} to 120 °C and followed by a second gradient of 2 °C min^{-1} to 243 °C and, finally, increased at 30 °C min^{-1} to 260 °C and held at this temperature for 5 min.

Electron impact ionization (EI) positive-mode was used in the ion-trap mass

Compound name	Formula	Mw	Retention time	Segment	Ions for quantification

spectrometer. The instrumental parameters were set at the following values: the manifold, trap and transfer line temperatures were set at 60, 170 and 200 °C, respectively. The analyses were performed with a filament–multiplier delay of 11 min while the emission current of the filament was set at 80 μA. Data acquisition was performed in full scanning and single ion monitoring (SIM) modes. The mass range in full scan mode was *m/z* 40–650 with a background mass of *m/z* 45. The scan time for data acquisition was set at 1.0 s with 3 microscans s^{-1} in full scan mode, while in SIM mode the scan time was optimized for each segment as a function of the range of mass under study. The values of *m/z* fixed for each monitored ion in SIM mode are listed in Table 1.

Statistical analysis

Concentration data for each target analyte were log transformed for normalization, when appropriate, to meet the assumption of the statistical tests. Descriptive statistical analysis was carried out by univariate and multivariate methods by using Statgraphics Plus software version 5.10 from Manugistics (Rockville, MD, USA) and Unscrambler software version 7.6 (Oslo, Norway).

Table 1. GC–MS parameters for determination of fatty acids as methyl ester derivatives

Methyl laurate	C12:0	214.32	17	2	74, 86.9, 129, 143, 175, 215
Methyl myristate	C14:0	242.37	22.07	3	87, 143, 199, 243
Methyl palmitate	C16:0	270.42	26.1	4	87, 143, 227, 271
Methyl palmitoleate n9	C16:1n9	268.41	30	5	81, 264, 269
Methyl palmitoleate n7	C16:1n7	298.41	30	5	
Methyl stearate	C18:0	296.48	32.21	8	67, 95, 143, 249
Methyl elaidate	C18:1n9t	296.46	34.46	9	87, 141, 264, 297
Methyl oleate	C18:1n9c	296.46	36.8	9	87, 141, 264, 297
Methyl *cis* vaccenate	C18:1n7c	296.46	38.18	9	87, 141, 264, 297
t9t12 methyl linoleate	t9t12 C18:2	294.45	39.2	10	67,81,95
c9t12 methyl linoleate	c9t12 C18:2	294.45	39.77	10	
t9c12 methyl linoleate	t9c12 C18:2	294.45	40.1	10	
c9c12 methyl linoleate	c9c12 C18:2	294.45	40.4	10	
Methyl nonadecenoate n10	C19:1 (IS)	312.53	41.8	11	67, 81, 95, 245, 263
t9t12t15 methyl linolenate	t9t12t15 C18:3	292.46	42.54	12	67, 81, 95, 241
t9t12c15 methyl linolenate	t9t12c15 C18:3	292.46	42	12	
t9c12t15 methyl linolenate	t9c12t15 C18:3	292.46	42	12	
c9t12t15 methyl linolenate	c9t12t15 C18:3	292.46	42.45	12	
c9t12c15 methyl linolenate	c9t12c15 C18:3	292.46	42.95	12	
t9c12c15 methyl linolenate	t9c12c15 C18:3	292.46	42.1	12	
c9c12c15 methyl linolenate	c9c12c15 C18:3	292.46	42.5	12	
Methyl eicosenoate	C20:1n9	324.51	44.6	12	67, 79, 81, 261,288
Methyl eicosadienoate	C20:2n6	322.50	46.8	13	67, 81, 95, 290, 322
Methyl eicosatrienoate	C20:3n6	320.48	47.4	14	67,81, 95, 149, 310
Methyl behenate	C22:0	354.58	48.8	14	67, 79, 81, 95, 149, 169
Methyl araquidonate	C20:4n6	326.53	50	15	67, 79, 91, 105, 203, 319
Methyl docosadienoate	C22:2n6	719.19	52.1	17	79, 91, 105, 131, 350
Methyl eicosapentaenoate	C20:5n3	316.45	52.8	17	79, 91, 105, 133
Methyl tetracosenoate	C24:0	380.62	54.1	18	73, 131, 355
Methyl docosatetraenoate	C22:4n6	346.50	56.2	19	91, 131, 143, 199, 293, 384
Methyl docosahexanoate	C22:6n3	342	56.1	21	91, 119, 199, 294, 437
Methyl docosapentanoate	C22:5n3	344.50	59.1	20	91, 131, 143, 199, 293, 384
Methyl octacosanoate	C28:0	438.74	60.0	22	91, 119, 199, 291, 445

Results and discussion

Optimization of gas chromatographic separation

Chromatographic separation was carried out with an SP™-2380 fused silica capillary column allowing separation of *cis/trans* isomers. Optimum separation was tested by using a commercial standard mixture of the target analytes. Identification of the chromatographic peaks from the samples was supported on comparison of mass spectra and retention times and compared with the method reported by Sánchez-Ávila *et al.* [24]. The complete separation of the target analytes was obtained after 63 min, as shows the chromatogram in Fig. 1 obtained by analysis of serum from a specific individual sampled at 4 h after the intake of each breakfast. Nevertheless, some groups of analytes such as C16:1n9/C16:1n7, t9t12t15 C18:3n3/t9c12t15 C18:3n3, and c9t12t15 C18:3n3/c9c12t15 C18:3n3 were partially overlapped; therefore, they were quantified together. Table 1 lists the main parameters for quantitative analysis of fatty acids applied to the determination of EFAs and NEFAs.

Characteristics of the cohort selected for the study in basal state

Table 2 lists the characteristics of the selected cohort in basal state, which includes the anthropometric characteristics (weight, height, age, gender, body mass index and waist perimeter) as well as the mean concentration, standard deviation and concentration range detected for EFAs and NEFAs before intake of the prepared breakfasts. The most common parameter to evaluate the occurrence of obesity is the body mass index (BMI), which takes into account weight and height of individuals. Pre-obese state is associated to overweight individuals with BMI between 25 and 30 Kg m^{-2}, while the obese state is ascribed to BMI equal or above 30 Kg m^{-2} [25]. Data are presented depending on the individuals' gender, which enables to set concentration ranges for obese individuals. The samples were injected in triplicate resulting in RSD values lower than 9.5%, which are in agreement with the precision

test carried out by Sánchez-Ávila *et al*. [24]. The concentrations of EFAs and NEFAs in basal state were characterized by a normal distribution with standardized skewness and kurtosis coefficients below ±2. Supported on normal distribution, the *t*-Student test allowed detecting non-significant differences in fatty acids levels for male and female individuals. Therefore, it was unnecessary to split the data set into two sub-groups to continue statistical analysis with gender discrimination.

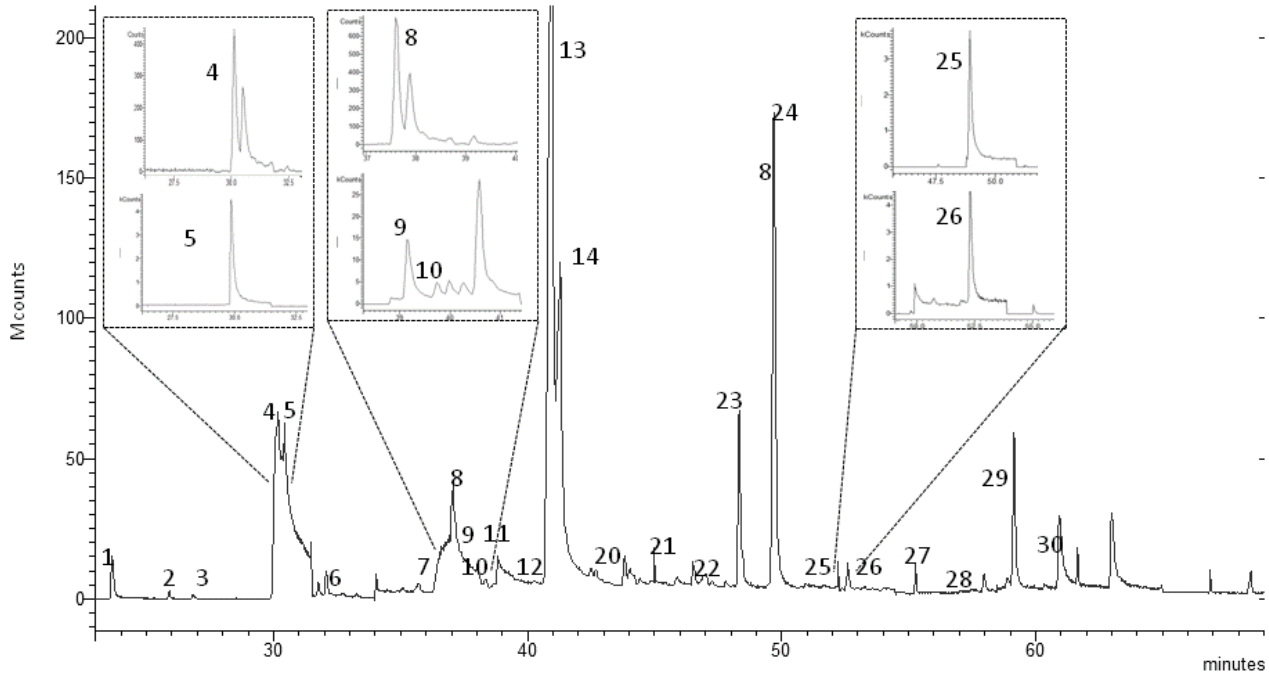


Figure. 1*. Total ion chromatogram obtained by GC–MS analysis of serum from an individual sampled at 4 h after intake of each breakfast: (1) C12:0; (2) C14:0; (3) C16:0; (4) C16:1n9 and C16:1n7; (5) C18:0; (6) C18:1n9t; (7) C18:1n9c; (8) C18:1n7; (9) t9t12 C18:2; (10) c9t12 C18:2; (11) t9c12 C18:2; (12) c9c12 C18:2; (13) IS, C19:1; (14) t9t12t15 C18:3; (15) t9t12c15 C18:3 and t9c12t15 C18:3; (16) c9t12t15 C18:3 and c9c12t15 C18:3; (17) c9t12c15 C18:3; (18) t9c12c15 C18:3; (19) c9c12c15 C18:3; (20) C20:1n9; (21) C20:2n6; (22) C20:3n6; (23) C22:0; (24) C20:4n6; (25) C22:2n6; (26) C20:5n3; (27) C24:0; (28) C22:4n6; (29) C22:5n3; (30) C28:0. EIC chromatograms for seven metabolites are zoomed.*

Fatty acids concentration is strongly dependent on both diet and habits. Among the different methods used for determination of fatty acids most of them have been aimed at EFAs profiling, which only required the first derivatization step of the whole protocol. The potential source of variability caused by the derivatization step should be taken into account, particularly when the Human Metabolome Database (HMDB) is used as it only includes fatty acids levels in

serum obtained by GC–MS using a silylation procedure. Therefore, it could be particularly complicated to compare serum levels of fatty acids reported from different studies if they were carried out with different protocols.

After this preliminary evaluation, correlation analysis was carried out to find associations between the concentrations of the target fatty acids in serum with statistical significance set above 99% confidence level ($p < 0.01$). High correlation was found for most pairs of fatty acids, which is in agreement with that observed in other studies such as that carried out by Yang *et al.* [26] who found significant correlation in serum fatty acids levels (high values in obese individuals) taking into account age, gender and BMI.

Multifactor analysis of variance (Multi-ANOVA)

Statistical analysis was applied to detect differences in the profile of fatty acids in the cohort selected as a function of the ingested breakfast. The first test was to evaluate the influence of anthropometric variables such as age, gender and BMI, together with the type of breakfast ingested, on the variability of serum levels of fatty acids. For this purpose, a multifactor ANOVA was selected to evaluate the contribution of each variable due to normal distribution of all analytes. Two of the variables, age and BMI, were stratified to divide individuals into four and five groups, respectively, with similar size. Thus, individuals were classified into 4 groups according to age: 39–52, 53–57, 58–64 and 64–70 years old, while BMI was stratified as follows: 29.37–33.31, 33.96–34.96, 36.14–38.16, 39.08–41.29 41.66–46.88 Kg m^{-2}. This strategy allowed obtaining Gaussian distributions for all fatty acids monitored in this study (skewness and kurtosis coefficients in the range 0 ± 2), which are essential for application of the statistical plan.

Table 2. Anthropometric characteristics and average concentrations of the target analytes found in basal state for the selected cohort. Serum concentrations of target metabolites are expressed as µg/mL (expressed as mean ± standard deviation).

Parameter/analyte	EFAs				NEFAs			
	Female* Mean ± SD	Range	Male* Mean ± SD	Range	Female* Mean ± SD	Range	Male* Mean ± SD	Range
Age (years)		39 – 63		48 – 70		39 – 63		48 – 70
Weight (Kg)		79 – 125		69 – 120		79 – 125		69 – 120
Height (cm)		164 – 176		146 – 164		164 – 176		146 – 164
Waist perimeter (cm)		104 – 145		88 – 140		104 – 145		88 – 140
Body mass index (Kg m^{-2})		29.4 – 41.3		30.7 – 46.9		29.4 – 41.3		30.7 – 46.9
C12:0	4.9 ± 1.1	3.7 – 5.6	4.5 ± 0.2	4.1 – 4.9	1.4 ± 0.2	1.0 – 1.8	1.3 ± 0.2	0.7 – 2.5
C14:0	116.9 ± 2.7	111.3 – 120.7	108.8 ± 7.1	105.2-116.9	31.1 ± 4.1	28.2 – 44.0	28.6 ± 4.0	25.9 – 34.8
C16:0	275.4 ± 4.4	270.1 – 280.4	213.6 ± 5.8	205.5 – 221.2	80.7 ± 5.2	70.0 – 87.5	73.4 ± 6.2	60.5 – 81.2
C16:1n9 + C16:1n7	107.5 ± 6.7	100.1 – 115.3	95.3 ± 2	92.0 – 98.4	20.9 ± 1.5	18.0 – 31.2	22.8 ± 2.3	18.0 – 27.5
C18:0	417.7 ± 8.3	407.2 – 428.9	458.3 ± 5.2	452.0 – 564.2	58.8 ± 6.3	50.1 – 68.2	51.3 ± 7.5	40.2 – 62.0
C18:1n9t	169.8 ± 3.2	165.0 – 174.1	171.2 ± 4.2	165.7 – 177.8	43.1 ± 3.2	36.5 – 50.1	39.4 ± 0.7	35.4 – 43.1
C18:1n9c	183.3 ± 4.7	172.5 – 190.2	174.7 ± 7.1	163.5 – 182.0	44.2 ± 1.7	40.1 – 52.1	40.5 ± 1.4	37.5 – 44.0
C18:1n7	23.3 ± 1.7	20.5 – 25.8	14.1 ± 0.2	13.0 – 15.2	7.4 ± 0.6	6.0 – 10.9	6.4 ± 0.5	5.1 – 7.2
t9t12 C18:2	38.6 ± 2.5	35.4 – 42.1	54.2 ± 1.7	50.4 – 56.4	10.5 ± 0.3	9.6 – 11.4	13.4 ± 1.3	11.6 – 15.2
c9t12 C18:2	0.3 ± 0.1	0.1 – 0.6	0.1 ± 0.1	0 – 0.3	1 ± 0.1	0.5 – 1.4	0.9 ± 0.4	0.2 – 2.3
t9c12 C18:2	7.8 ± 0.9	6.8 – 9.0	8.7 ± 0.8	6.0 – 10.2	83.1 ± 1.7	80.5 – 87.0	72.6 ± 6.8	60.3 – 90.3
c9c12 C18:2	123.3 ± 8.3	114.5 – 134.0	99.3 ± 6.2	92.0 – 106.7	74.1 ± 2.2	70.2 – 78.1	67.5 ± 8.3	53.3 – 88.3
t9t12t15 C18:3	0.7 ± 0.2	0.3 – 1.0	0.6 ± 0.2	0.3 – 0.9	19.1 ± 0.8	15.2 – 21.3	20.7 ± 1.3	18.5 – 23.7
t9t12c15 C18:3+t9c12t15 C18:3	0.2 ± 0.1	0.1 – 0.5	0.2 ± 0.1	0.1 – 0.8	59.6 ± 1.3	56.2 – 62.1	52 ± 3	43.6 – 56.8
c9t12t15 C18:3+c9c12t15 C18:3	1.4 ± 0.3	1.0 – 1.9	1 ± 0.2	0.6 – 1.5	2.7 ± 0.5	1.8 – 3.8	2.3 ± 0.5	1.0 – 3.6
c9t12c15 C18:3	1.3 ± 0.3	0.8 – 2.1	1 ± 0.3	0.5 – 1.6	44.5 ± 0.7	41.0 – 47.4	40.8 ± 4.7	35.0 – 48.2
t9c12c15 C18:3	82.5 ± 1.6	79.2 – 85.8	67.3 ± 2.5	63.0 – 70.2	7.5 ± 1.2	5.2 – 10.0	6.4 ± 3.1	2.7 – 10.2
c9c12c15 C18:3	395.7 ± 7.3	384.8 – 404.1	334 ± 9.3	322.1 – 347.0	10.6 ± 0.3	9.4 – 11.2	13.6 ± 0.7	10.5 – 16.7
C20:1n9	4.2 ± 0.7	4.0 – 4.8	3.5 ± 0.8	2.2 – 5.7	0.1 ± 0.1	0.0 – 0.6	0.1 ± 0.06	0.01 – 0.3
C20:2n6	12.5 ± 0.5	10.1 – 15.3	14.5 ± 0.3	14.0 – 15.1	2.1 ± 0.5	1.0 – 3.5	1.9 ± 0.7	1.0 – 3.8
C20:3n6	5.8 ± 0.9	3.8 – 7.1	8.1 ± 1.2	6.0 – 10.3	28.4 ± 2.6	22.3 – 39.1	25.1 ± 5.8	18.0 – 36.3
C20:4n6	14.3±0.4	13.0 – 15.8	16.8±0.7	15.0 – 18.6	0.04±0.02	0.01 – 0.3	0.1±0.08	0.01 – 0.7
C20:5n3	325.9±3.1	320.2 – 331.8	317.4±6.1	311.5 – 324.1	0.2±0.2	0.07 – 0.21	0.1±0.1	0.08 – 0.14
C22:0	4 ± 0.4	3.0 – 5.1	3.1 ± 0.4	2.0 – 3.9	0 ± 0.1	0.0 – 0.5	N.D	N.D
C22:2n6	4.3±0.2	3.8 – 5.5	3.2±0.1	3.0 – 3.6	1±0.4	0.2 – 2.7	0.9±0.2	0.2 – 2.7
C22:4n6	11.3±0.6	10.3 – 12.4	7±0.5	6.4 – 7.9	91.1±7.2	82.2 – 100.3	84.3±9.4	72.5 – 100.1
C22:5n3	5.1±0.4	4.0 – 5.8	4.8±1.1	3.2 – 6.1	1.3±0.4	0.5 – 3.5	1±0.3	0.6 – 1.4
C22:6n3	92±0.5	90.7 – 93.1	105.3±5	99.2 – 115.9	3.9±0.3	2.5 – 5.2	4.2±1.6	1.7 – 7.6
C22:6n3	92±0.5	90.7 – 93.1	105.3±5	99.2 – 115.9	3.9±0.3	2.5 – 5.2	4.2±1.6	1.7 – 7.6
C24:0	10.2±1.2	8.1 – 12.2	7.7±4.1	3.2 – 12.1	16±1.3	12.3 – 20.8	13.5±2.1	10.0 – 17.3
C28:0	4.3±0.1	4.0 – 5.1	5.4±0.7	3.6 – 6.5	1.4±0.3	0.4 – 2.1	1.6±0.7	0.7 – 2.9

Concentrations below the detection limits were considered non-detected (N.D).

The multifactor ANOVA test was applied to the data set to estimate the contribution of each variability source in the profile of fatty acids in the studied cohort. The contribution of each factor is measured by removing the influence of all other factors and expressing it as a sum of squares. The values of sum of squares and the percent they represent in the total variability observed for each compound are shown in Table 3. This multivariate test revealed very significant contribution of the breakfast on the concentration of all EFAs and NEFAs detected in serum. In fact, the type of breakfast was the most influential variable to explain the variability of the fatty acid levels found in the cohort under study. Contribution of individuals' age was observed only for C22:5n3 and C20:5n3 as EFAs, and C22:5n3 and C22:2n6 as NEFAs. Gender and BMI variables did not contribute significantly to explain the levels of the monitored compounds.

Influence of the breakfast intake on serum levels of fatty acids

After identification of breakfast type as the main source of variability, multivariate analysis was used for qualitative comparison of EFAs and NEFAs profiles in the obese cohort. The first test was aimed at evaluating how the type of breakfast contributed to differentiate levels of fatty acids as the multifactor ANOVA concluded. First of all, a PCA was carried out including samples in basal state and after intake of the different breakfasts. The scores plot in Fig. 2 shows, a clear discrimination into four clusters of samples. Each cluster was formed by samples obtained after intake of each breakfast but also by samples in the basal state. As previously mentioned, breakfasts were administered following a randomized strategy; therefore, trends associated to the order of breakfast intake can be discarded. For this reason, this differentiation in the profile of fatty acids could be associated to two relevant effects. Firstly, a breakfast effect could be involved since samples corresponding to post-basal states are clearly separated.

Table 3. Multifactor ANOVA revealing the contribution of the different factors to explain serum levels of EFAs and NEFAs. Results are expressed as sum of squares and the percent each one represents in the total observed variability.

	EFAS				NEFAS			
Formula	**Age**	**Diet**	**Gender**	**Body mass index**	**Age**	**Diet**	**Gender**	**Body mass index**
C12:0	0.31(2.26)	0.08(0.57)***	1(7.35)	0(0.02)	0.16(0.07)	1.09(0.48)**	126.54(56.07)	5.99(2.65)
C14:0	0(0)	0.02(0)***	0(0)	301.15(65.64)	0(0.02)	0.09(0.76)***	0.74(5.97)	0.25(2.03)
C16:0	0(0)	0.21(0.2)***	0.11(0.11)	67.03(65.53)	0(0)	0.04(0.53)***	0.51(6.12)	0.24(2.84)
C16:1n9+c16:1n7	34035.4(0.26)	432927(3.28)***	128029(0.97)	7900010(59.81)	0(0.01)	0.03(0.32)***	0.43(5.45)	0.25(3.17)
C18:0	0.24(2.43)	0.07(0.67)***	0.62(6.32)	0(0.02)	1.56(0.3)	1.36(0.26)***	333.06(64.17)	3.92(0.75)
C18:1n9t	0.31(2.4)	0.74(5.74)**	0.11(0.88)	0(0.02)	2.7(1.11)	155.14(63.77)***	0.38(0.15)	0.52(0.22)
C18:1n9c	0.24(3.14)	0.03(0.34)***	0.43(5.53)	0(0)	0.05(0.01)	317.82(65.8)***	0.42(0.09)	1.41(0.29)
C18:1n7	0.23(2.85)	0.04(0.47)***	0.49(6.07)	0(0)	0.18(0.04)	287.12(66.2)***	0(0)	0.01(0)
c9t12 C18:2	0.25(2.04)	0.75(5.98)**	0(0.02)	0.09(0.75)	0.37(0.24)	102.32(66.81)***	0.01(0.01)	0.01(0.01)
c9c12 C18:2	0.4(3.7)	0.04(0.4)***	0.66(6.18)	0(0)	0.2(0.04)	340.35(65.95)***	0(0)	0.01(0)
t9t12c15 C18:3+t9c12t15 C18:3	1.46(2.21)	3.79(5.73)***	0.35(0.53)	0(0)	0.04(0.14)	0.29(1.05)***	21.25(10.91)	0.77(2.82)
c9t12t15 C18:3+c9c12t15 C18:3	0.23(2.41)	0.06(0.67)***	0.61(6.39)	0(0.02)	6.06(2.34)	146.36(56.53)***	0.55(0.21)	2.21(0.85)
t9c12c15 C18:3	0(0)	0.26(2.79)***	0.05(0.56)	0.57(6.18)	0(0)	0(0)***	304.03(66.07)	0.17(0.04)
c9c12c15 C18:3	0.26(2.79)	0.05(0.56)***	0.57(6.18)	0(0)	0.17(0.02)	748.37(66)***	0(0)	0(0)
C20:1n9	0.27(3.03)	0.04(0.47)***	0.53(5.93)	0(0)	0.98(2.46)	25.04(63.28)***	0(0.01)	0.16(0.39)
C20:2n6	0.3(2.46)	0.11(0.87)***	0.72(5.88)	0(0.02)	1.84(0.37)	327.97(65.87)***	0.07(0.01)	0.85(0.17)
C20:3n6	1.66(2.27)	0.41(0.56)***	4.3(5.88)	0.01(0.01)	12.03(2.97)	241.99(59.68)***	0.15(0.04)	2.2(0.54)
C20:4n6	0.3(2.48)	0.77(6.32)***	0(0.02)	0.08(0.66)	5.26(1.02)	340.17(66.14)***	0.04(0.01)	1.96(0.38)
C20:5n3	6.07(8.94)***	0.39(0.57)***	21.99(32.39)	0.06(0.08)	1.46(0.21)	468.92(66.58)***	0.02(0)	0.14(0.02)
C22:0	0.27(2.02)	0.1(0.77)**	0.81(6.15)	0.01(0.06)	2.92(1.02)	170.81(59.59)***	0.14(0.05)	0.8(0.28)
C22:2n6	0.26(3.35)	0.02(0.3)***	0.4(5.13)	0(0.01)	304.21(64.75)*	0.45(0.1)***	0.28(0.06)	1.25(0.27)
C22:4n6	190.3(46.69)	8.51(2.09)***	3.51(0.86)	1.85(0.45)	1.3(0.27)	330.92(69.32)***	0.08(0.02)	0.41(0.09)
C22:5n3	1.83(6.89)*	2.29(8.62)*	0.2(0.75)	0.09(0.34)	89.16(38.67)*	1.3(0.56)***	0.04(0.02)	8.64(3.75)
C22:6n3	0.27(2.88)	0.06(0.68)***	0.55(5.93)	0(0)	2.32(0.51)	272.07(59.87)***	0.11(0.02)	0.66(0.14)
C24:0	0.21(1.8)	0.04(0.3)***	0.64(5.37)	0.01(0.05)	0.31(0.11)	4.34(1.49)***	1.82(0.63)	194.74(66.86)
C28:0	0.25(2.85)	0.05(0.61)***	0.53(6.14)	0(0)	2.27(3.11)	27.64(37.76)***	0.12(0.16)	0.89(1.22)

*0.01 <P < 0.05; ** 0.001 < P< 0.01; *** P < 0.001

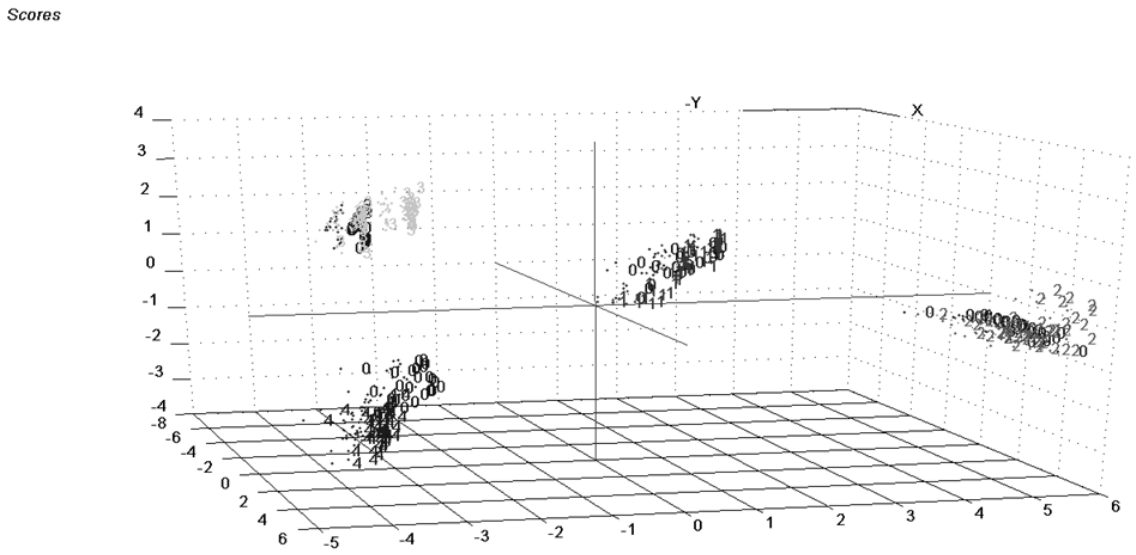
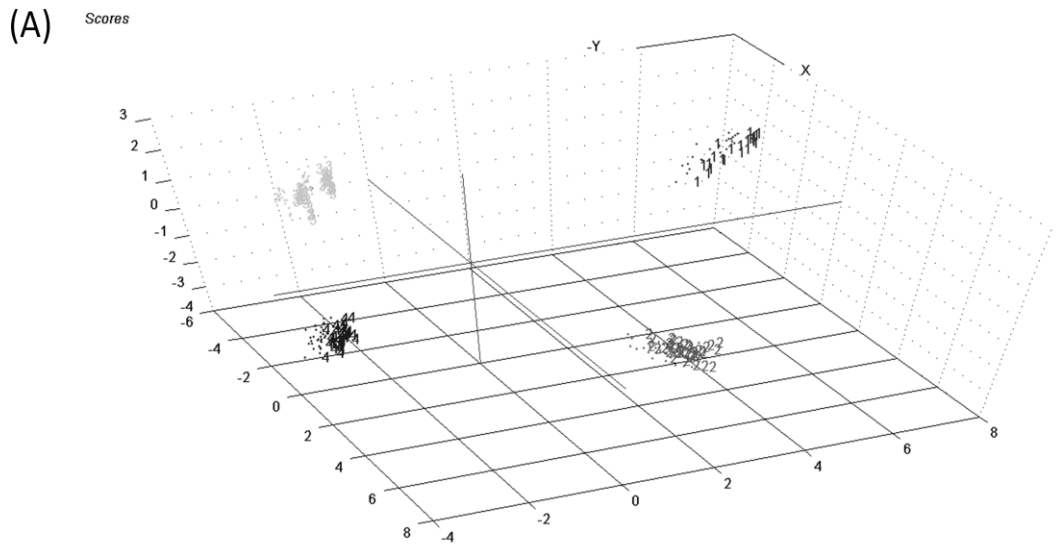


***Figure. 2**. Principal component analysis of serum samples from individuals after intake of prepared breakfast versus control individuals attending to levels of the monitored fatty acids. Number code is as follows: control inviduals before breakfast intake (0), and intake of breakfasts prepared with virgin olive oil (1), with sunflower oil enriched with antioxidants from olive pomace (2), with sunflower oil enriched with dimethylsiloxane (3), and with pure sunflower oil (4) after subjecting the oils to a simulated frying process.*

Nevertheless, there could also be due to within-individuals variability associated to the planned experimental study since basal states previous to intake of each breakfast were also discriminated. Attending to these results, the next statistical step was aimed at separation of both effects. For this purpose, samples from individuals in basal states were subtracted from samples in post-basal states to compare metabolic changes occurring as a result of breakfast intake. Figure 3.A shows a clear discrimination of individuals after intake of the four breakfasts. This separation was produced by combination of PC1–PC2, which explained a 84% of the total variability. Thus, discrimination of individuals after consumption of breakfasts containing hydrophilic oxidation inhibitors (VOO or PSO) from those individuals after intake of breakfasts prepared with sunflower oil (SO) or sunflower oil enriched with dimethylsiloxane (DSO) was observed along PC1 (*y* axis in plot). Complementarily, PC2 (*x* axis in plot) also

showed other discrimination by separation of individuals after intake of breakfasts prepared with DSO and VOO from those who consumed SO- or ASO-based breakfasts. If the loadings plot is examined (Fig. 3.B), the main compounds contributing to discrimination can also be divided into two groups depending on their contribution to explain variability in PC1 or PC2. The main compounds contributing to the discrimination observed along PC1 were esterified forms of omega-6 fatty acids such as C20:2n6, C20:3n6 and C20:4n6 (arachidonic acid); omega-3 fatty acids such as C20:5n3 and C22:6n3 (EPA and DHA, respectively); a monounsaturated fatty acid (oleic acid, C18:1n9); two saturated fatty acids such as stearic acid (C18:0) and octacosanoic acid (C28:0); and two *trans* isomers of C18:2 and C18:3 (t9t12 C18:2 and t9t12c15 C18:3, respectively). Apart from them, two non-esterified fatty acids such as C20:5n3 and C22:4n6 also contributed to explain the variability along PC1. Concerning the discrimination observed for PC2, only three esterified compounds were involved in the variability previously explained. Among them, C22:4n6 showed a behavior opposite to that of t9c12 C18:2 and t9c12c15 C18:3.



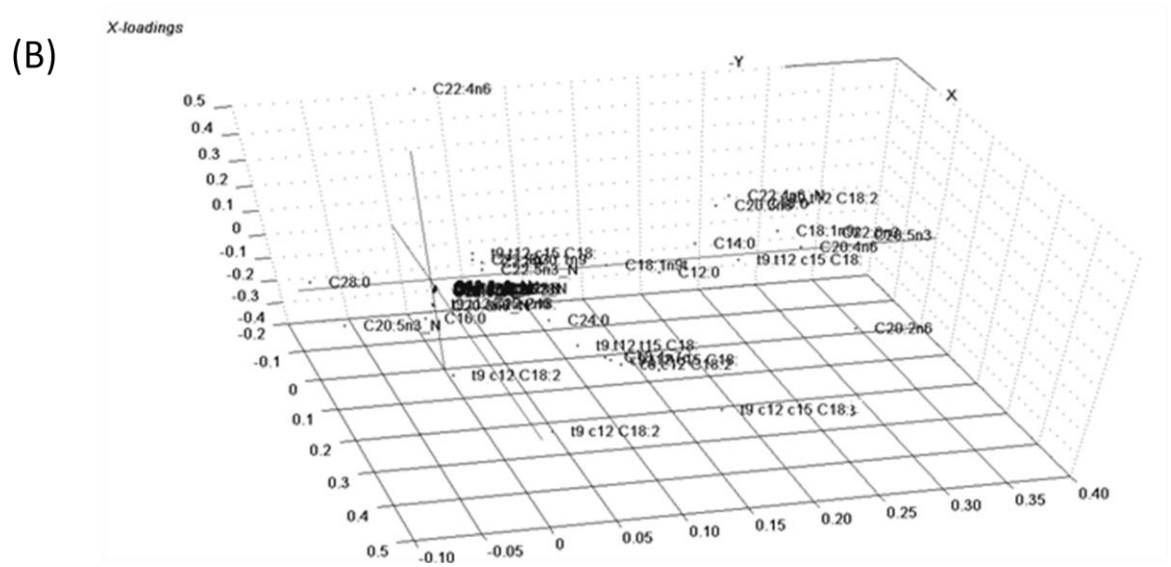
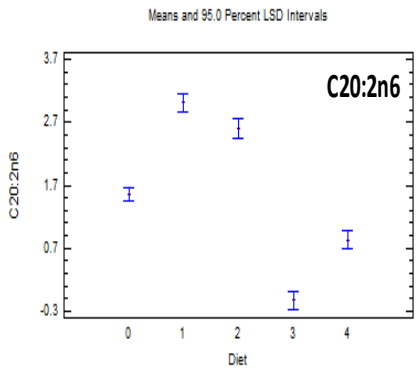


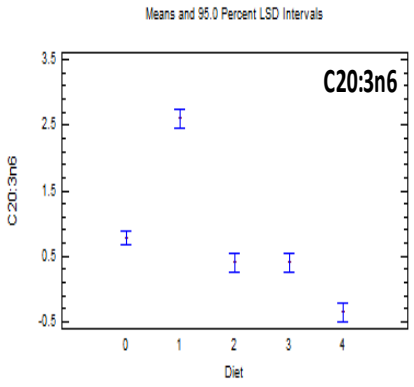
Figure 3. *Principal component analysis of serum samples from individuals after intake of prepared breakfasts attending to the levels of the monitored fatty acids. Scores graph (A). Number code is as follows: breakfasts prepared with extra-virgin olive-oil (1), sunflower oil enriched with antioxidants from olive pomace (2), sunflower oil enriched with dimethylsiloxane (3), Pure sunflower oil (4). Loadings graph (B).*

The discrimination capability obtained by PCA was confirmed by a supervised method based on partial least squares–discriminant analysis (PLS-DA) with cross-validation as validation strategy. Supplementary Fig. 1 shows the scores plot with the four clusters corresponding to each intervention breakfast. The resulting model was quite consistent since classification parameters were 98.54 and 98.41% of prediction capability for training and validation sets with RMSE (root mean square error) 0.1285 and 0.1354 for training and validation sets.

The discrimination capability of these compounds was independently evaluated by average levels plots comparing basal state and post-basal states for each breakfast. Figure 4 shows the plots for relevant EFAs pertaining to omega-6 and omega-3 families. As can be seen, all these fatty acids presented higher levels in individuals after intake of the breakfast prepared with heated VOO. These levels were even greater than those observed in individuals before

intake of breakfasts (control individuals). Additionally, serum levels of this panel of omega-6 and omega-3 fatty acids were higher in control individuals than in those who consumed breakfasts prepared with heated sunflower oil (SO, PSO and DSO), except for C20:2n6 and C22:6n3 (DHA). These two EFAs were present in individuals after intake of PSO-based breakfast at similar levels to those individuals after ingestion of VOO-based breakfast. Therefore, the enrichment with phenolic compounds seems to contribute to this particular trend. The lowest levels of C20:2n6, C20:4n6 (arachidonic acid) and C20:5n3 (EPA) were detected in individuals after intake of breakfast prepared with heated sunflower oil enriched with dimethylsiloxane followed by individuals after intake of the SO-based breakfast. Arachidonic acid and EPA are the precursors of eicosanoids such as leukotrienes, prostaglandins and hydroxyeicosatetraenoic acids (HETEs), which are involved in the inflammation cascade, affecting symptoms such as pain, fever and swelling [27,28]. Lower levels of arachidonic acid and EPA would be indicative of their metabolism by oxydation enzymes (ciclooxygenases, lipoxygenases amd P450 complex) to produce eicosanoid metabolites. However, it is now recognized that the interactions are complex, and prostanoids can act both in a pro- and anti-inflammatory manner according to the nature of the inflammatory stimulus and the specific prostanoid produced [28,29].





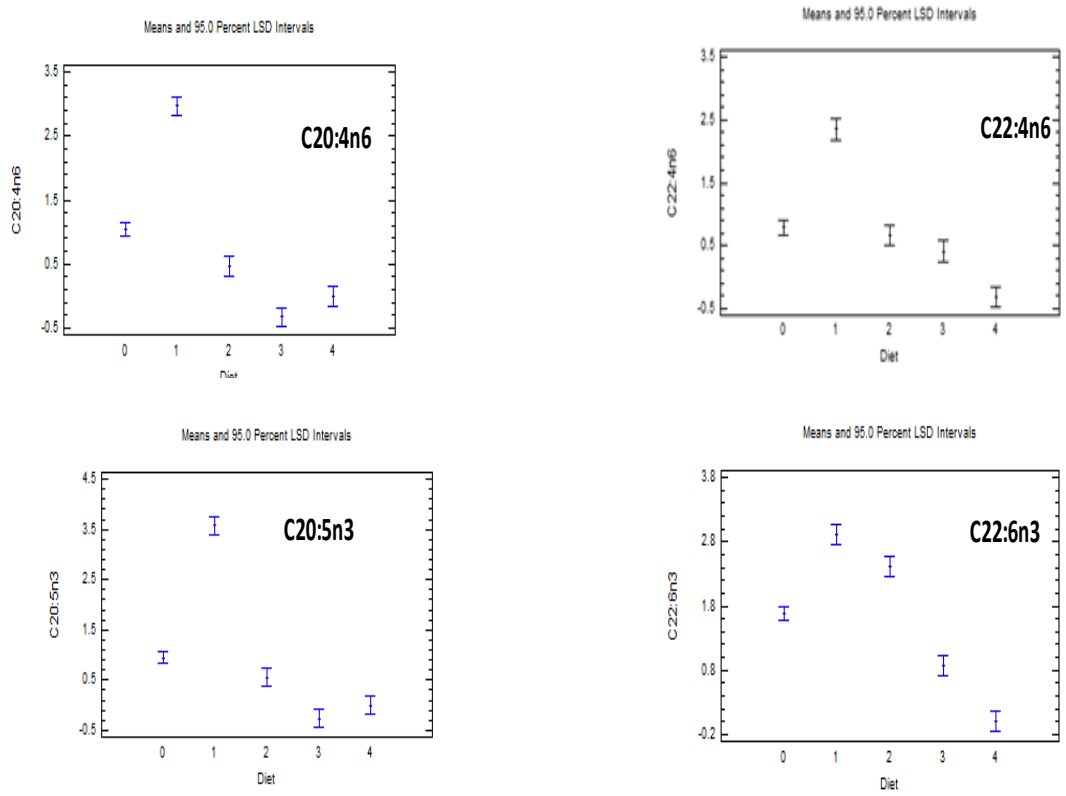
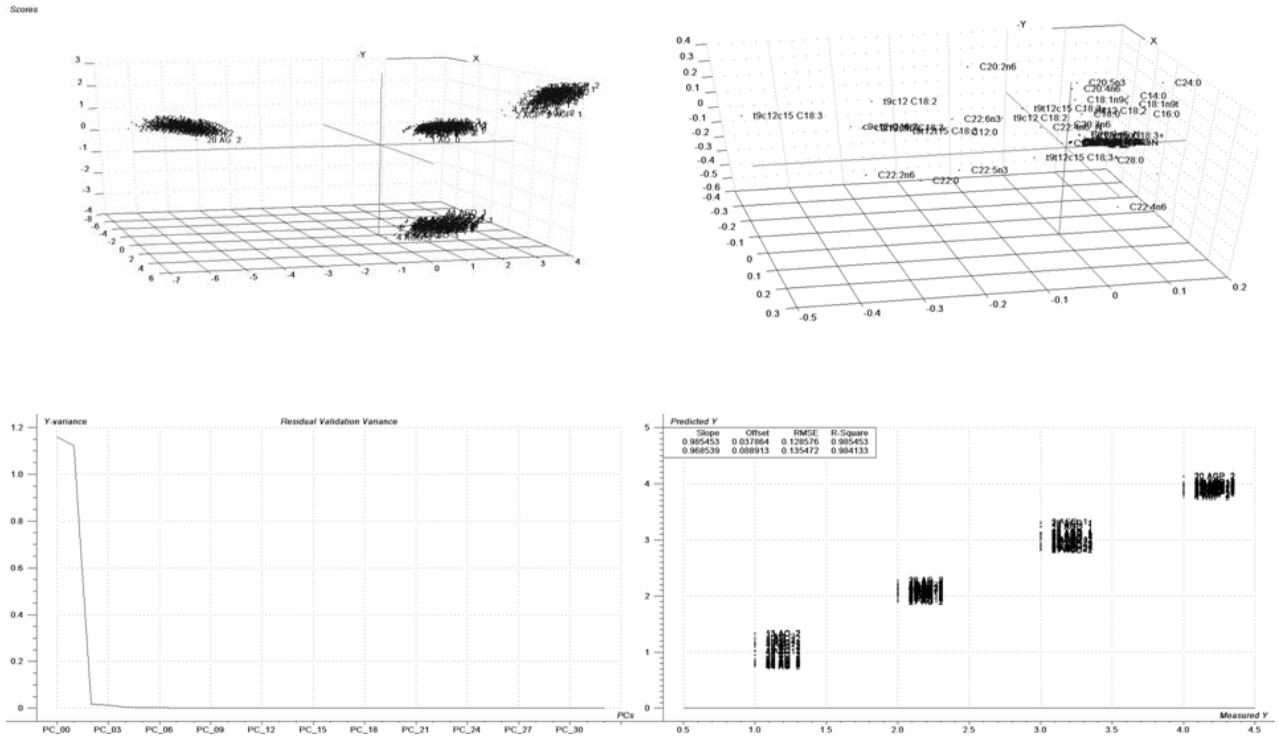


Figure 4. *Average levels plots for omega-6 and omega-3 EFAs detected in serum obtained from individuals at: (0) basal state; (1) VOO-based breakfast; (2) ASO-based breakfast; (3) DSO-based breakfast; SO-based breakfast.*

On the other hand, the lowest levels of esterified forms of C20:3n6 (dihomo-γ-linolenic acid), C22:4n6 (docosatetraenoic acid) and C22:6n3 (DHA) were detected in individuals after intake of SO-based breakfast. C20:3n6 is the precursor of arachidonic acid by action of a desaturase enzyme, while C22:4n6 is its metabolite formed by action of an elongase enzyme. The behavior of C20:3n6, C20:4n6 and C22:4n6 in the different classes revealed that the omega-6 pathway for metabolism of PUFAs was not affected by intake of breakfast prepared with VOO, while in the individuals after intake of SO- and DSO-based breakfasts there were evidences revealing changes in this metabolic pathway. Concerning DHA, this is the final product in the metabolism of omega-3 PUFAs. Similarly to the omega-6 pathway, that of omega-3 seems to be affected in those individuals after intake of SO-based and DSO-based

breakfasts since the serum levels of EPA and DHA in these two classes of individuals presented an opposite trend. Omega-3 fatty acids are characterized by their role in cell membrane functions, eicosanoid production and regulation of gene expression. Although there are studies which suggest a positive association between dietary C22:5n-3 and heart health [6,7], there are only a limited number of studies which have investigated the biological effects of pure C22:5n-3 and most of these studies have been conducted using either endothelial cells or platelets. Other biological effect of C22:6n3 and C20:5n3 is that both reduce the *in vivo* age-related oxidative changes [8].



Supplementary Figure 1. *PLS scores graph of serum samples from individuals after intake of each prepared breakfast supported on EFAs and NEFAs levels (A). Name code is as follows: intake of breakfasts prepared with extra-virgin olive-oil (VOO), sunflower oil enriched with antioxidants from olive pomace (PSO), sunflower oil enriched with dimethylsiloxane (DSO), pure sunflower oil (SO). Loadings graph (B). Residual validation variance scores (C). Response in y explained by the PLS model (D).*

It is also worth mentioning the role of other fatty acids that contribute to discrimination among the four groups of individuals. Thus, Figure 5 shows the discrimination capability of the esterified forms of C18:0 (stearic acid), C18:1n9 (oleic acid) and the *trans* isomer of linoleic

acid (t9t12 C18:2). Similar results were obtained for the three fatty acids by comparison of the levels detected in serum from individuals pertaining to the four classes considered as compared to control individuals. Thus, the highest levels were detected in individuals after intake of VOO-based breakfast while the lowest levels were detected for individuals after intake of SO-based breakfast, except for oleic acid which was detected at lower concentrations in individuals after intake of PSO- and DSO-based breakfasts.

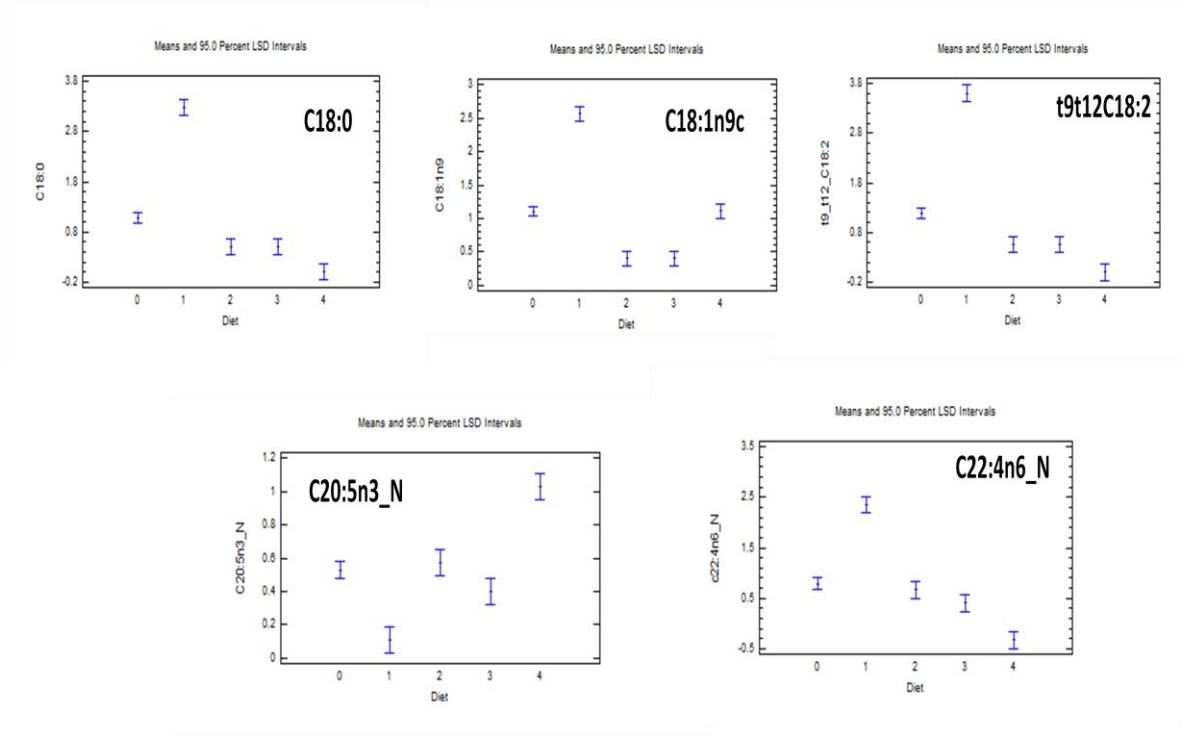
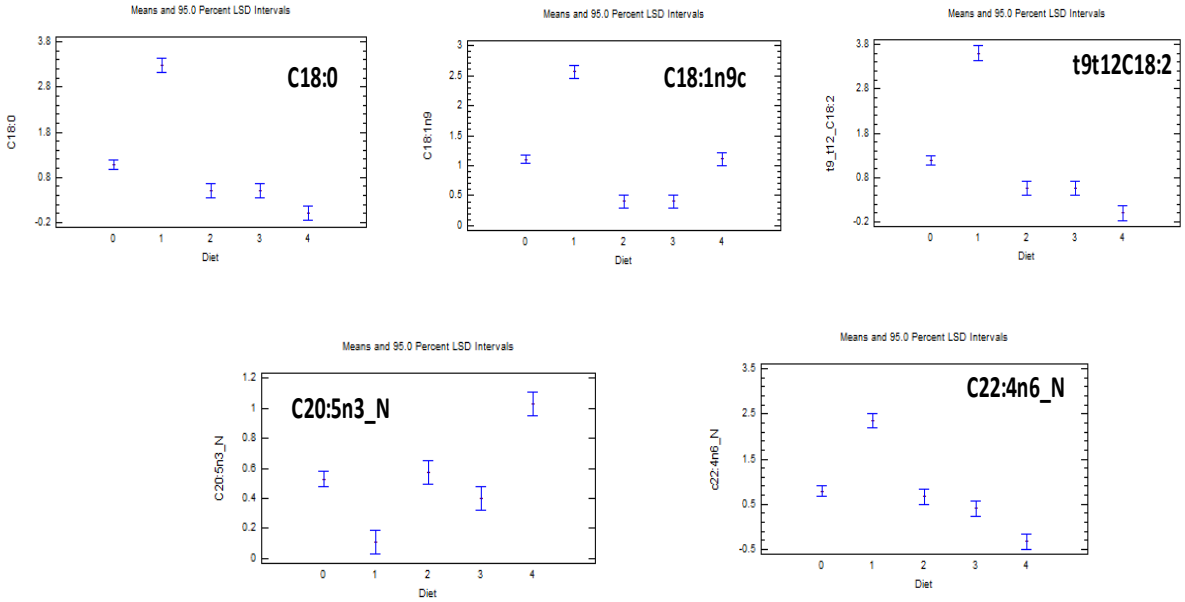


Figure 5*. Average levels plots for other representative fatty acids contributing to explain the observed variability in serum obtained from individuals at: (0) basal state; (1) VOO-based breakfast; (2) ASO-based breakfast; (3) DSO-based breakfast; SO-based breakfast.*

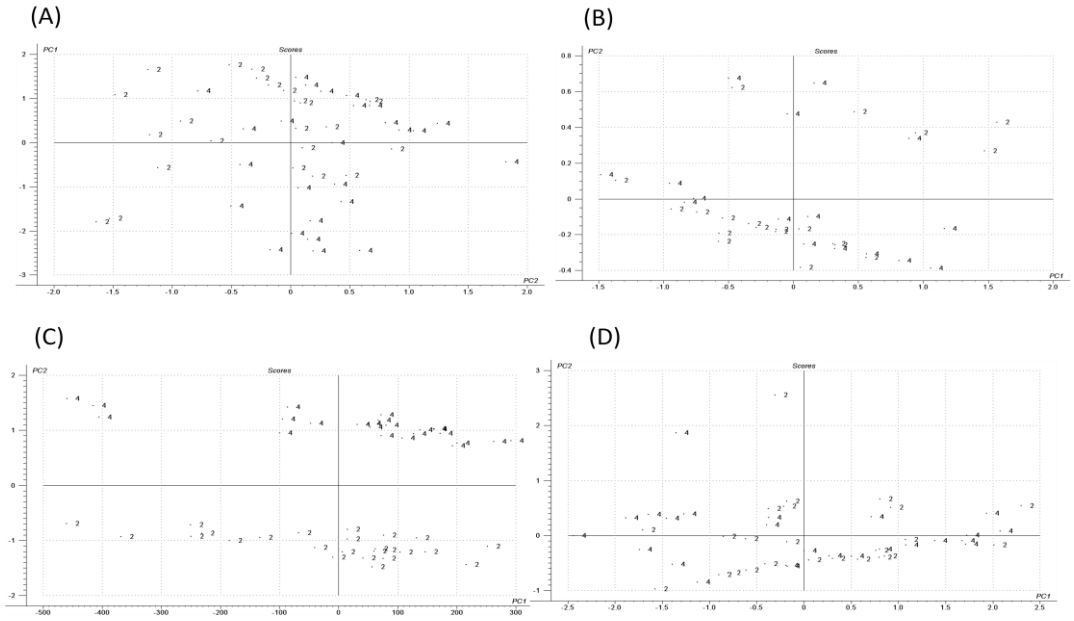
Concerning NEFAs only two compounds were detected with contribution to explain the variability observed: C20:5n3 and C22:4n6. The NEFA form of C22:4n6 presented a behavior similar to that of its EFA form. The situation was different for the NEFA form of C20:5n3 since this was more concentrated in individuals after intake of SO-based breakfast. Supplementary Fig. 2 shows the average level plots for other relevant compounds contributing to explain variability but without discrimination capability.



Supplementary Figure 2. *Average level plots for other representative fatty acids in serum obtained from individuals at: (0) basal state; (1) VOO-based breakfast; (2) ASO-based breakfast; (3) DSO-based breakfast; SO-based breakfast.*

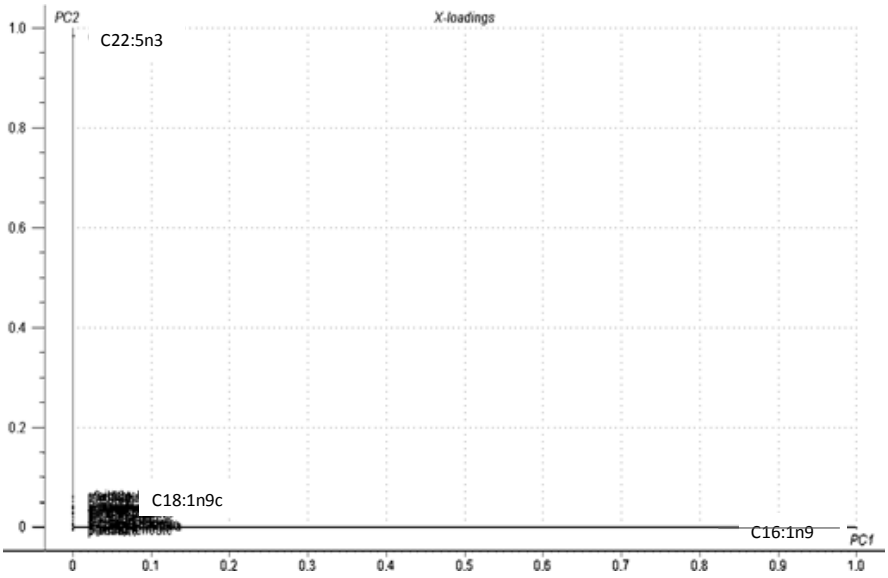
Influence of sampling time after breakfasts consumption on serum levels of fatty acids

Metabolic differences in serum levels of fatty acids were studied in samples corresponding to both post-basal states. Independent PCAs were obtained for each intervention breakfast by representing samples taken at post-basal states after subtraction of values in basal states. Supplementary Figure 3 illustrates the different scores plots obtained for each PCA. As can be seen, discrimination between samples obtained in both post-basal states was only observed in the study dealing with the intervention breakfast prepared with sunflower oil enriched with phenolic compounds. Therefore, only in this case the levels of EFAs and NEFAs had discrimination capability to separate samples taken in both post-basal states.



Supplementary Figure 3. *PCA scores graphs of serum samples from individuals after intake of each prepared breakfast attending to fatty acids levels. Distinction was made between the two sampling times after breakfast intake. Number code is as follows: sampling time 2 h after breakfast intake (2), sampling time 4 h after breakfast intake (4). Name code is as follows: intake of breakfasts prepared with extra-virgin olive-oil (A), sunflower oil enriched with antioxidants from olive pomace (B), sunflower oil enriched with dimethylsiloxane (C), pure sunflower oil (D).*

The loadings plot for PCA representing post-basal states after intake of the breakfast prepared with sunflower oil enriched with phenolic compounds revealed a significant contribution of C22:5n3 to explain discrimination (Supplementary Fig. 4). This compound, also known as docosapentaenoic acid (DPA), is an elongated metabolite of EPA and an intermediate product between EPA and docosahexanoic acid (DHA). It has also been reported as an effective (even more than EPA and DHA) in inhibition of aggregation in platelets obtained from rabbit blood. In addition, there is evidence that DPA possesses 10-fold greater endothelial cell migration ability than EPA, which is important in wound-healing processes [8]. For this reasons, the contribution of DPA in the statistical model is of importance.



Supplementary Figure 4. *PCA loadings graph of serum samples from individuals after intake of PSO-based breakfast at two sampling times attending to the levels of fatty acids.*

Conclusions

Serum levels of EFAs and NEFAs are important biomarkers of intervention diets. The effect of the intake of breakfasts prepared with four edible oils subjected to a simulated deep frying protocol has been assessed in terms of serum fatty acids levels. Statistical analysis has revealed the discrimination capability of omega-6 and omega-3 PUFAs. Among them, serum levels of arachidonic acid and EPA allowed interpreting the metabolic differences observed in the profile of fatty acids from individuals after intake of the four breakfasts.

Acknowledgements

The Spanish Ministerio de Ciencia e Innovación (MICINN) and European FEDER program are thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

References

1. Ascherio A, Hennekens C, Buring J, Master C, Stampfer M, Willett W (1994) Trans-fatty acids intake and risk of myocardial infarction. Circulation 89 (1):94-101

2. Simon JA, Hodgkins ML, Browner WS, Neuhaus JM, Bernert JT, Hulley SB (1995) Serum Fatty Acids and the Risk of Coronary Heart Disease. Am J Epidemiol 142 (5):469-476

3. Paravar T (2005) The Mediterranean Diet: A Protective Effect on Coronary Heart Disease. Nutr Bytes 10 (2): 1-6

4. González-Périz Ana CJ (2010) Resolution of Adipose Tissue Inflammation. TheScientificWorldJournal 10:832-856. http://escholarship.org/uc/item/4d9121sf

5. Burlingame B, Nishida C, Uauy R, Weisell R (2009) Fats and Fatty Acids in Human Nutrition: Inntroduction. Nutr Metab 55: 5-7

6. Oda E, Hatada K, Katoh K, Kodama M, Nakamura Y, Aizawa Y (2005) A case-control pilot study on n-3 polyunsaturated fatty acid as a negative risk factor for myocardial infarction. Int Heart J 46 (4):583-591

7. Rissanen T, Voutilainen S, Nyyssönen K, Lakka TA, Salonen JT (2000) Fish Oil–Derived Fatty Acids, Docosahexaenoic Acid and Docosapentaenoic Acid, and the Risk of Acute Coronary Events. Circulation 102 (22):2677-2679

8. Kaur G, Cameron-Smith D, Garg M, Sinclair AJ (2011) Docosapentaenoic acid (22:5n-3): A review of its biological effects. Prog Lipid Res 50 (1):28-34

9. Perez-Herrera A, Delgado-Lista J, Torres-Sanchez LA, Rangel-Zuñiga OA, Camargo A, Moreno-Navarrete JM, Garcia-Olid B, Quintana-Navarro GM, Alcala-Diaz JF, Muñoz-Lopez C, Lopez-Segura F, Fernandez-Real JM, Luque de Castro MD, Lopez-Miranda J, Perez-Jimenez F (2012) The postprandial inflammatory response after ingestion of heated oils in obese persons is reduced by the presence of phenol compounds. Mol Nutr Food Res 56 (3):510-514

10. Lewis T, Nichols PD, McMeekin TA (2000) Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. J Microbiol Meth 43 (2):107-116

11. Haslam DW, James WPT (2005) Obesity. The Lancet 366 (9492):1197-1209

12. Shinohara E, Kihara S, Yamashita S, Yamane M, Nishida M, Arai T, Kotani K, Nakamura T, Takemura K, Matsuzawa Y (1997) Visceral fat accumulation as an important risk factor for obstructive sleep apnoea syndrome in obese subjects. J Intern Med 241 (1):11-18

13. Filipovský J, Ducimetiére P, Eschwége E, Richard JL, Rosselin G, Claude JR (1996) The relationship of blood pressure with glucose, insulin, heart rate, free fatty acids and plasma cortisol levels according to degree of obesity in middle-aged men. J Hypertens 14 (2):229-235

14. Kusunoki M, Tsutsumi K, Nakayama M, Kurokawa T, Nakamura T, Ogawa H, Fukuzawa Y, Morishita M, Koide T, Miyata T (2007) Relationship between serum concentrations of saturated fatty acids and unsaturated fatty acids and the homeostasis model insulin resistance index in Japanese patients with type 2 diabetes mellitus. J Med Invest 54 (3-4):243-247

15. Guallar-Castillón P, Rodríguez-Artalejo F, Lopez-Garcia E, León-Muñoz LM, Amiano P, Ardanaz E, Arriola L, Barricarte A, Buckland G, Chirlaque M-D, Dorronsoro M, Huerta J-M, Larrañaga N, Marin P, Martínez C, Molina E, Navarro C, Quirós JR, Rodríguez L, Sanchez MJ, González CA, Moreno-Iribas C (2012) Consumption of fried foods and risk of coronary heart disease: Spanish cohort of the European Prospective Investigation into Cancer and Nutrition study. Brit Med J 344. doi: 10.1136/bmj.e363

16. Guallar-Castillón P, Rodríguez-Artalejo F, Fornés NS, Banegas JR, Etxezarreta PA, Ardanaz E, Barricarte A, Chirlaque M-D, Iraeta MD, Larrañaga NL, Losada A, Mendez M, Martínez C, Quirós JR, Navarro C, Jakszyn P, Sánchez MJ, Tormo MJ, González CA (2007) Intake of fried foods is associated with obesity in the cohort of Spanish adults from the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr 86 (1):198-205

17. Peairs A, Rankin J, Lee Y (2011) Effects of acute ingestion of different fats on oxidative stress and inflammation in overweight and obese adults. Nutr J 10 (1):122

18. Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R (2005) Metabolic Syndrome. Circulation 111 (11):1448-1454

19. Yang W-C, Adamec J, Regnier FE (2007) Enhancement of the LC/MS Analysis of Fatty Acids through Derivatization and Stable Isotope Coding. Anal Chem 79 (14):5150-5157

20. Klingler M, Demmelmair H, Koletzko B, Glaser C (2011) Fatty Acid Status Determination by Cheek Cell Sampling Combined with Methanol-Based Ultrasound Extraction of Glycerophospholipids. Lipids 46 (10):981-990

21. Hallmann C, van Aarssen BGK, Grice K (2008) Relative efficiency of free fatty acid butyl esterification: Choice of catalyst and derivatisation procedure. J Chromatogr A 1198–1199 (0):14-20

22. Woo K-L, Kim J-I (1999) New hydrolysis method for extremely small amount of lipids and capillary gas chromatographic analysis as N(O)-tert.-butyldimethylsilyl fatty acid derivatives compared with methyl ester derivatives. J Chromatogr A 862 (2):199-208

23. Girón MaV, Ruiz-Jiménez J, Luque de Castro MD (2009) Dependence of Fatty-Acid Composition of Edible Oils on Their Enrichment in Olive Phenols. J Agr Food Chem 57 (7):2797-2802

24. Sánchez-Ávila N, Mata-Granados JM, Ruiz-Jiménez J, Luque de Castro MD (2009) Fast, sensitive and highly discriminant gas chromatography–mass spectrometry method for profiling analysis of fatty acids in serum. J. Chromatogr. A. 1216 (40):6864-6872

25. Sweeting H (2007) Measurement and definitions of obesity in childhood and adolescence: a field guide for the uninitiated. Nutr J 6 (32). doi: 10.1186/1475-2891-6-32

26. Yang R-Z, Lee M-J, Hu H, Pollin TI, Ryan AS, Nicklas BJ, Snitker S, Horenstein RB, Hull K, Goldberg NH, Goldberg AP, Shuldiner AR, Fried SK, Gong D-W (2006) Acute-Phase Serum Amyloid A: An Inflammatory Adipokine and Potential Link between Obesity and Its Metabolic Complications. PLoS Med 3 (6): 884-894

27. Comba A, Pasqualini ME (2009) Primers on Molecular Pathways — Lipoxygenases: Their Role as an Oncogenic Pathway in Pancreatic Cancer. Pancreatology 9 (6):724-728

28. Di Mari JF, Saada JI, Mifflin RC, Valentich JD, Powell DW (2007) HETEs enhance IL-1-mediated COX-2 expression via augmentation of message stability in human colonic myofibroblasts. Am J Physiol-Gastr L 293 (4):G719-G728

29. Comba A, Pasqualini ME (2009) Primers on Molecular Pathways – Lipoxygenases: Their Role as an Oncogenic Pathway in Pancreatic Cancer. Pancreatology 9 (6):724-728

CAPÍTULO 8

Método automatizado de determinación de fenoles del aceite de oliva y sus metabolitos en plasma humano y su aplicacion en estudios de intervención

Automated method for determination of olive oil phenols and metabolites in human plasma and application in intervention studies

M.I. Orozco-Solano[a,b], C. Ferreiro-Vera[a,b], F. Priego-Capote[a,b], M.D. Luque de Castro[a,b*]

[a]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain

[b]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain

Journal of Chromatography A, 1258 (2010) 108–116

Automated method for determination of olive oil phenols and metabolites in human plasma and application in intervention studies

ABSTRACT

The interest for olive oil phenols (OOPs) is a growing trend thanks to their contribution to prevent or improve diseases associated to oxidative damage. OOPs ingested in the diet are found at low concentrations in blood either as free forms (*e.g.* hydroxytyrosol, tyrosol, vanillin, ferulic acid, coumaric acid) or conjugated as sulfate and glucuronide derivatives. Therefore, the identification/quantitation of OOPs in plasma to study their biological effects and elucidate their metabolism requires selective and sensitive methods. The present research describes the development, validation and application of an automated method based on on-line coupling of solid-phase extraction and liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) for quantitation of conjugated and free OOPs in human plasma. This approach minimizes sample handling —thus reducing analyte losses and degradation by contact with the atmosphere— and increases analysis throughput, which is crucial in intervention studies dealing with cohorts formed by numerous individuals. The fundamental of the approach is the retention of OOPs and metabolites in an SPE anionic cartridge with subsequent on-line elution to an LC–MS/MS system. Quantitative analysis of OOPs (relative quantitation for conjugated OOPs) was carried out by selected reaction monitoring mode that reported relative limits of detection and quantitation between 0.02–0.28 ng/mL (16.6 to 232 pg on-column) and 0.05–0.83 ng/mL (41.5 to 689 pg on-column), respectively . The accuracy of the method, estimated as recovery factor, ranged from 84.2 to 99.4%, and precision, expressed as relative standard deviation, was below 3.8%. The resulting method has been applied to the determination of OOPs and metabolites in plasma samples from individuals who ingested a breakfast prepared with virgin olive oil. The proposed method has an excellent potential for high-throughput use in both clinical and research laboratories.

Keywords: Olive oil, Hydroxytyrosol, Conjugated phenolic compounds, Plasma, LC–MS/MS, On-line SPE.

1. Introduction

The Mediterranean diet, associated for centuries with human health benefits, has evidenced its preventive character against diseases associated with oxidative damage such as coronary heart disease and cancer [1–3]). Numerous clinical studies have reported the antioxidant health benefits attributed to virgin olive oil (VOO) consumption as the main fat source in this diet. Thus, Bonani *et al.* [4] showed that VOO consumption reduces inflammatory markers and increases plasma antioxidant capacity at postprandial state. Different studies have correlated a VOO-rich Mediterranean diet with a reduced inflammatory response of peripheral blood mononuclear cells during postprandial state mediated by transcription factor NF-κB [3, 5-6]. These effects are responsible for a decrease in mortality index in individuals affected by cardiovascular diseases. Protector properties of VOO have been essentially attributed to its content in monounsaturated fatty acids (MUFAs) and to minor components with well-known health benefits [8,9]. This minor fraction, constituted approximately by a set of 230–250 compounds, is the unsaponifiable fraction, which encompasses groups of compounds such as triterpenes, fatty alcohols, sterols, and phenolic compounds, among others. Olive oil phenols (OOPs), which are characterized by a polar and mid-polar character, have been the most studied VOO components in the last years [10]. In fact, several reports have demonstrated that the VOO phenol fraction can improve endothelial dysfunction and haemostatic profile during postprandial state [11,12], ameliorate lipid profiles and decrease *in vivo* oxidative stress [13,14]. Therefore, the phenolic fraction can be considered as responsible for the prevention effect of VOO against oxidative damage.

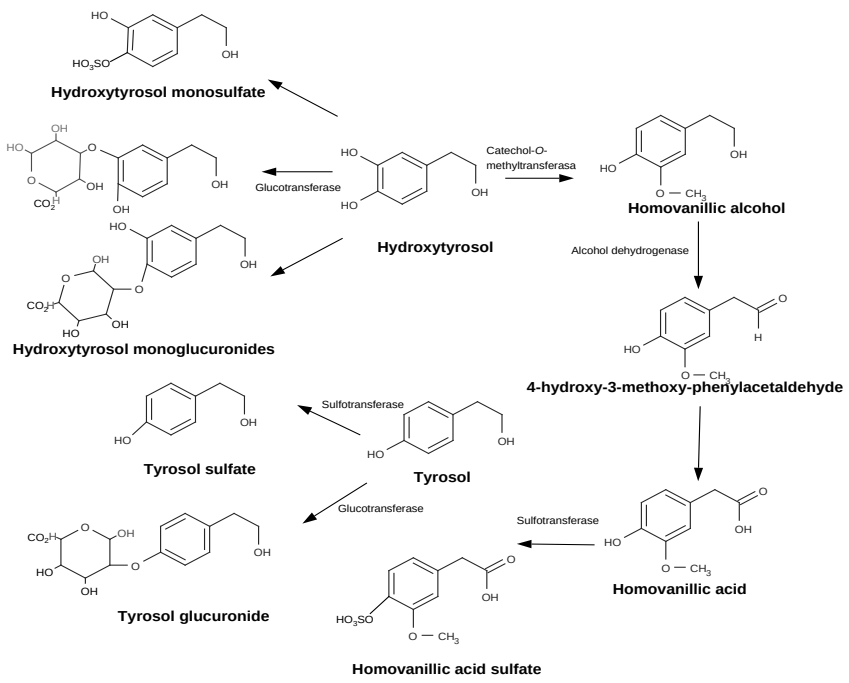
Olive oil phenols may range from simple phenols to very complex structures endowed with one or more hydroxyl groups and with possibility to be bound to sugar molecules. Major OOPs are simple phenols (*i.e.*, hydroxytyrosol, tyrosol), phenolic acids and aldehydes (ferulic acid, coumaric acids, vanillic acid, vanillin), secoiridoids (oleuropein-aglycone di-aldehyde, oleuropein-aglycone mono-aldehyde, ligstroside-aglycone di-aldehyde, ligstroside-aglycone mono-aldehyde), and lignans (pinoresinol

and acetoxypinoresinol) [15–17]. Tyrosol, hydroxytyrosol and secoiridoid derivatives represent around 30% of OOPs [15, 18], which commonly range between 130 and 350 mg/kg.

Apart from the characterization of OOPs, the study of their bioavailability and metabolism is a crucial task with very important implications for human health. The natural biotransformation of OOPs is currently a subject of intensive research. From a nutritional point of view, the absorption of OOPs is affected by their polarity. Polar phenols are primarily absorbed in the stomach and in the gastrointestinal tract [19]; then, they undergo extremely extensive first-pass intestinal/hepatic metabolism in the body [18,20]. On the other hand, OOPs such as secoiridoids should be previously metabolized to generate more polar phenolic compounds such as hydroxytyrosol and tyrosol. In the body, the metabolism of secoiridoid aglycones releases hydroxytyrosol or tyrosol and elenolic acid by enzymatic hydrolysis in the gastrointestinal tract [16,20], which can be absorbed in tissues. Following absorption, OOPs can be transformed by three main types of conjugation mechanisms (*viz.* methylation, sulfation, and glucoronidation) catalyzed by different enzymes acting in liver, kidneys, and small intestinal tissues [21,22]. The postulated enzymatic pathways involving hydroxytyrosol and tyrosol metabolites are shown in Supplementary Fig. 1.

A great number of studies have reported the multiple benefits of OOPs to human health. These studies could be complemented by knowing the bioavailability of OOPs in humans in order to assess their *in vivo* physiological behavior as well as their capacity to protect against oxidation when the consumption of VOO is increased [9]. The bioavailability of OOPs is also influenced by numerous physiological factors such as fat and protein content [9,23]. The maximum concentration of OOPs in plasma is generally reached within 30–60 min after intake of VOO, but the half time for their disappearance depends on the polarity of each phenol (glycoside, aglycone, conjugation reactions, etc.).

Several analytical procedures have been published for determination of phenolic compounds in biological fluids and in animal and human tissues. They have been usually based on chromatographic separation systems coupled to common spectrometric detectors (absorption or fluorescence detectors) [24,25] and to electrochemical detectors (coulometric, amperometric or voltammetric detectors) [26–28]. However, the best



Supplementary Figure 1. *Tentative enzymatic pathways proposed by Tuck et al. for the in vivo metabolism of hydroxytyrosol and tyrosol (Reproduced with permission from Elsevier, ref.[17]).*

approach for quantitation of phenolic metabolites in biological matrices is LC–MS/MS [29–32]. In a method developed by Suárez *et al.* for application in human plasma, absolute detection and quantitation limits for determination of hydroxytyrosol were 15.4 and 77.1 ng/mL, respectively, using 200 µL of plasma which were treated by off-line SPE on microelution plates [32]. Also hydroxytyrosol was determined in fractions of low density lipoproteins isolated from human plasma, reporting detection and quantitation limits of 0.32 and 1.08 ng/mL respectively, although in this case 1 mL of sample was used [31]. All these methods were based on manual sample preparation protocols, which limit in certain way their implementation in studies involving cohorts formed by a great number of individuals. Apart from that, the reduced bioavailability of OOPs (less than 1% of the consumed amount) demands for highly-sensitive methods without increasing sample volumes. The aim of the present research was to develop a method for the determination of simple and conjugated forms of OOPs in human plasma

Código de campo cambiado

with analytical characteristics suitable for application to intervention clinical studies. Appropriate tools to achieve this purpose were considered, namely: (i) an automatic solid-phase extraction (SPE) system for efficient cleanup of the sample matrix and preconcentration of the target analytes without contact with the atmosphere, thus avoiding degradation by light and/or air; (ii) on-line connection of SPE with the chromatographic step to allow eluting the target analytes from the cartridge with the chromatographic mobile phase inserting completely the eluate into the chromatographic column; thus achieving maximum sensitivity; (iii) a triple quadrupole (QqQ) mass analyzer for selective and sensitive detection of the target metabolites supported on selected reaction monitoring (SRM) mode.

2. Experimental

2.1. Chemicals

LC–MS grade methanol and formic acid were from Scharlab (Barcelona, Spain). Deionized water (18 mΩ·cm) from a Millipore Milli-Q water purification system was used for preparation of all aqueous solutions. The OOPs (*i.e.*, hydroxytyrosol, tyrosol, oleuropein, luteolin, apigenin, apigenin-7-glucoside, vanillin, vanillic acid, homovanillic acid, *p*- and *o*-coumaric acids, ferulic acid and caffeic acid), and also syringic acid, used as internal standard, IS, were from Extrasynthese (Genay, France) or Sigma (St. Louis, USA). All chemicals were HPLC grade and used without further purification.

Working solutions were prepared by dilution of appropriate volumes of stock methanolic solutions in plasma pool aliquots, which were used for optimization of the different steps of the method. All stock standard solutions and spiked samples were dispensed into glass vials and stored at −20 ºC until use.

2.2. Subjects and samples

Plasma samples for validation of the method belonged to three healthy subjects from the Lipids and Atherosclerosis Unit at University Hospital Reina Sofía (Córdoba, Spain). All subjects fulfilled three or more of the criteria proposed by the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation

and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) for MetS. The subject's anthropometric variables such as body mass index, waist perimeter, systolic blood pressure, diastolic blood pressure, plasma glucose levels, among others, were within normal ranges [7]. All subjects gave their informed consent for blood extraction.

Each of the volunteers received 40 mL of VOO with high content in phenolic compounds (400 mg/L) and 60,000 IU of vitamin A per m^2 of body surface. This was administered as a single dose accompanied by a standard piece of bread (60 g). Throughout the duration of the study session, subjects performed no physical activity, nor consumed anything but water. Venous blood samples were collected at 60 min after breakfast consumption in tubes containing 1 g/L of EDTA and stored in containers with ice in the dark. Special care was taken to avoid exposure to air, light and ambient temperature. Plasma was separated from whole blood by low speed centrifugation at 1500 × g for 15 min at 4°C within the one hour after extraction. After separation, plasma samples were stored at or below –80 °C until analysis. A plasma pool was prepared with aliquots collected from individuals after intake of the VOO-based breakfast. This plasma pool was used for optimization of the different steps involved in the analytical protocol devoted to the analysis of OOPs.

Control samples were obtained from five volunteers who previously excluded from their diets phenol-rich food, vitamins, soy supplements, or any drug, 6 weeks before the study started. A second plasma pool (blank plasma) was prepared with aliquots from control samples to ensure the absence of target OOPs and metabolites.

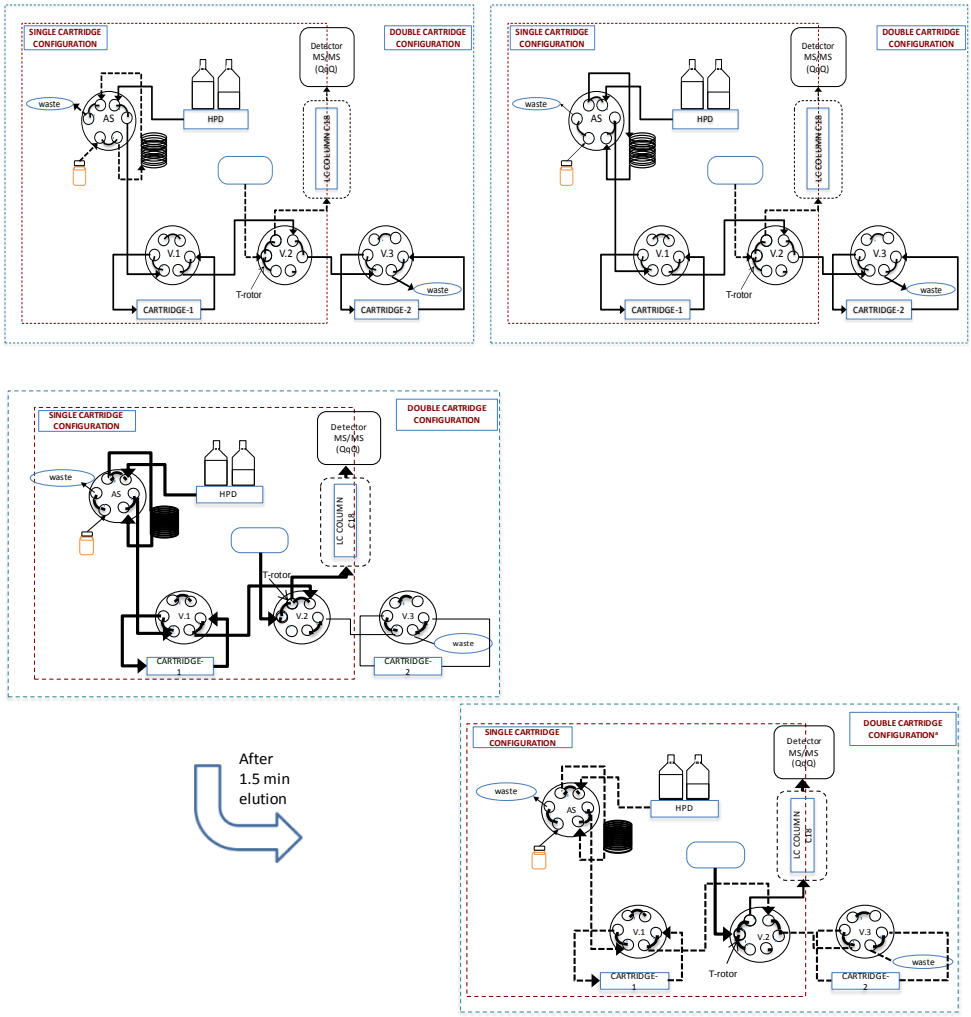
2.3. The SPE–LC–MS/MS approach

A scheme of the configuration device is illustrated in Supplementary Fig. 2. A Midas autosampler furnished with a 500-µL sample-loop connected to a Prospekt-2 system (Spark Holland, Emmen, The Netherlands) was used to automate solid-phase extraction (SPE). The Prospekt system consists of an automatic cartridge exchanger and a high pressure dispenser (HPD) that enables a fully automated performance of sample preparation controlled by Sparklink version 2.10 software. The automatic cartridge exchanger possesses two clamp positions (in the loop of valves 1 and 3, V.1 and V.3, respectively) for on-line coupling, if required, of two SPE cartridges. Hysphere mixed-mode (MM) anionic cartridges (8 µm, 10 × 2.0 mm, Spark Holland) were used for SPE

step. Other Hysphere cartridges (10 × 2 mm) were assayed in optimization of the solid-phase extraction step —*viz.* CN (silica-based cyanopropyl phase), C2 (silica-based ethyl phase), C8(EC) (end-capped silica-based octyl phase), C18(EC) (end-capped silica-based octadecyl phase), resin GP (polymeric polydivinylbenzene phase) and resin SH (strong-hydrophobic modified polystyrene-divinylbenzene). The Prospekt-2 system was on-line connected to an Agilent (Palo Alto, CA, USA) 1200 Series LC system, which consists of a binary pump and a vacuum degasser. The eluate from the chromatographic column was directly introduced into an Agilent 6410 triple quadrupole mass analyzer (QqQ), furnished with an electrospray ion (ESI) source. Agilent MassHunter Workstation was the software for data acquisition and treatment with qualitative and quantitative purposes. A C18 Mediterranea Sea® analytical column (5.0 µm, 150 x 4.6 mm i.d) from Teknokroma (Barcelona, Spain) of 5.0 µm, 150 x 4.6 mm i.d was used in the developed method.

2.4. Analytical protocol for determination of conjugated and free phenols in plasma

The sample preparation process was initiated with 1:5 dilution of the plasma sample (100 µL) with water in an amber injection vial placed in the autosampler tray. The entire procedure was automatically performed in the sequence of steps schemed in Supplementary Fig. 2 (A)-to-(C), it is and summarized as follows: (A) the analytical sample (500 µL) was placed into the Prospekt-2 system for starting SPE–LC–MS/MS analysis. Simultaneously, the SPE anionic cartridge in the ACE unit was independently clamped in the loop of valve 1 (V.1), solvated and equilibrated by the HPD as follows: 4 mL methanol at 5 mL/min for solvation (step 1) and 2 mL aqueous solution at pH 8.5 adjusted with 1 M ammonia at 0.7 mL/min to equilibrate the cartridge (step 2). Then, the valve of the autosampler (AS) switched to initiate the sample loading. (B) 2 mL of loading solution (aqueous solution at pH 8.5 adjusted with 1 M ammonia) was circulated and passed through the sample loop to cartridge at 0.7 mL/min. For the washing step, the cartridge was subsequently rinsed with 0.5 mL pure water at 0.5 mL/min (step 3). Once the sorbent was washed, an input/output signal was established between the SPE workstation and the LC unit to trigger the chromatographic run. (C) The latter was started by switching the left clamp valve (valve 1) to eluate the target metabolites. For this purpose, the LC pump initiated the chromatographic gradient at 0.3 mL/min with 60:40 (v/v) methanol–water solution containing 0.01% formic acid as



Supplementary Figure. 2. *Scheme of the device based on an SPE workstation coupled to an LC–MS/MS system using a T-rotor in valve 2 (V.2). A) Solvation/equilibration of the cartridge; B) sample loading and washing; and C) elution of the compounds retained in the cartridge using the focusing mode. HPD, high pressure dispenser, QqQ, triple quadrupole mass detector.*

mobile phase. Simultaneously, the HPD pumped 0.5 mL of 90:10 (v/v) methanol–water and pH 3.5 adjusted with 1 M formic acid at 0.5 mL/min. Both solutions were merged in the T-shaped rotor situated in valve 2 (V.2) and, subsequently mixed in the mix-tubing located before the cartridge (total flow rate 0.8 mL/min). This process took 1.5 min, after which the valve 2 (with T-rotor) was switched allowing only passage of the programmed mobile phase at 0.8 mL/min to the chromatographic column. The

chromatographic gradient was as follows: the initial mobile phase was maintained for 2 min; then, a linear gradient was programmed to obtain 100% methanol in 12 min, maintained for 4 minutes until the end of the chromatographic separation step. The temperature of the column compartment was set at 25 ºC. Tandem mass spectrometry detection was used after ESI in negative mode. The capillary voltage and the source temperature were set at 2.7 kV and 300 ºC, respectively. The pressure nebulizer was 55 psi with nitrogen as desolvation gas flowing at 8 mL/min.

Once the chromatographic run was finished (step 4), a step for cleaning cartridges with 5-mL methanol (step 5) and 5-mL water (step 6) started. The SPE cartridge was thus ready for being reused. The entire analytical process was completed in 25 min, which enabled to synchronize the SPE protocol with the chromatographic step and overlap the chromatographic step of one sample with the SPE of the next sample.

2.5. SRM-based quantitation of target analytes

Calibration curves were built by SPE–LC–MS/MS analysis of both multistandard solutions and spiked aliquots of the blank plasma pool (1:5 plasma–water) with different levels of standard solutions (6 levels). The concentrations of standards at the lowest and highest levels of each phenol were 1 and 1000 ng/mL. Syringic acid was used as internal standard (at 0.1 μg/mL) to obtain the calibration model for each OOP. Three concentration levels (high, medium and low values, within the calibration range) were injected in triplicate to set confidence levels. The calibration plots of non-conjugated phenols was used for relative quantitation of the conjugated compounds, the standards of which are not commercially available.

3. Results and discussion

The optimization sequence followed in this study consisted of two steps: the first one was focused on the best separation–determination of the target compounds using LC–MS and, the second step, on the most efficient preconcentration–cleanup of the target compounds by the automated SPE system. The optimization of the different steps was performed with multistandard solutions and the plasma pool prepared with samples from individuals after intake of the VOO-based breakfast.

3.1. Tandem mass spectrometry detection

Negative ionization has clearly been the preferred mode for MS detection of phenols [29-31, 33] and, thus, it was selected in this research. Due to the incidence of the ESI process on the sensitivity of LC–MS detection, variables such as ionization temperature, pressure and nebulizer voltage were studied by a multivariate response surface design consisting of 16 experiments and 2 central points. For this purpose, precursor ions of the commercially available phenols, listed in Table 1, were monitored in single ion monitoring mode (SIM). The optimum values of temperature, pressure and capillary voltage were 300 ºC, 40 psi, and 3000 V. Optimization of electrospray ionization was performed by direct injection of individual standard solutions with different ionization agents. The influence of the ionization agent was studied by addition of different concentrations of acetic acid, formic acid and ammonium acetate within the concentration range 0.01–1% (v/v or w/v in the case of the salt), with pH values ranging between 2.5 and 4 to a 60:40 methanol–water mobile phase. The best results were obtained with 0.01% formic acid. Under these ESI conditions, the tandem-mass spectrometry parameters were optimized for efficient isolation of the precursor ions and their sensitive and selective fragments. The optimum collision energy, studied within the range 10–60 eV, was between 10 and 30 eV for all phenols. The selected voltage for efficient filtration of precursor ions in the first quadrupole, collision energy to fragment the precursor ions by collision induced dissociation (CID) and the product ions selected for quantification in the selected reaction monitoring (SRM) mode are shown in Table 1. Only one transition was selected to monitor each phenol with quantification purposes owing to the low sensitivity of secondary transitions which were used for qualitative purposes. The dwell time was fixed at 50 ms for each SRM transition.

Table 1. Tandem mass spectrometry parameters and retention times for qualitative and quantitative determination of olive-oil phenols and metabolites in human plasma.

Analyte	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Voltage MS1 (V)	Collision energy (eV)	Quantitation transition
Hydroxytyrosol	3.1	152.9	123.1 94.9	60	10	152.9→123.1
Hydroxytyrosol monosulfate	4.8	233.0	153.0	60	10	233.0→153.0
Hydroxytyrosol monoglucuronide (I)	5.9	329.0	153.0	60	10	329.0→153.0
Hydroxytyrosol monoglucuronide (II)	7.4	329.0	153.0	60	10	329.0→153.0
Tyrosol	5.4	137.0	119.0	80	10	137.0→119.0
Tyrosol sulfate	7.2	217.0	137.0	80	10	217.0→137.0
Tyrosol glucuronide	13.9	313.0	137.0	80	10	313.0→137.0
Vanillin	4.6	151.0	136.1 92.1	60	10	151.0→136.1
Homovanillic acid	7.7	181.0	137.0	60	10	181.0→137.0
Homovanillic acid sulfate	7.8	261.0	181.0	60	10	261.0→181.0
Syringic acid (IS)	4.9	197.0	182.0 167.0	60	10	197.0→182.0
Ferulic acid	11.0	193.0	134.0	80	10	193.0→134.0
Caffeic acid	7.3	179.0	135.1	120	10	179.0→135.0
Vanillic acid	5.8	167.0	152.0 123.1	100	10	167.0→152.0
o-Coumaric acid	9.5	163.0	119.1 93.0	60	10	163.0→119.1
p-Coumaric acid	9.7	163.0	119.1 93.0	80	10	163.0→119.1
Oleuropein	7.5	539.1	275.1 149.0	200	30	539.1→275.1
Luteolin	15.4	285.0	133.0 151.0 175.0	180	20	285.0→133.0
Apigenin	16.0	269.0	117.0 151.1	180	20	269.0→117.0

The panel of conjugate metabolites was selected according to previous studies reported in the literature [8,16,17,31,34,35], which pointed out the occurrence of the main phenolic metabolites derived from VOO consumption. Concerning the conjugated phenols, the precursor ions were estimated from their molecular weights by monitoring tentative adducts such as $[M-H]^-$, $[M+Cl]^-$, $[M+COOH]^-$ and $[M-H_3O]^-$ ions. All

conjugate phenols reported preferentially the $[M-H]^-$ precursor ion. Thus, the precursor ion for hydroxytyrosol monosulfate (Mw 234 g/mol) was *m/z* 233. For SRM-based detection their transitions were defined according to the mass of the non-conjugated phenol. Thus, the transition for quantitation of hydroxytyrosol monosulfate was from *m/z* 233 as precursor ion to *m/z* 153 formed after cleavage of the sulfate group. This behavior was common for all conjugated phenols since the conjugation bond is the most labile part of the molecule. The same strategy was adopted to define the transitions for glucuronide conjugates. Thus, hydroxytyrosol and tyrosol glucuronides, with precursor ions *m/z* 329 and 313, respectively, were monitored by transitions to *m/z* 153 and 137, respectively. These transitions are in agreement with other studies reported in the literature dealing with determination of conjugate phenols in biological samples [31,33].

3.2. Optimization of the automated solid-phase extraction step

In this study, SPE directly coupled to LC–MS was used for direct analysis of plasma avoiding protein precipitation, time-consuming concentration of target metabolites and reconstitution steps. Maximum extraction efficiency was the parameter considered in the optimization of on-line sample preparation. The SPE sorbent, sample volume, loading solvent (type, volume and flow rate) and elution flow rate were studied by a univariate approach because of the number of non-continuous variables involved (*i.e.* SPE sorbent, loading- and washing solvents). Both a multistandard solution 0.1 µg/mL and a plasma pool were used for optimization of the SPE process. The plasma pool was prepared by mixing aliquots of the individuals after intake of VOO-based breakfast and, then, it was spiked with a multistandard solution containing the commercial phenols at 0.1 µg/mL.

3.2.1. Selection of the SPE sorbent

Eight types of SPE sorbents (described in the experimental section) encompassing medium and low polar materials as well as resin phases with mixed retention mechanisms were assayed in the optimization of the SPE step. The different sorbents (mentioned in section 2.3) were tested with a standard protocol based on plasma sample loading with an aqueous solution and subsequent elution with the corresponding chromatographic mobile phase. Only in the case of the MM anion sorbent, different pH values were assayed to favor ionic interactions. Figure 1 shows SRM chromatograms of two representative analytes, hydroxytyrosol and apigenin, eluted at the beginning and at

the end, respectively, of the chromatographic profile obtained from a spiked plasma pool. The retention capability in the case of hydroxytyrosol followed the order: MM anionic > Resin GP > Resin SH > C2 > C8(EC) > C18(HD) > C18(SE) > CN. This sequence was common for more polar OOPs such as conjugates, phenolic acids and vanillin. However, for less polar OOPs such as oleuropein, luteolin and apigenin and its glucoside the general sequence was as follows: MM anionic > CN > C18(SE) > C8(EC) > C18(HD) > Resin GP > Resin SH > C2. As can be seen the MM anionic sorbent led to the best results in retention/elution capacity for OOPs independently of the analytes polarity. This was supported on the mixed mode mechanisms for retention of metabolites: ionic interactions for those metabolites that acquired a charge state at the operation pH, and polymeric interactions for neutral metabolites at the operation pH. The pKa *versus* pH curves for free and conjugated phenolic metabolites were studied to select the optimum pH range, and pH values above 8 were used to obtain –1 and –2 as preferential charged states for phenolic metabolites. Under these conditions, the ionic interactions between metabolites and sorbent were enhanced and the recovery of target metabolites with negative charge at the working pH was increased.

Despite most SPE approaches require an additional sample pretreatment step to avoid clogging of the SPE column by *in situ* protein precipitation [21,22], the approach used in this research involved only dilution of the plasma sample with water to evaluate the matrix incidence. For this purpose, different dilution ratios from 1:1 to 1:8 (v/v) were tested by injecting the same amount of plasma. Figure 2 shows 1:5 (v/v) plasma–water dilution as the best for hydroxytyrosol as representative of the behavior of all target metabolites.

3.2.2. Optimization of the step for sample loading

Methanol–water and acetonitrile–water mixtures at different ratios and pHs were tested as loading solutions. The pH of the solutions was a critical parameter to enhance ionic interactions and ensure release of OOPs bound to plasma transporters and other interferents. Water and water–organic solutions with different pHs between 7 and 12 were prepared using a 0.5 M ammonia solution. The optimum loading solution was obtained with pure water at pH 8.5 as small concentrations of organic solvent promoted partial elution of simple OOPs. Higher pH values did not lead to improved retention,

probably due to the increased ionic strength and also to the minimized mixed-mode interactions associated to this sorbent.

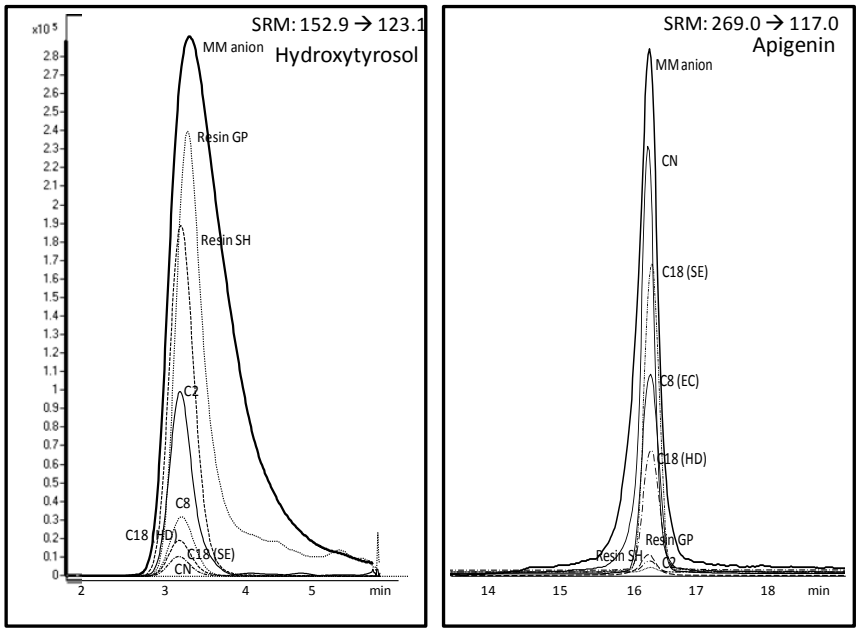


Figure 1. *Evaluation of the retention capability of SPE sorbents by comparison of SRM chromatograms for hydroxytyrosol (left) and apigenin (right): CN (silica-based cyanopropyl phase), C8(EC) (end-capped silica-based octyl phase), C18(EC) (end-capped silica-based octadecyl phase), C18(HD) (high density phase), Resin GP (polymeric polydivinylbenzene phase) and Resin SH (strong-hydrophobic modified polystyrene-divinylbenzene), C2 (silica-based ethyl phase) and MM anion (polymer mixed-mode ion exchangers).*

The volume of the loading solution and its flow rate were studied from 0.5 to 4 mL and 0.5 to 5 mL/min, respectively, by setting the minimum value as twice the sum of the sample loop, transport tubing and internal cartridge volume. 2 mL and 0.7 mL/min were selected as the highest volume and flow rate without losses of analytes in the retention step.

3.2.3. Optimization of the washing step

Deionized water solutions containing methanol within the range 0–30% (v/v) were evaluated as washing solutions of the SPE cartridge. Volumes within 0–4 mL were

tested to remove non-retained plasma compounds and salts. Hence, 0.5 mL of pure water at 0.5 mL/min was adopted as washing solution. A cleaner chromatogram was obtained by implementing a washing step as compared with that obtained in its absence.

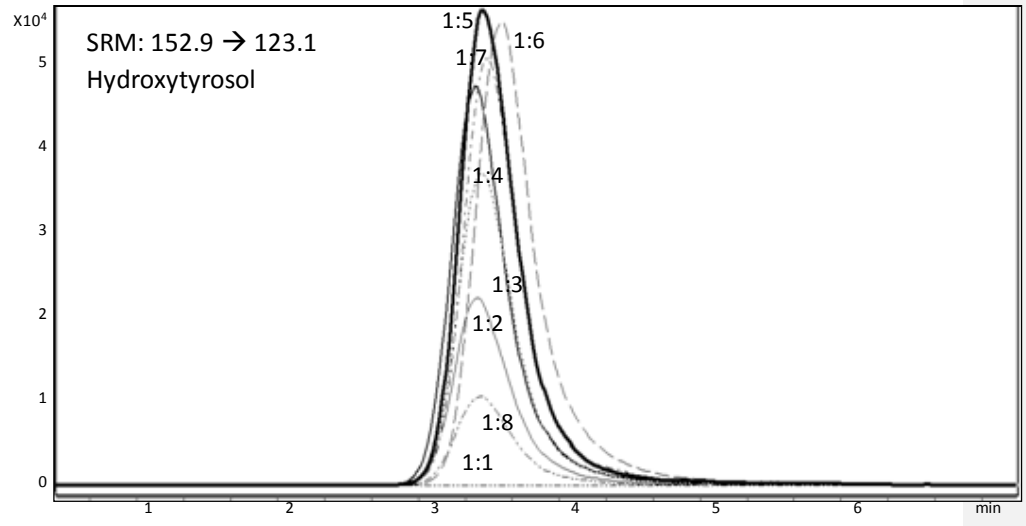


Figure 2. *Comparison of the dilution effect tested in analytical samples spiked with 50 ng/mL of a multistandard of phenols. Ratios tested: 1:1 to 1:8 (v/v) plasma–water. The shown peak corresponds to the SRM chromatograms of hydroxytyrosol.*

3.2.4. Mode and time for elution of target metabolites

The elution step required an acid solution to protonate the analytes and, thus, to eliminate the ionic interactions with the sorbent. This could be carried out with the chromatographic mobile phase during the gradient program. However, differences in polarity among the target metabolites led to peak-broadening effects in those compounds eluted later. For this reason, a focusing elution mode was adopted. In this mode the mobile phase is mixed with a suited solution for a short period of time to attain elution of the metabolites without altering the chromatographic separation. As shows Supplementary Fig. 2, a T-shaped rotor is inserted in valve 2 (V.2) to operate in this elution mode. The composition of the auxiliary solution was optimized by testing different water–methanol mixtures with pH within the range 2–5 adjusted with formic acid. Optimum elution was obtained with 90% methanol in water at pH 3.5. This

solution was mixed with the mobile phase consisting of 60:40 (v/v) methanol–water at pH 3.5 with 0.01% formic acid. The mixture ratio of both solutions was set by changing the flow rates. The optimum flow rate ratio for both solutions was 0.5:0.3 mL/min for the auxiliary solution/chromatographic mobile phase.

The elution time, during which the right clamp valve was switched to the LC analytical column, was studied to ensure complete elution of OOPs. This was achieved after 1.5 min. After this, the valve was switched again to continue with the chromatographic separation with aflow rate of 0.8 mL/min. Longer times led to peak band-broadening effects and caused elution of interferents with potential ionization suppression in the electrospray unit of the mass analyzer.

3.3. Validation of the method

Non-spiked and spiked human plasma samples were used to validate the method and evaluate potential matrix effects. The plasma pool used for this task was that prepared with the aliquots from individuals who ingested VOO-based breakfast. The recovery factor was first studied by using a dual SPE cartridge configuration with non-spiked plasma aliquots. For this purpose, two identical cartridges were on-line coupled by using a third valve (V.3) so the amount of analytes no retained in the first cartridge could be retained in the second. After retention and washing, the OOPs retained in both cartridges were sequentially eluted to the chromatographic column for two independent analyses. The recovery factor for each analyte was calculated as amount in cartridge 1/[amount in cartridge 1+amount in cartridge 2], where the first and second cartridges were MM anionic. Recovery percentages for all free and conjugated OOPs are listed in Table 2. As can be seen, the recovery factors were above 84.2%, which proves the quantitative retention in the cartridge and therefore, supports the development of quantitative calibration models.

Table 2. Recovery factors for each analyte calculated with a two-cartridge configuration ([a]), and single-cartridge configuration ([b])

Analyte	Recovery [a]	Recovery [b]
Hydroxytyrosol	98.7	99.2
Hydroxytyrosol monosulfate	98.5	-
Hydroxytyrosol monoglucuronide (I)	92.9	-
Hydroxytyrosol monoglucuronide (II)	93.9	-
Tyrosol	89.0	97.4
Tyrosol sulfate	88.0	-
Tyrosol glucuronide	87.2	-
Vanillin	94.0	98.6
Homovanillic acid	95.5	98.7
Homovanillic acid sulfate	94.7	-
Ferulic acid	95.9	99.3
Caffeic acid	98.8	99.0
Vanillic acid	96.4	97.3
o-Coumaric acid	95.5	98.5
p-Coumaric acid	84.4	99.1
Oleuropein	99.4	98.4
Luteolin	84.2	99.2
Apigenin	94.0	98.7

[a]Expressed as percent of material retained in cartridge 1/[material retained in cartridge 1+material retained in cartridge 2]. [b]Expressed as percent of [final concentration–initial concentration]/added concentration.

Calibration plots were run by analysis of aliquots of the blank plasma pool spiked with different levels of the standard solutions. The ratio between analyte peak area and IS peak area was plotted as a function of the standard concentration of each compound. Analytical replicates (n=3) of low, intermediate and high levels of target OOPs (10, 50 and 100 ng/mL, respectively), within the linear range, were injected to establish the confidence levels. It is worth mentioning that linearity ranges were estimated for the different calibration curves, which encompassed from the limits of quantitation to 1 µg/mL for most phenols. For this reason, with practical purposes, the same dynamic range was selected for the different calibration curves. Table 3 shows the regression coefficient for each calibration curve together with other characteristics of

the method. Thus, the limits of detection (LOD) and quantitation (LOQ) were calculated as the concentration providing signals three and ten times higher, respectively, than the noise background signal. The LODs ranged between 0.02 and 0.28 ng/mL (16.6 and 232 pg on column), while LOQs were from 0.05 and 0.83 ng/mL (41.5 to 689 pg on-column); values clearly lower than the ranges reported in human plasma in previous studies (from 15.42 to 193.42 ng/mL for LODs and 65.83 to 663.17 ng/mL for LOQs) for non-conjugated phenols such as hydroxytyrosol, tyrosol or luteolin [32]. Higher detection and quantitation limits than those obtained with the method proposed here were also reported for hydroxytyrosol in rat plasma [36]: 1.7 and 5.0 ng/mL, respectively.

The accuracy of the method was studied by analysis of four replicates of plasma aliquots spiked at 10, 50 and 100 ng/mL of each available phenol according to the FDA validation protocol [37]. This test was developed with the one-cartridge configuration. Four replicates of plasma aliquots of the same pool without spiking were also analyzed as control. In the one-cartridge configuration, the recovery was calculated as [final concentration–initial concentration]/added concentration using the developed calibration curves. The application of this formula only allowed evaluating spiked OOPs. The recovery factors were not dependant on the spiked concentration and were above 97.3% for all analytes (average concentration for the three spiked levels), as shown in Table 2.

To evaluate within-day and between-days variability a single experimental set-up was planned using human plasma spiked at three concentrations (10, 50 and 100 ng/mL). Two analyses per day of samples at each concentration were carried out on 7 days [37,38]. As Table 3 shows, the repeatability, expressed as relative standard deviation (RSD), ranged from 1.7 to 2.9%; and within-laboratory reproducibility, also expressed as RSD, was from 2.7 to 3.8%. The specificity of the method was studied by analysis of plasma collected from five control volunteers who previously excluded phenol-rich food from their diets. As expected, no OOPs were detected in any sample from these individuals.

Table 3. Analytical features of the method.

Analyte	**LOD (ng/mL)**	**LOQ (ng/mL)**	**Correlation coefficient**	**Repeatability[a] (%)**	**Reproducibility[a] (%)**
Hydroxytyrosol	0.25	0.46	0.999	1.7	3.2
Hydroxytyrosol monosulphate	0.25	0.46	0.999	1.7	3.2
Hydroxytyrosol monoglucuronide (I)	0.25	0.46	0.999	1.7	3.2
Hydroxytyrosol monoglucuronide (II)	0.25	0.46	0.999	1.7	3.2
Tyrosol	0.25	0.46	0.999	1.7	3.2
Tyrosol sulphate	0.25	0.46	0.999	1.7	3.2
Tyrosol glucuronide	0.25	0.46	0.999	1.7	3.2
Vanillin	0.12	0.50	0.999	2.5	3.3
Homovanillic acid	0.25	0.46	0.999	1.7	3.2
Homovanillic acid sulphate	0.25	0.46	0.999	1.7	3.2
Ferulic acid	0.09	0.30	0.997	2.5	3.1
Caffeic acid	0.17	0.60	0.999	2.9	3.8
Vanillic acid	0.23	0.82	0.997	2.3	3.1
o-Coumaric acid	0.08	0.12	0.999	2.6	2.9
p-Coumaric acid	0.02	0.05	0.998	1.8	2.7
Oleuropein	0.07	0.25	0.999	2.1	2.8
Luteolin	0.28	0.83	0.996	1.8	3.6
Apigenin	0.11	0.37	0.998	2.4	3.1

3.4. Application of the method to human plasma samples

The proposed method was applied to a cohort of three individuals after intake of a breakfast based on VOO. None of the subjects was taking medication or supplementary vitamins with influential effect on plasma metabolome. Blood samples were extracted from each individual at 60 min after intake. As controls, five volunteers who previously

excluded phenol-rich food from their diets were also analyzed. As expected, no OOPs were detected in any of the volunteers. Concerning the individuals who ingested the VOO-based breakfast, the mean concentration and standard deviation for each metabolite are listed in Table 4. It is worth emphasizing that concentrations of conjugated OOPs are relative values. As can be seen, hydroxytyrosol was found both as free and conjugated forms. Thus, hydroxytyrosol monosulfate was the most concentrated form with levels between 5.2 and 6.7 ng/mL, although the sum of the two monoglucuronide derivatives reported levels from 5.9 to 8.8 ng/mL. Non-conjugated hydroxytyrosol was quantified in human plasma at lower levels: 2.6 to 3.9 ng/mL. Figure 3 shows the SRM chromatograms for hydroxytyrosol and metabolites by analysis of plasma from control individuals (blank) and those who ingested the VOO-based breakfast. As can be seen, no detectable levels were found in control individuals. In the case of tyrosol, only the conjugates were found at concentrations ranging from 2.0 to 2.9 ng/mL for the sulfate conjugate and from 1.7 to 2.3 ng/mL for the glucuronide conjugate. Tyrosol and its glucuronide derivative were not detected in any individual, which is quite logical since this phenol is always below the concentration of hydroxytyrosol in VOO. Figure 4.A illustrates the SRM chromatograms for tyrosol sulfate in control individuals and those after VOO consumption. One other frequent metabolite previously reported as marker of VOO consumption is homovanillic acid monosulfate, which was detected at concentrations from 6.6 to 11.5 ng/mL (see Fig. 4.B). The detection of six conjugated forms (albeit at low concentration in some cases) in plasma from the three individuals after VOO consumption should be attributed to the breakfast intake, although some metabolites such as homovanillic acid could have an endogenous origin associated to the dopamine metabolism [38,40].

The target phenolic acids were also detected in the three patients being caffeic acid and *p*-coumaric acid the most and less concentrated phenols in plasma. Concerning vanillin, the phenolic ketone was present at levels from 3.1 to 7.8 ng/mL. Flavonoids such as apigenin and its glucoside, and luteolin were not detected in plasma. A similar case was oleuropein that was not detected either in plasma, which is logical since this metabolite is detected in VOO at low concentration. Apart from that, oleuropein is metabolized in the human body to release hydroxytyrosol, which is more polar.

Table 4. Profile of the target analytes in human plasma at 60 minutes after olive oil ingestion (expressed as mean ± standard deviation. ng/mL)

Analyte / Sample	Voluntary 1_t60	Voluntary 2_t60	Voluntary 3_t60
Hydroxytyrosol	3.94 ± 0.4	2.61 ± 0.1	2.99 ± 0.1
Hydroxytyrosol monosulfate	5.29 ± 0.6	6.68 ± 1.6	5.23 ± 0.9
Hydroxytyrosol monoglucuronide (I)	4.18 ± 0.2	3.41 ± 0.3	2.78 ± 0.1
Hydroxytyrosol monoglucuronide (II)	4.67 ± 0.8	4.15 ± 0.5	3.09 ± 0.5
Tyrosol	ND[a]	ND	ND
Tyrosol sulfate	2.24 ± 0.2	2.93± 0.4	2.06 ± 0.8
Tyrosol glucuronide	1.7 ± 0.3	2.3 ± 0.5	2.02 ± 0.4
Vanillin	7.83 ± 0.6	3.1 ± 0.6	5.08 ± 0.5
Homovanillic acid	ND	ND	ND
Homovanillic acid sulfate	6.56 ± 0.3	11.48 ± 1.2	6.84 ± 0.7
Ferulic acid	5.94 ± 0.5	3.94 ± 0.4	4.95 ± 0.2
Caffeic acid	10.84 ± 1.2	6.54 ± 0.7	8.87 ± 1.2
Vanillic acid	2.41 ± 0.8	1.99 ± 0.2	1.97 ± 0.5
o-Coumaric acid	4.64 ± 0.6	3.59 ± 0.5	4.05 ± 0.3
p-Coumaric acid	1.17 ± 0.2	1.04 ± 0.7	1.08 ± 0.3
Oleuropein	ND	ND	ND
Luteolin	ND	ND	ND
Apigenin	NQ[b]	ND	ND

[a]*Non-detected;* [b]*Non-quantified.*LE FALTA LA CABECERA DE LA PÁGINA

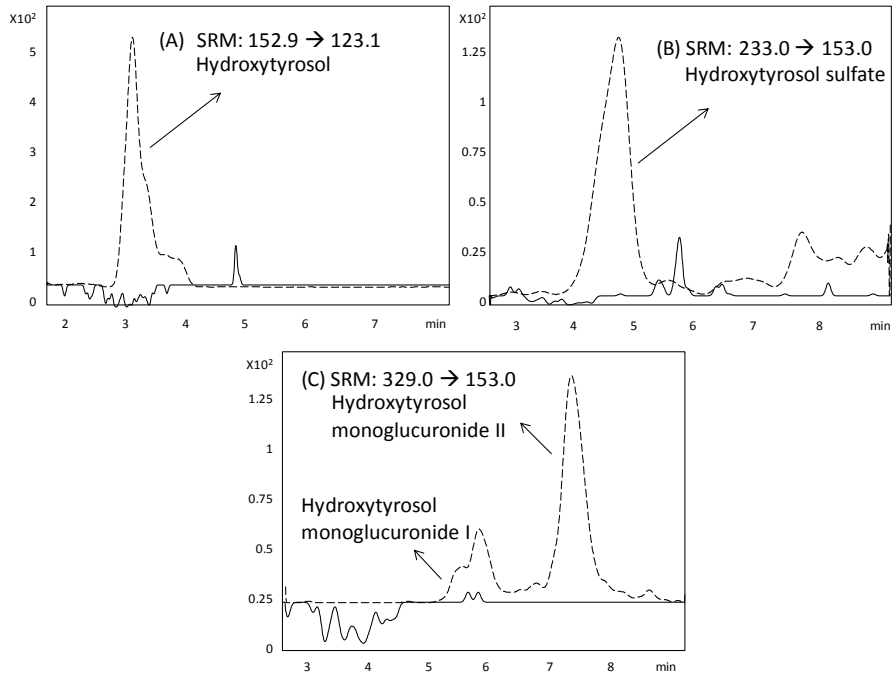


Figure 3. *SRM chromatograms for hydroxytyrosol and metabolites in plasma from control individuals (continuous line) and individuals after intake of VOO-based breakfast (dashed line). (A) Hydroxytyrosol; (B) hydroxytyrosol sulfate; (C) hydroxytyrosol monoglucuronide I and II.*

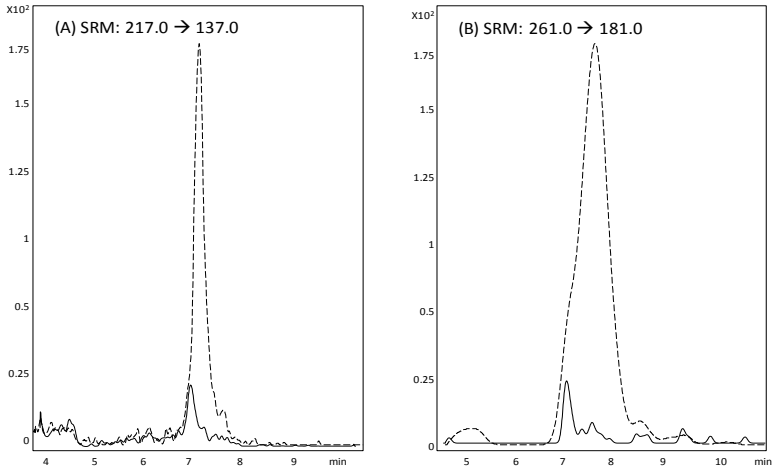


Figure 4. *SRM chromatograms for tyrosol sulfate (A) and homovanillic acid sulfate (B) in plasma from control individuals (continuous line) and individuals after intake of VOO-based breakfast (dashed line).*

4. Conclusion

An automated and fast method based on the on-line coupling between SPE as sample preparation approach and LC–MS/MS with a triple quadrupole mass analyzer has been developed for detection and quantitation of OOPs and metabolites in human plasma. In the case of conjugated metabolites, the standards of which are not commercially available, their quantitation was relative to that of the free metabolites,for which commercial standards exist. With the resulting method the analyst intervention is limited to samples location in the autosampler of the SPE workstation. The method has been characterized in terms of sensitivity, accuracy and precision in order to evaluate its capability for application in intervention studies dealing with cohorts formed by numerous individuals. The suitability for high-throughput analyses makes this method a competitive alternative to be implemented in clinical studies destined to elucidate the health benefits of VOO consumption, which is supported on the antioxidant capacity ascribed to OOPs.

Acknowledgments

The Spanish Ministerio de Ciencia e Innovación (MICINN) and European FEDER program are thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

References

[1] M. P. Mena, E. Sacanella, M. Vazquez -Agell, M. Morales, M. Fitó, R. Escoda, M. Serrano -Martínez, J. Salas -Salvadó, N. Benages, R. Casas, R. M. Lamuela -Raventós, F. Masanes, E. Ros, R. Estruch, Am. J. Clin. Nutr. 89 (2009) 248.

[2] M. A. Carluccio, L. Siculella, M. A. Ancora, M. Massaro, E. Scoditti, C. Storelli, F. Visioli, A. Distante, R. De Caterina, Arterioscl. Throm. Vas. 23 (2003) 622.

[3] M. Meydani, Nutr. Rev. 63 (2005) 312.

[4] P. Bogani, C. Galli, M. Villa, F. Visioli, Atherosclerosis. 190 (2007) 181.

[5] P. Pérez-Martínez, J. López-Miranda, L. Blanco -Colio, C. Bellido, Y. Jiménez, J. A. Moreno, J. Delgado-Lista, J. Egido, F. Pérez-Jiménez, Atherosclerosis. 194 (2007) e141.

[6] K. Esposito, R. Marfella, M. Ciotola, C. Di Palo, F. Giugliano, G. Giugliano, M. D'Armiento, F. D'Andrea, D. Giugliano, Jama J. Am. Medical. Assoc. 292 (2004) 1440.

[7] A. Camargo, J. Ruano, J. Fernández, L. Parnell, A. Jiménez, M. Santos-González, C. Marín, P. Pérez-Martínez, M. Uceda, J. López-Miranda, F. Pérez-Jiménez, BMC Genomics. 11 (2010) 253.

[8] R. de la Torre, Inflammopharmacology. 16 (2008) 245.

[9] M. Fitó, M. Cladellas, R. de la Torre, J. Martí, M. Alcántara, M. Pujadas - Bastardes, J. Marrugat, J. Bruguera, M. C. López -Sabater, J. Vila, M. I. Covas, Atherosclerosis. 181 (2005) 149.

[10] F. Pérez-Jiménez, J. Ruano, P. Pérez-Martínez, F. López-Segura, J. López-Miranda, Mol. Nutr. Food Res. 51 (2007) 1199.

[11] J. Ruano, J. López-Miranda, F. Fuentes, J. A. Moreno, C. Bellido, P. Pérez-Martínez, A. Lozano, P. Gómez, Y. Jiménez, F. Pérez -Jiménez, J. Am. Coll. Cardiol. 46 (2005) 1864.

[12] J. Ruano, J. López -Miranda, R. de la Torre, J. Delgado-Lista, J. Fernández, J. Caballero, M. I. Covas, Y. Jiménez, P. Pérez-Martínez, C. Marín, F. Fuentes, F. Pérez-Jiménez, Am. J. Clin. Nutr. 86 (2007) 341.

[13] M. J. Oliveras-López, G. Berná, E. M. Carneiro, H. López-García de la Serrana, F. Martín, M. C. López, J. Nutr. 138 (2008) 1074.

[14] D. Eletto, A. Leone, M. Bifulco, M. F. Tecce, Nutr. Metab. Cardiovasc. Dis. 15 (2005) 13.

[15] R. W. Owen, W. Mier, A. Giacosa, W. E. Hull, B. Spiegelhalder, H. Bartsch, Food Chem. Toxicol. 38 (2000) 647.

[16] E. Miró-Casas, M. I. Covas, M. Farré, M. Fitó, J. Ortuño, T. Weinbrenner, P. Roset, R. de la Torre, Clin. Chem. 49 (2003) 945.

[17] K. L. Tuck P. J. Hayball, J. Nutr. Biochem. 13 (2002) 636.

[18] M. N. Vissers, P. L. Zock, M. B. Katan, Eur. J. Clin. Nutr. 58 (2003) 955.

[19] F. Ursini, A. Zamburlini, G. Cazzolato, M. Maiorino, G. B. Bon, A. Sevanian, Free Radical Bio. Med. 25 (1998) 250.

[20] J. J. Moreno, Free Radical Bio. Med. 35 (2003) 1073.

[21] C. Manach, G. Williamson, C. Morand, A. Scalbert, C. Rémésy, Am. J. Clin. Nutr. 81 (2005) 230S.

[22] M. N. Vissers, P. L. Zock, A. J. C. Roodenburg, R. Leenen, M. B. Katan, J. Nutr. 132 (2002) 409.

[23] E. Miniati, Annali dell Istituto Superiore di Sanita. 43 (2007) 362.

[24] H.W. Tan, K. L. Tuck, I. Stupans, P. J. Hayball, J. Chromatogr. B. 785 (2003) 187.

[25] K. Nakashima, S. Kinoshita, M. Wada, N. Kuroda, W. R. G. Baeyens, Analyst. 123 (1998) 2281.

[26] A. Bolarinwa J. Linseisen, J. Chromatogr. B. 823 (2005) 143.

[27] A. Romani, M. Minunni, N. Mulinacci, P. Pinelli, F. F. Vincieri, M. Del Carlo, M. Mascini, J. Agr. Food Chem. 48 (2000) 1197.

[28] M. J. Lee, S. Prabhu, X. Meng, C. Li, C. S. Yang, Anal. Biochem. 279 (2000) 164.

[29] A. Bonanome, A. Pagnan, D. Caruso, A. Toia, A. Xamin, E. Fedeli, B. Berra, A. Zamburlini, F. Ursini, G. Galli, Nutr. Metab. Cardiovasc. Dis. 10 (2000) 111

[30] P. Del Boccio, A. Di Deo, A. De Curtis, N. Celli, L. Iacoviello, D. Rotilio, J. Chromatogr. B. 785 (2003) 47.

[31] K. de la Torre-Carbot, J. L. Chávez-Servín, O. Jaúregui, A. I. Castellote, R. M. Lamuela-Raventós, M. Fitó, M. I. Covas, D. Muñoz -Aguayo, M. C. López -Sabater, Anal. Chim. Acta. 583 (2007) 402.

[32] M. Suárez, M. P. Romero, A. Macià, R. M. Valls, S. Fernández, R. Solà, M. J. Motilva, J. Chromatogr. B. 877 (2009) 4097.

[33] K. de la Torre-Carbot, O. Jaúregui, A. I. Castellote, R. M. Lamuela-Raventós, M. I. Covas, I. Casals, M. C. López-Sabater, J. Chromatogr. A. 1116 (2006) 69.

[34] M. I. Covas, K. de la Torre, M. Farré-Albaladejo, J. Kaikkonen, M. Fitó, C. López-Sabater, M. A. Pujadas-Bastardes, J. Joglar, T. Weinbrenner, R. M. Lamuela-Raventós, R. de la Torre, Free Radical Bio. Med. 40 (2006) 608.

[35] T. Weinbrenner, M. Fitó, R. d. l. Torre, G. T. Saez, P. Rijken, C. Tormos, S. Coolen, M. F. Albaladejo, S. Abanades, H. Schroder, J. Marrugat, M. I. Covas, J. Nutr. 134 (2004) 2314.

[36] F. N. Bazoti, E. Gikas, A. Tsarbopoulos, Biomed. Chromatogr. 24 (2010) 506.

[37] Food and Drug Administration. Guidance for industry: bioanalytical method validation, 2001. Available from: http://www.fda.gov/cder/guidance/4252fnl.pdf.

[38] V. B. Massart DL, Buydens LMC, De Jong S, Lewi PJ, Smeyers Verbeke J, Handbook of Chemometrics and Qualimetrics. Data Handling in Science and Technology, Elsevier Science, Amsterdam, Vol. 1. 1997.

[39] E. Miró-Casas, M. I. Covas, M. Fito, M. Farré-Albadalejo, J. Marrugat, R. de la Torre, Eur. J. Clin. Nutr. 57 (2003) 186.

[40] O. Khymenets, M. Farré, M. Pujadas, E. Ortiz, J. Joglar, M. I. Covas, R. de la Torre, Food Chem. 126 (2011) 306.

PARTE III

Desarrollo de plataformas orientadas en metabolómica clínica: Variedad de muestras

Con la investigación recogida en esta Parte III de la Memoria se ha pretendido destacar la versatilidad de la metabolómica para su aplicación a muestras clínicas de muy diversa naturaleza, además de miniaturizar y automatizar todas las etapas implicadas en el método en cuestión.

Con estas premisas, se ha puesto a punto un método basado en un sistema LOV para el desarrollo de las etapas de SPE y derivatización de ácidos siálicos; una separación mediante μ-LC y detección de los fluoróforos formados mediante su emisión inducida por láser. El método así desarrollado se ha aplicado, con muy ligeras modificaciones, a la determinación de los analitos en muestras de suero, orina, saliva y leche materna con excelentes resultados; todo lo cual se recoge en el Capítulo 9.

Un método para los mismos analitos (ácidos siálicos en suero y en orina en este caso) con un mayor grado de sofisticación y un mayor coste de adquisición del equipamiento, pero también más rápido, más sensible —límites de detección y cuantificación un orden de magnitud más bajos que los del método anterior— y con nula implicación del usuario, ha estado basado en una plataforma SPE–LC–MS/MS, tal como se recoge en el Capítulo 10.

CAPÍTULO 9

Etapas de hidrólisis asistida por ultrasonidos/extracción en fase sólida en un sistema "lab-on-valve"/derivatización asistida por ultrasonidos previas a la determinación de los ácidos N-acetilneuramínico y N-glicolilneuramínico en fluidos biológicos mediante μ-LC–LIF

Ultrasound-assisted hydrolysis/lab-on-valve solid-phase extraction/ultrasound-assisted derivatization prior to μ-LC-LIF for the determination of *N*-acetylneuraminic and *N*-glycolylneuraminic acids in human biofluids

M.I. Orozco-Solano, F. Priego-Capote*, M.D. Luque de Castro*

Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain

Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain

Enviado a Analytica Chimica Acta para su publicación

ELSEVIER

Ultrasound-assisted hydrolysis/lab-on-valve solid-phase extraction/ultrasound-assisted derivatization prior to μ-LC-LIF for the determination of *N*-acetylneuraminic and *N*-glycolylneuraminic acids in human biofluids

M.I. Orozco-Solano, F. Priego-Capote, M.D. Luque de Castro

ABSTRACT

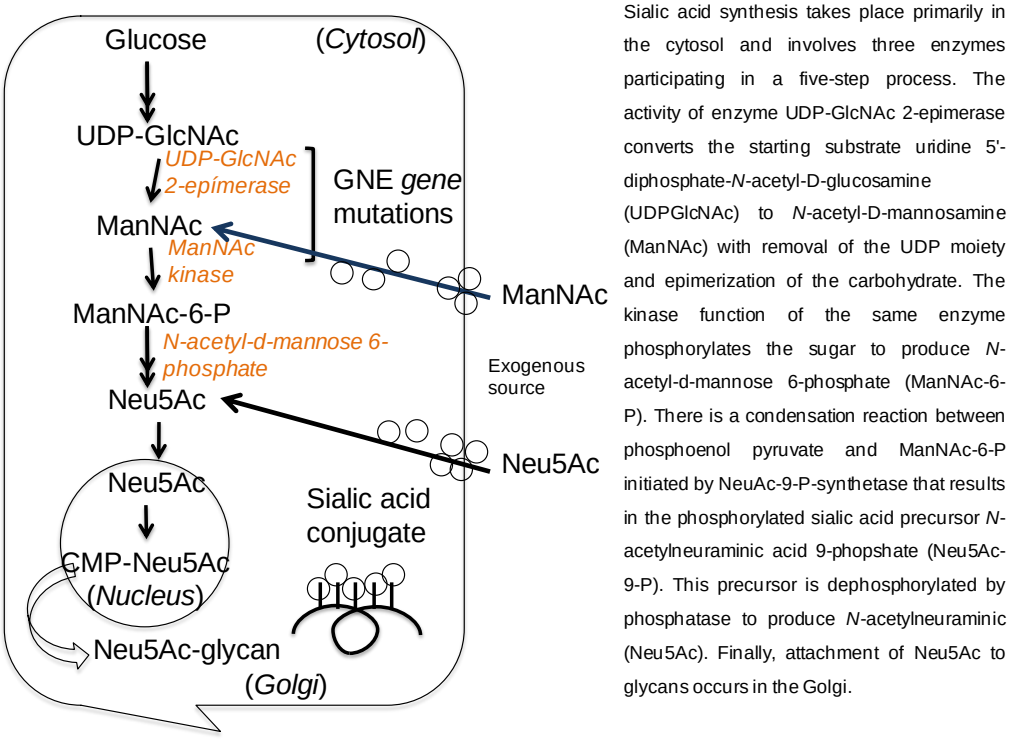
The determination of sialic acids (SIAs) has recently gained interest because of their potential role as markers of inflammatory disorders or chronic diseases. Hydrolysis of conjugated derivatives, solid-phase extraction (SPE) and derivatization steps constitute sample preparation prior to insertion of the analytical sample into a □-liquid chromatograph–laser induced fluorescence detector (μ-LC–LIF) in the present method for the determination of two representative SIAs of human metabolism. Ultrasound-accelerated hydrolysis released free SIAs, which were efficiently concentrated in a dynamic manner using a lab-on-valve (LOV) module that allows automation of SPE for preconcentration and cleanup. This step was on-line connected with DMB-labeling of SIAs (derivatization), which was shortened from 180 min required with the conventional heating method to 20 min with ultrasound assistance. Individual separation of the target analytes was achieved within 20 min by μ-LC, while LIF detection endowed the overall method with high sensitivity. The LODs and LOQs provided by the method ranged 0.1–0.8 ng mL^{-1} and 0.4–1.0 ng mL^{-1} (between 0.1–0.8 pg and 0.4–1.0 pg expressed as on-column amount), respectively. High efficiency for interferents removal by SPE enabled the application of the method to four different biofluids — serum, urine, saliva and breast milk— for determination of the target metabolites.

Keywords: Sialic acids, Lab-on-valve, μ-LC-laser induced fluorescence, Solid-phase extraction, Ultrasound-assisted hydrolysis and derivatization.

1. Introduction

Sialic acids (SIAs) comprise a family of 43 naturally occurring derivatives of the nine-carbon sugar neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galactononulsonic acid). One branch of the SIAs family is N-acetylated to form N-acetylneuraminic acids (Neu5Ac), which are the most widespread forms of SIAs and almost the only found in humans. The other branch is based on N-glycolylneuraminic acids (Neu5Gc), which are common in many animal species (better investigated in porcine tissues, but not found in humans, except for individuals affected by certain types of cancers such as colon and breast cancers [1-3].

While a small portion of SIAs occurs as free metabolites in human biofluids, ahe major part of them is bound to glycosidic forms. Thus, SIAs are commonly present as components of oligosaccharide chains of mucins, glycoproteins, and glycolipids [4]. They usually occupy terminal, non-reducing positions of oligosaccharide chains in complex carbohydrates on outer and inner membrane surfaces by various linkages, mainly to galactose, N-acetylgalactosamine, and other sialic acid moieties. Cells from higher animals and various microorganisms produce SIAs in a long pathway starting from glucose. An outline of the multiple reactions involving SIAs is shown in Supplementary Fig. 1. Sialic acids behave very different in model animals; in young rats the levels of SIAs in the liver are initially low, rising to a maximum 14–21 days after birth [5]; in mice and guinea pigs SIAs concentration is correlated with age and developmental stage [6]. It has been postulated that human infant liver may also have a limited capacity for synthesizing SIAs during early postnatal life [7]. Human milk also contains a high concentration of SIAs attached to the terminal end of free oligosaccharides, but their metabolic fate and biological role are practically unknown. High concentrations of SIAs in serum have been reported in patients with some inflammatory disorders or chronic diseases, but the range is lowered in healthy humans [8, 9].



Supplementary Figure 1. *Scheme of* **N***-acetylneuraminic acid (Neu5Ac) enzymatic synthesis and attachment to glycan structure.*

The discovery of the role of SIAs as potential biomarkers for different pathologies has increased their clinical interest. Methods for determination of SIAs in biological samples involve two major steps: first, release from individual monosaccharides is achieved by acid or enzymatic hydrolysis; then, the resulting mixtures of monosaccharides can be analyzed by different methods: fractionation, characterization, and quantitation by high-performance liquid chromatography using anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [10], conventional HPLC coupled to fluorescence detection [11] or mass spectrometry detection [12, 13], or by gas chromatography with flame ionization detection (GC–FID) [14-16]. Concerning sample preparation, the implementation of a solid-phase extraction step (SPE) to attain higher sensitivity and selectivity for quantitative analysis of SIAs in biofluids is frequent [17]. This is usually carried out by reverse-phase sorbents such as C18 and C8 to take benefit from non-polar interactions with SIAs [18, 19]. Nevertheless, no single cleanup procedure based on polar interactions between the hydroxyl group of the target analytes and a polar sorbent has so far been described.

In this research, an ultrasound-assisted hydrolysis step was planned to precede a cleanup/preconcentration step based on a dynamic lab-on-valve (LOV) system. The

latter was coupled on-line to an ultrasound-assisted derivatization step with the aim of proposing a semi-automatic method for sample preparation of SIAs in biological samples with easy implementation in the clinical area. The resulting treated sample was inserted into a μ-LC–LIF approach for individual separation and quantitation of *N*-acetylneuraminic and *N*-glycolylneuraminic acids.

2. Materials and methods

2.1. Instruments and apparatus

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450W) equipped with a cylindrical titanium–alloy probe (12.70 mm diameter), which was immersed into a lab-made stainless-steel container with eight compartments to place test tubes.

Figure 1 illustrates the experimental setup used for sample preparation based on three steps: ultrasound-assisted hydrolysis step (A), automated SPE (B) and derivatization of SIAs (C). SPE was carried out with a sequential injection LOV microfluidic system. The LOV system consists of a single-channel high precision bi-directional syringe pump of 1 mL, a holding coil and a multiposition valve. The pump allows aspirating and dispensing micro-volumes of sample and reagents; the valve, a six-port valve made of Plexiglass, has the ports interconnected and also with the syringe pump by a central port. The connections are as follows: port 1-waste, port 2-buffer, port 3-sample, port 4-flow cell (it was not used), port 5-eluant flow-through to SPE, port 6-air. The holding coil (holding coil 1 in Fig. 1 —1 mL, 0.3 mm i.d.) is located in between the syringe pump and the six-port valve. A two-position selection valve allows wasting all solutions different from the analytes fraction, which is led to holding coil 2 for derivatization. Therefore, SPE and derivatization were on-line coupled for automated development. Polyetheretherketone (PEEK) tubing (0.5 mm × 0.8 mm i. d.) from Análisis Vínicos (Ciudad Real, Spain) was used to connect the LOV and additional components. Polytetrafluorethylene (PTFE) tube (1.5 mm i. d.) from Análisis Vínicos was used to construct the SPE mini-column, which was packed with aminopropyl (40 μm homogeneous particle size) from Varian (Madrid, Spain). Other tested sorbent material was Chromabond C18 (size 40 μm particle diameter) from Macherey–Nagel (Durem, Germany). The two-position selection valve (VICI, Valco, Houston, USA),

connected to the microcolumn channel, enabled to lead the eluate after the SPE step to holding coil 2 (500 µL, 0.5 mm i. d.) in which the eluate was subjected to ultrasound assistance, thus favoring derivatization. The LOV and the selection valve were fully automated and controlled by the FIAlab for Windows version 5.0 software.

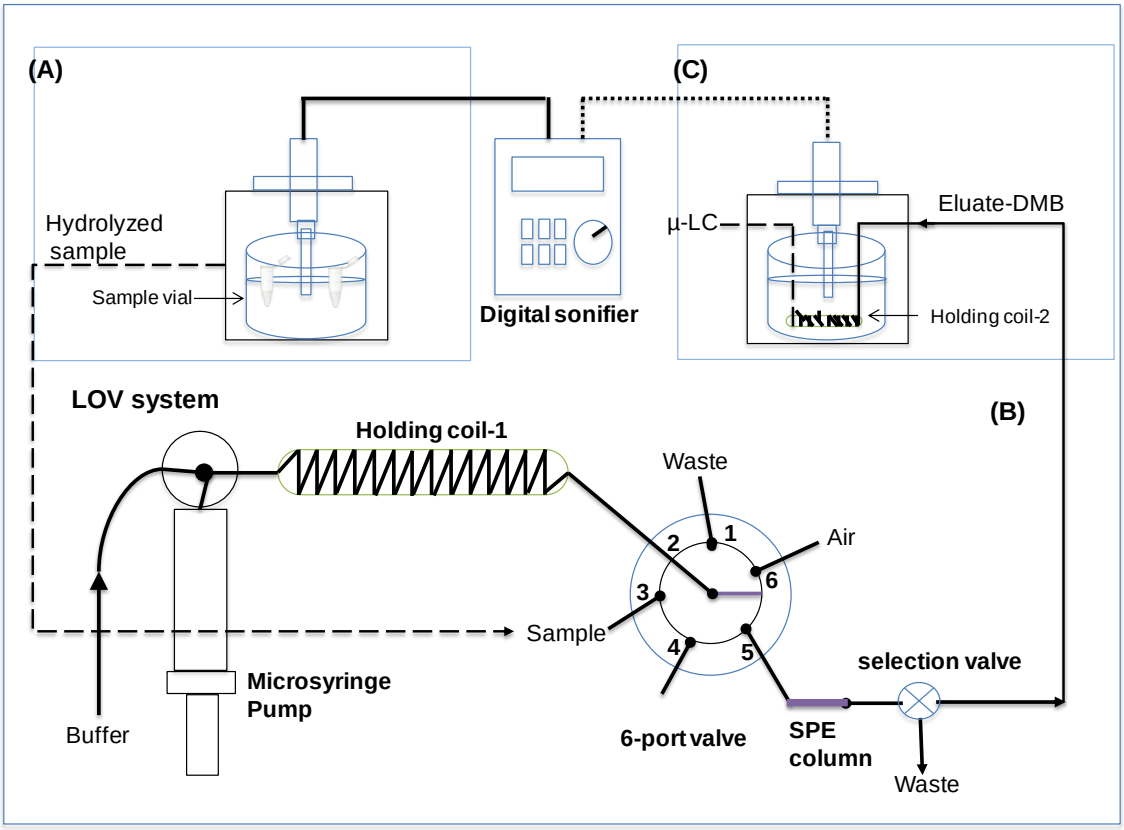


Figure 1. *Experimental scheme for sample preparation: (A) and (C) ultrasound-assisted hydrolysis and derivatization, respectively. Dashed line, non-automated process; dotted line, exchangeable position of the probe for derivatization. (B) LOV system for SPE . Continuous line, automated process.*

Separation of SIAs was performed by an Agilent (Palo Alto, CA, USA) 1100 micro-liquid chromatograph (µ-LC) equipped with a binary capillary pump and an automatic injection valve (1 µL sample loop). The analytical column was a reversed-phase Zorbax SB-C18 (150 mm × 0.5 mm i. d., 5 µm) from Agilent. The overall system was mounted with capillary tubes 75 µm i.d. × 375 µm outer diameter from Teknokroma (Barcelona, Spain). After chromatographic separation, the derivatized analytes were detected by a ZETALIF 2000 325 nm/CE LIF detector from Picometrics (Toulouse, France). The µ-LC was connected to the LIF detector by capillary tubing in which a detection window of 5 mm length was made for collecting the emitted light.

Signal acquisition from the LIF detector was monitored and then integrated by the Agilent Chemstation software.

2.2. Chemicals and sock solutions

N-Acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), *N*-acetylneuraminic acid methyl ester (Neu5Ac-methyl ester) and 1,2-diamino-4,5-methylene dioxybenzene (DMB) were purchased from Sigma–Aldrich (St. Louis MO, USA). Stock standard solutions at 1000 μg mL^{-1} of Neu5Ac and Neu5Gc were prepared in deionized water (DI water) (18 MΩ·cm) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA). Neu5Ac-methyl ester, used as internal standard, was dissolved at 500 μg mL^{-1} in LC–MS grade methanol from Panreac (Barcelona, Spain). Multistandard solutions were prepared by diluting the stock solutions in water. All the above solutions were stored at –20 °C in glass vials and kept in the dark until use.

LC grade methanol and acetonitrile from Scharlab (Barcelona, Spain) were used to prepare the chromatographic mobile phases. The solution for conditioning the SPE cartridge was 1 M sodium phosphate solution at pH 3. The activation solution was a 90:10 acetonitrile–water mixture. Hydrochloric acid (12 mM) from Panreac was used to hydrolyze the samples.

A 4 mM DMB solution was prepared by dissolving solid DMB in the following mixture: 1.5 M acetic acid, 0.8 M 2-mercaptoethanol (Merck, Darmstadt, Germany) and 14 mM sodium hydrosulfite in DI water. This solution was used for conventional derivatization. In the method proposed here, the DMB solution was daily prepared in 20:80 methanol–water and then stored at –20 °C in the dark until use owing to its sensitivity to light.

2.3. Samples

Serum samples were provided by the Blood Donors Department of Reina Sofia Hospital (Córdoba, Spain). All steps from blood extraction to storage were developed according to the guidelines dictated by the World Medical Association Declaration of Helsinki (2004). The ethical review board (ERB) of the Hospital supervised and approved these experiments. Individuals selected for this study were informed to obtain consent prior to

sample extraction. Venous blood was collected into a plastic Vacutainer® tube from Becton Dickinson (Franklin Lakes, NJ, USA). The serum was obtained from blood samples processed within 1 h after collection and centrifuged at 4000 × g for 10 min; then placed in plastic tubes and stored at –80 ºC until analysis.

Breast milk, saliva and urine samples were kindly donated by healthy volunteers. No restrictions about diet, age or sex were taken into account but none of the subjects was taking medication, and all of them were non smokers. The breast milk samples were centrifuged for 5 min at 5 ºC to separate serum from the lipid fraction. For saliva samples, the procedure described by Siskos *et al.* [20] was carried out to collect the samples, which were also centrifuged at 4000 × g for 5 min to separate bubbles. Pools of the four biofluids, which were used in the optimization of the method, were prepared by mixing aliquots of the different individuals. The internal standard (0.5 ng mL^{-1}) was added to samples before the hydrolysis step of conjugated SIAs.

2.4. Conventional protocols

2.4.1. Hydrolysis

Conventional hydrolysis was carried out by placing 100 µL of the target sample in a test Eppendorf tube to which 1.9 mL of 10 mM hydrochloric acid was added according to the method described by Shaw *et al.* [21]. The tube was heated for 60 min at 80 ºC in a heater block (Thermoblock, from Fisher scientific, Pittsburgh, PA), after which the sample was centrifuged for 5 min at 2000 × g to separate solid particles, thus yielding a supernatant phase of 2 mL, which was used for the SPE step.

2.4.2. Derivatization

The protocol described by Martin *et al*. [22] was applied for conventional derivatization. The sample–DMB mixture was incubated at 50 °C for 180 min, cooled and stored in the dark until analysis

2.5. Proposed method

The proposed method can be divided into four steps: (1) ultrasound-assisted hydrolysis of SIAs in the target sample; (2) preconcentration and cleanup of the target metabolites by SPE developed following the LOV program; (3) derivatization of the target analytes to yield quinoxalinone derivatives; (4) individual separation of the derivatives and determination by μ-LC–LIF.

2.5.1. Ultrasound-assisted hydrolysis

Deconjugation of SIAs was carried out by placing 100 μL of the target sample in a test Eppendorf tube to which 1.9 mL of 12 mM hydrochloric acid was added. The tube was placed in the stainless-steel container, then immersed into the water bath at 40 ºC, where ultrasonic irradiation under the optimal working conditions —duty cycle 50% (*viz*. ultrasound application 0.5 s s^{-1}), output amplitude 40% of the converter (180 W) and irradiation time for 20 min— was applied. The sample with the hydrolyzed metabolites was centrifuged as in section 2.4.1.

2.5.2. Solid-phase extraction

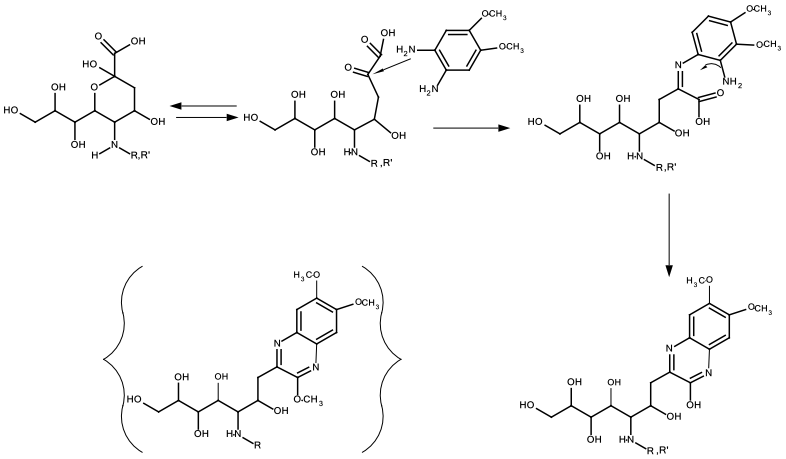
All sub-steps of SPE were carried out automatically by the LOV manifold (see Fig. 1), in a programmed sequence that can be summarized as follows: (a) activation of the mini-column (prepared by package of 30 mg of aminopropyl sorbent in 50 mm PTFE tube) with 1 mL 90:10 acetonitrile–water solution and conditioned with 500 μL of 1 M phosphate solution of pH 3 (both steps at 3 mL min^{-1}); (b) equilibration of the column with 500 μL phosphate solution at 1 mL min^{-1}; (c) loading of sample (1 mL hydrolyzed sample) at 1.2 mL min^{-1}; (d) washing of the mini-column with 250 μL of conditioning solution at 1 mL min^{-1}, and flushing air to eliminate the rest of sample matrix; (e) derivatization simultaneous with elution from the sorbent cartridge by the eluant (4 mM DMB in 20:80 methanol–water) at 50 μL min^{-1}. The first 100 μL of eluate, which did not contain the derivatized analytes, was discarded to prevent their dilution; then, (f) the external selection valve was switched and (g) 50 μL of the eluted fraction containing the derivatized analytes was led to holding coil 2, where the reaction mixture was subjected to ultrasound.

2.5.3. Ultrasound-assisted derivatization

The derivatization process started with elution from the sorbent mini-column by the eluant–derivatizing solution containing the fluorogenic reagent. The selection valve was in the waste position for the first 1.4 min elution; then, it was switched for 1 min to drive 50 µL of the eluted fraction to holding coil 2. After this time, the selection valve was switched again to the waste position and the LOV system was ready for the washing step and, then, for receiving the next hydrolyzed sample. Simultaneously, the digital sonifier was activated and the probe irradiated the water bath (part C in Fig. 1) for 20 min —duty cycle 50%, output amplitude 60% of the converter (270 W)— at 40 ºC, in which holding coil 2 was immersed. In this way, the labeling reaction (see Supplementary Fig. 2) was favored. Then, the DMB-labeled analytes were collected in an Eppendorf vial and analyzed by µ-LC–LIF within 24 h.

2.5.4. µ-LC–LIF separation and determination

Once the SPE and derivatization steps were finished, the µ-LC system interfaced to the LIF detector was used for individual separation and determination of the target analytes. The derivatized SIAs (1 µL injection volume) were separated by an SB-C18 analytical column at 20 ºC and 1.5 µL min^{-1} flow rate of a gradient program using mobile phases A (DI water) and B (6:4, v/v methanol–acetonitrile). The gradient was as follows: 0–2 min, 4% B; 2–12 min linear gradient to 100% B held for 8 min. The analytical column was equilibrated for a post-run time of 10 min. The excitation wavelength of the laser source was 325 nm (emission of the labeled analytes, 448 nm).



Supplementary Figure 2. *Fluorescent-quinoxalinone derivatives formation by derivatization of SIAs with DMB*

2.6. Statistical analysis

A Student t-test was used to evaluate statistically the results obtained in the different studies, for which the significance level was set at $\alpha = 0.05$. All analyses were done with the Statgraphics Centurion XV.I Statpoint Technologies, Inc. (Warranton, VA).

3. Results and discussion

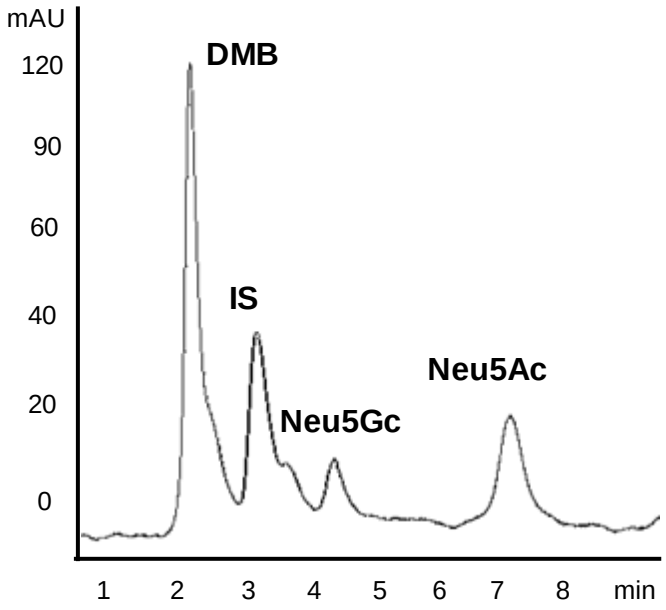
Spiked and non-spiked pools of each biofluid and multistandard solutions were used in the optimization study, the sequence of which consisted of four steps. First, the influential variables on individual chromatographic separation of the labeled SIAs by μ-LC–LIF were optimized for proper monitoring of sample preparation. In these μ-LC–LIF experiments the DMB-labeling reaction was performed without ultrasound assistance, as described in the conventional method described by Martin *et al.* [22]. Then, biofluids were used to optimize clean-up of matrix effects by SPE using the conventional hydrolysis protocol described by Shaw *et al.* [21]. Finally, the assistance of ultrasonic energy was tested to accelerate the kinetics of the two reactions involved in the analytical method: hydrolysis of SIA conjugated and the derivatization based on formation of DMB-labeled products.

3.1. Optimization of the determination step

The optimization study of the chromatographic variables was carried out with multistandard solutions resulting in the operating conditions described in the experimental section. The conventional derivatization protocol [22] was used to attain an optimum separation of the quinoxalinone derivatives of SIAs. Neu5Ac-methyl ester was used as internal standard, after checking its absence in the samples, since its physical and chemical behavior was similar to that of the target analytes.

Concerning detection, the excitation wavelength was 325 nm, close to the optimum excitation wavelength for the quinoxalinone derivatives (373 nm [23]), and the fluorescence emitted from the flow-cell was collected at the maximum emission wavelength (448 nm). Identification of the target analytes using commercially available

standards was based on comparison of the retention times. Complete separation of the target compounds was achieved within 20 min, as shows Supplementary Fig. 3.



Supplementary Figure 3. *Chromatogram obtained by analysis of an aqueous multistandard solution of the target analytes (10 ng mL^{-1}) after derivatization using the conventional protocol described by Martin et al. [22].*

3.2. Automated solid-phase extraction step

The different variables related to automated sample preparation by SPE were optimized by using a univariate approach to assess cleanup and analytes preconcentration. The variables under study, the tested range and the optimum values are shown in Supplementary Table 1. For this study, human biofluids were spiked with the target analytes at 10 ng mL^{-1} each (0.5 μg mL^{-1} external standard). Every hydrolyzed sample was circulated through the LOV manifold for cleanup and preconcentration of the target analytes and the eluate was off-line derivatized by the conventional derivatization protocol prior to μ-LC–LIF analysis [22]. The SPE study included optimization of the sorbent material, sample loading rate, eluant, volume of discharged eluate and elution volume. Two sorbents were tested taking into account the chemical properties of the analytes under study (combination of polar and non-polar functional groups), namely: C18 (40 μm particle size), and aminopropyl NH2 BondElut (40 μm particle size), which is a polar sorbent based on both hydrogen bonding and anion exchange interactions. Due to the anionic character of the target analytes, the best retention capability was obtained by the aminopropyl sorbent because of the two types of interactions this

material provides. The phosphate solution (pH= 3) used for solvation and equilibration of the sorbent favored anion interactions with the target analytes.

Supplementary Table 1. Optimization study of the SPE step

Variable	Tested range	Optimum value
Sorbent	C18, Aminopropyl	Aminopropyl
Sample loading rate (μL min^{-1})	0–5	1.2
Volume of eluate discharged with non- analytes (μL)	0–500	100
Volume of eluate containing the analytes (μL)	10–500	50

Finally, the composition of the eluant and the volume of discharged eluant were optimized to achieve maximum concentration of the analytes in the minimum volume of eluate. Concerning the composition of the eluate, a 20:80 methanol–water mixture provided the best desorption of analytes from the sorbent. The composition of the eluate was studied by off-line collection of 50 μL fractions, after minicolumn washing. Figure 2 shows the chromatograms obtained by injecting 1 μL of each of the 50-μL fractions obtained from the spiked serum pool in the μ-LC–LIF system. As can be seen, the higher concentration of the retained compounds was found in the third 50 μL of eluate; so, the first 100 μL were discharged to avoid dilution of the target analytes. Despite Fig. 2B shows that the IS is partially eluted in the second fraction, the intensity and precise signal of the peak in the third fraction allowed its use as such IS. On the other hand, the results shown in Fig. 2 demonstrated the high efficiency of sorbent retention and the fast desorption kinetics provided by the eluant. The chromatogram in Fig. 2.C shows the absence of peaks different from those of either the analytes or IS; therefore, an excellent cleanup was achieved in the SPE step.

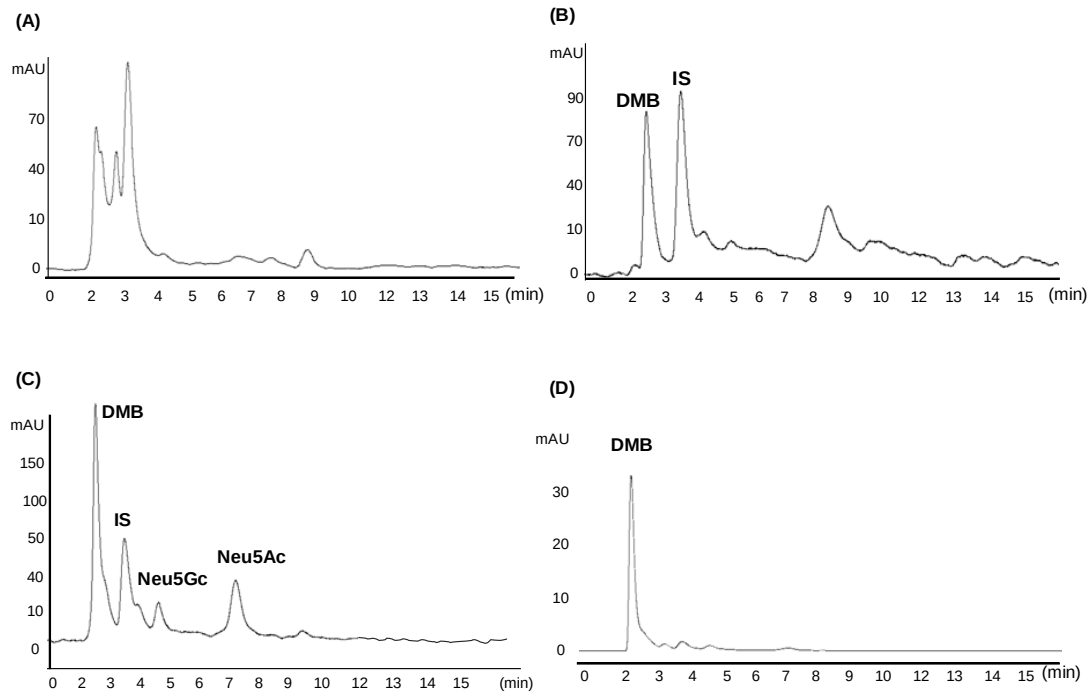


Figure 2. *Optimization of the elution volume in the SPE step. Chromatograms obtained for the elution with four consecutive 50-µL fractions of a spiked serum (10 ng mL^{-1}). Hydrolysis and derivatization were carried out by conventional protocols reported in [21] and [22], respectively. Fractions: first (A); second (B); third (C) and fourth (D).*

The efficiency of the SPE step was evaluated with a dual analysis of non spiked and spiked biofluids (10.0, 50.0 and 250.0 ng mL^{-1} each analyte) by calculation of the recovery factor. The two biofluids with a more complex matrix were selected for this study, namely, human serum and breast milk. The results, shown in Table 1, reported recovery factors for Neu5Ac and Neu5Gc above 92% for both biofluids, which support the efficiency of this step.

3.3. Optimization of ultrasound-assisted hydrolysis

Taking into account that glucuronidation is one of the main biotransformation pathways of SIAs [24], the samples were subjected to acid hydrolysis with HCl. This selection was supported on the research reported by Shaw *et al.* [21], who carried out a comparative study using different acids to release the target compounds. This study demonstrated total release of SIAs using 10 mM HCl at 80 ºC for 1 h.

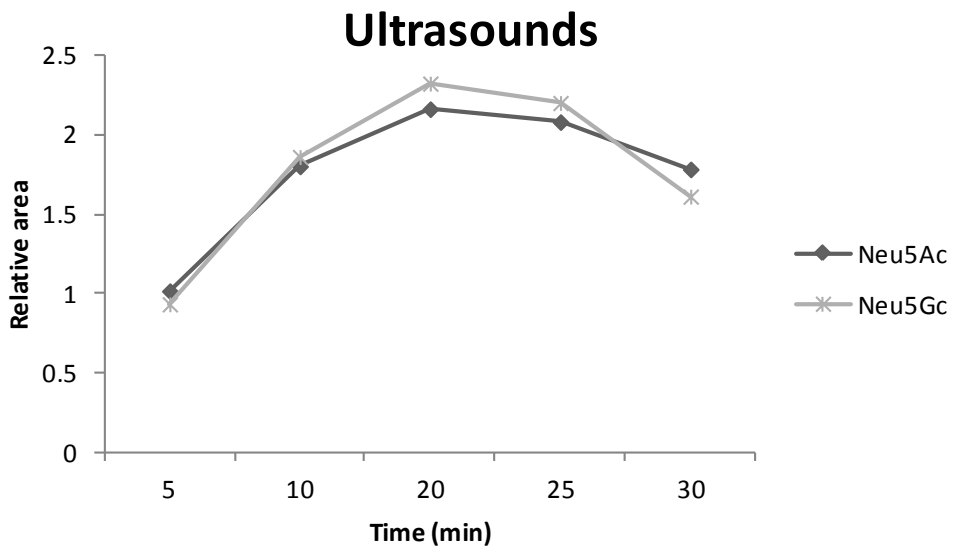
The shortened time reported in the literature for ultrasound-assisted hydrolysis, relative to traditional methods [25,26], justifies the experiments carried out here. These experiments were planned with both spiked and non-spiked biofluids to check the influence of ultrasonic energy on the hydrolysis reaction. After hydrolysis, the resulting solutions were treated by the optimized SPE and derivatized by the conventional protocol [22]. First of all, the influence of the two more critical variables of ultrasound on hydrolysis, irradiation amplitude and percent of ultrasound exposure or duty cycle, were optimized in the ranges 40–60% and 0.5–0.8 s s^{-1}, respectively. The bath temperature was kept at 40±2 ºC by addition of cold water to that in the bath previously heated at 40 ºC. The values given to each variable were selected from the experience gathered in preliminary experiments. In this study the ultrasonic irradiation was applied for 20 min while the concentration of HCl was set at 10 mM according to the protocol described by Shaw *et al.* [21], who ensured the stabilitiy of the target analytes.

The response variable was the ratio between the peak area of each compound and that of the IS. The irradiation amplitude (40%) and duty cycle (0.5 s s^{-1}) provided the highest response, although both variables were not significant; so, these values were used for subsequent studies. The reaction time was varied from 10 to 60 min to monitor the kinetics of the process. Significant increases in the response variable were observed for ultrasound-assisted hydrolysis times up to 25 min (p-value = 0.002). However, no significant differences existed between 20- and 25-min ultrasound-assisted experiments (p-value= 0.250). Therefore, 20 min was selected as optimum hydrolysis time. Concerning the HCl concentration, this was considered a key variable since the concentration of HCl could influence the stability of the analytes and the sample composition. For this reason, the HCl concentration was independently studied under optimum ultrasonic conditions in order to select the minimum HCl concentration offering quantitative results. The range of HCl concentration studied was varied from 10 to 32 mM resulting in an optimum concentration of 12 mM.

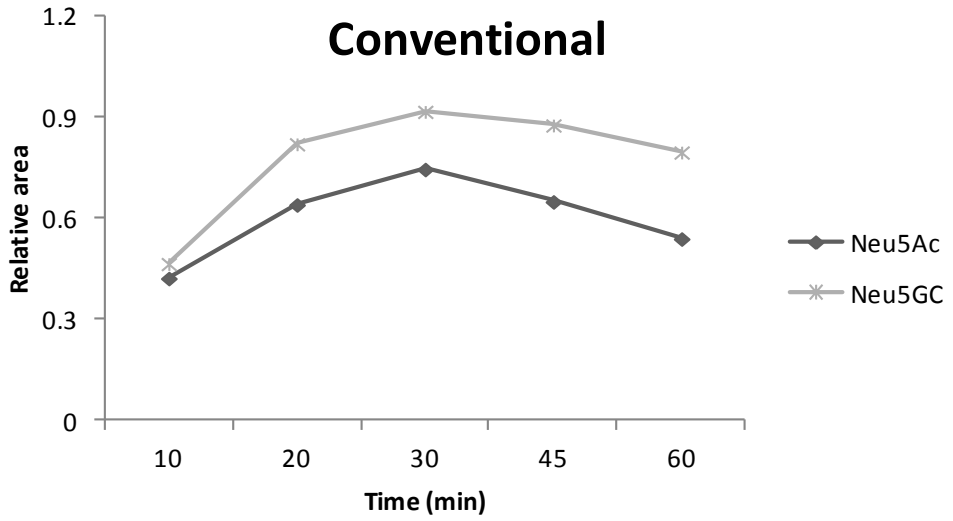
Apart from the shortening effect of the reaction time from 60 to 20 min, the US-assisted method provided a higher reaction yield than the conventional hydrolysis method [21]. As Supplementary Fig. 4 shows, the reaction yield was two times higher when assisted by auxiliary energy. The relevance of the hydrolysis step was proved by comparative analysis of non-spiked serum samples subjected or not to US-assisted hydrolysis. The non-hydrolyzed sample was directly treated by SPE. Figure 3 shows the chromatograms obtained in both cases for non-spiked serum. As can be seen, the

analytes were not detected in the chromatogram corresponding to the non-hydrolyzed sample, which showed the presence of unidentified chromatographic signals (Fig. 3.A). On the other hand, Neu5Ac was detected in the chromatogram of the hydrolyzed sample in which the background contribution was also clearly reduced (Fig. 3.B). The same study was applied to samples spiked with 20 ng mL^{-1}. Supplementary Fig. 5 shows the chromatograms obtained from the hydrolyzed and non-hydrolyzed samples. As can be seen, the signal of the IS was not affected by exposition to ultrasonic irradiation, but also the shape of the chromatographic peak corresponding to Neu5Ac was improved after US-assisted hydrolysis.

(A)



(B)



Supplementary Figure 4. *Kinetics study of the US-assisted hydrolysis and the conventional hydrolysis reported by Shaw et al. [21]. Both studies were carried out with human serum spiked at 30 ng mL^{-1} with the target analytes.*

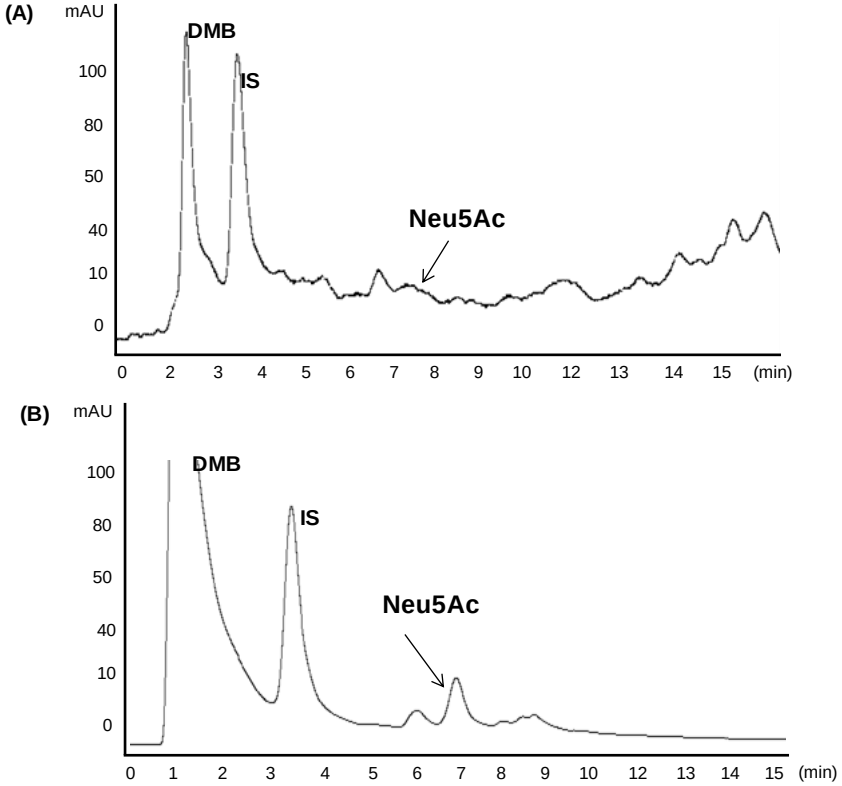


Figure 3. *Chromatograms provided by non-spiked human serum to assess the influence of the hydrolysis step: (A) non-hydrolyzed sample; (B) hydrolyzed sample with detection of Neu5Ac.*

3.4. Derivatization step

Ultrasound energy was also used to accelerate the DMB-labeling reaction of SIAs. Irradiation amplitude (40–80%), duty cycle (30–70%) and DMB concentration (0.05–10 mM) were the variables studied in a crossover design using 20 min as irradiation time. This study was carried out with all biofluids spiked with the analytes at 5 μg mL^{-1} each (0.5 μg mL^{-1} internal standard). The best conditions according to this experimental planning were 60% irradiation amplitude (250 W applied power), 50% duty cycle, and 4 mM DMB in the eluant. A kinetics study enabled to compare the conventional derivatization protocol and that proposed here with ultrasonic assistance. Figure 4 illustrates the enhancement effect of ultrasound irradiation, which reduced the

derivatization time from 180 min required in the conventional protocol to only 20 min with ultrasonic assistance. As can be seen, the same behavior was observed for both analytes.

It is worth mentioning that the concentration of Neu5Ac and Neu5Gc decreased with ultrasound exposition for times longer than 20 min (see Fig. 4B). This aspect can be due to degradation of SIAs under prolonged ultrasonic irradiation times. On the other hand, the DMB derivatives were stable for at least 6 h in daylight at room temperature.

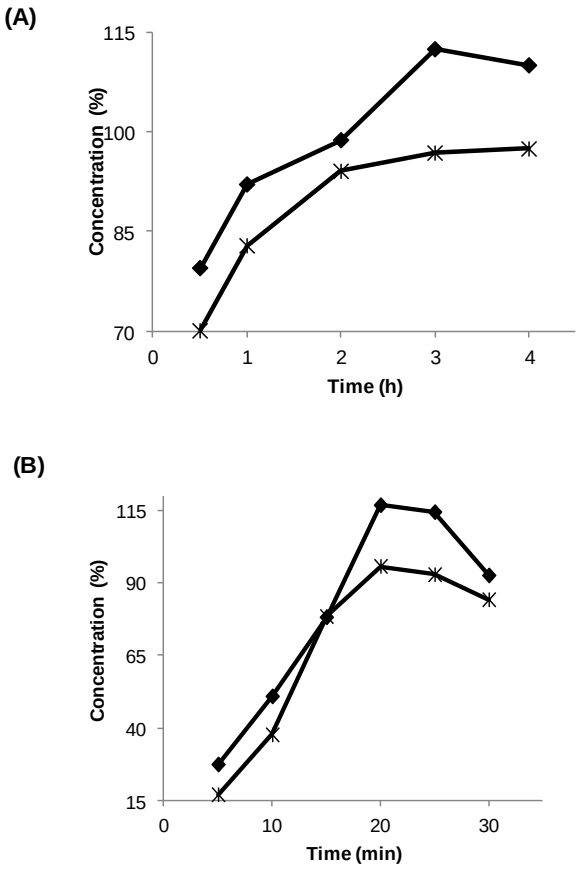


Figure 4. *Effect of the reaction time on the DMB derivatization of Neu5Ac (-♦-) and Neu5Gc (-*-) at 40 ºC with the conventional protocol described in [22] (A) and with US assistance with the protocol optimized here (B). The study was carried out with human serum spiked at 1 µg mL^{-1} with the target analytes.*

3.5. Analytical features of the method

3.5.1. Calibration

Different calibration models were prepared depending on the sample matrix. In the case of urine and saliva calibration plots were run by plotting the peak area ratio of

chromatographic signals for analytes and IS *versus* standard concentrations in aqueous multistandard solutions. On the other hand, the standard addition method was tested for serum and breast milk to evaluate matrix effects. The calibration curves were constructed by spiking serum and breast milk with known amounts of standard solutions. Three concentrations (high, medium and low —10.0, 50.0 and 250 ng mL^{-1}) within the calibration range were injected in triplicate to set confidence levels for Neu5Ac and Neu5Gc. Features of the calibration curves such as equation, intercept and regression coefficient for each calibration model are listed in Supplementary Table 2. Calibration graphs (n=7) exhibited excellent linearity for Neu5Ac and Neu5Gc, with regression coefficients higher than 0.993 over the concentration range 0.1–100 ng mL^{-1} and 0.5–500 ng mL^{-1}, respectively (Supplementary Fig. 6). The slope of the calibration models revealed that matrix effects were significant for serum and highly-significant for breast milk.

Supplementary Table 2. Calibration models built for Neu5Ac and Neu5Gc using aqueous multistandard solutions as well as spiked serum and breast milk

		Matrix		
Analyte	Parameter	Aqueous [a]	Serum [a]	Breast milk [a]
Neu5Ac	Calibration curve	Y= 0.1610X + 3.196	Y= 0.2133X + 4.123	Y= 1.209X + 2.021
	Intercept	0.0000 ± 0.0001	0.0000 ± 0.0001	0.0000 ± 0.0001
	R^2 [b]	0.996	0.993	0.995
Neu5Gc	Calibration curve	Y=0.1022X + 2.010	Y=0.57 ± 1.020	Y=1.63 ± 2.120
	Intercept	0.000 ± 0.002	0.0001 ± 0.0006	0.0002 ± 0.0003
	R^2 [b]	0.995	0.998	0.997

[a]*Number of points: n = 7.* [b]*Regression coefficient.*

The suitability of the calibration curve built with multistandard solutions was checked for urine and saliva. For this purpose, samples from the pools of both biofluids were spiked at three concentration levels (10.0, 50.0 and 250 ng mL^{-1} corresponding to

high, medium and low values within the calibration curves) and analyzed with the complete protocol. These analyses were compared with those provided by multistandard solutions at the same concentrations. Supplementary Table 3 shows the ratios of the response between aqueous standard solutions and those from spiked urine and saliva. The ratio values were within 1.0±0.04, which supports the applicability of the calibration model developed with standard solution for analysis of urine and saliva samples.

Supplementary Table 3. Ratios of responses obtained from spiked urine and saliva samples versus aqueous multistandard solutions at three concentration levels within the linear range of the calibration curves (1.0, 20.0 and 100 ng mL^{-1} representing low, medium and high concentrations, respectively).

		Concentration[a]		
Matrix	**Analyte**	**Low**	**Medium**	**High**
Urine	Neu5Ac	1.02	1.03	1.03
	Neu5Gc	1.03	1.04	1.01
Saliva	Neu5Ac	1.04	1.00	1.00
	Neu5Gc	1.04	1.01	1.00

3.5.2. Sensitivity and precision

The limits of detection (LOD) and quantitation (LOQ) for each analyte were expressed as the concentration of analyte that gave a signal 3σ and 10σ, respectively, above the mean blank signal (where σ is the standard deviation of the blank signal). The LOD and LOQ values listed in Table 2 were calculated for the four studied biofluids. Thus, LODs ranged from 0.1 to 0.8 pg (0.1–0.8 ng mL^{-1}), while LOQs were from 0.4 to 1.0 pg (and 0.4–1.0 ng mL^{-1}) in the different samples, expressed as amount injected on-column. These LODs are equivalent to urine concentrations of 0.2 ng mL^{-1} for Neu5Ac and 0.5 ng mL^{-1} for Neu5Gc; lower than those reported in most previous studies. Thus, Valianpour *et al.* [27] reported LOD for Neu5Ac in urine of 0.93 µg mL^{-1}; in serum,

Siskos *et al.* [17] reported LODs of 18.54 µg mL^{-1}. Therefore, the sensitivity has been improved in our method, despite the fact that only 1 µL of the analytical sample was injected in the chromatographic system. Nevertheless, it is worth pointing out that Hayama *et al.* have recently developed a method coupling derivatization and LC–MS/MS with a QTrap system and reported LODs of 3 and 16 pg mL^{-1} for Neu5Ac and Neu5Gc, respectively [29]. Therefore, if higher sensitivity is required, this LC–MS/MS method can be combined with the sample preparation strategy here reported.

Within-day and between-days variability studies were developed to evaluate the precision of the method by analysis of replicates in five consecutive days. Precision tests were carried out for each target SIA metabolite using serum and breast milk pools from four volunteers. The pool of each sample was spiked with Neu5Ac and Neu5Gc at 0.1 µg mL^{-1}. As can be seen in Table 3, within-day and between-days variability, expressed as RSD, ranged from 4.31 to 9.50% and 10.68 to 14.26%, respectively, depending on both the analyte and type of biofluid.

Table 2. Analytical features of the method.

Analyte	**Parameter**	**Sample**			
		Serum	Breast milk	Urine	Saliva
Neu5Ac	(ng mL^{-1})[a]	20	20	20	20
	$C \pm s$ (ng mL^{-1})[b]	23 ± 3	22 ± 2	21 ± 5	27 ± 2
	$t_{experimental}$ [c]	2.50	0.61	1.23	0.75
	Accuracy (%)	97.2 ± 12	92.1 ± 8	95.2 ± 2	91.2 ± 3
	On column LOD (pg)[d]	0.8	0.6	0.2	0.1
	On column LOQ (pg) [d]	1.0	0.8	0.5	0.4
Neu5Gc	(ng mL^{-1})[a]	20	20	20	20
	$C \pm s$ (ng mL^{-1})[b]	21± 3	22 ± 3	25 ± 4	21 ± 9
	$t_{experimental}$ [c]	1.29	2.55	1.25	1.72
	Accuracy (%)	92.1 ± 3	96.0 ± 2	94.2 ± 8	96.1 ± 2
	On column LOD (pg)[d]	0.3	0.7	0.5	0.3
	On column LOQ (pg) [d]	0.5	0.9	0.8	0.6

Table 3. Precision-related parameters for the determination of Neu5Ac and Neu5Gc in serum and breast milk samples.

	Analyte	Within-day variability, RSD (%)[a]	Between-day variability, RSD (%)[a]
Serum	Neu5Ac	4.31	14.26
	Neu5Gc	8.59	11.52
Breast milk	Neu5Ac	7.11	12.21
	Neu5Gc	9.50	10.68

Comparing the results obtained with those of other methods for determination of SIAs, the RSD values here reported are similar to those obtained by Hurum [29], who provided a 13% of between-day variability for both Neu5Ac and Neu5Gc in infant formula. The results obtained are also similar to those provided by Spichtig *et al.* [30]: RSD values lower than 7% for Neu5Ac in within-day variability, and 9.31% for between-days variability when determined in milk and milk-based products.

3.5.3. Accuracy

The accuracy of the method was evaluated for all samples with a dual analysis of non spiked and spiked biofluids at 10.0, 50.0 and 250.0 ng mL^{-1} each analyte. The results for each sample and the standard deviations are shown in Table 2. The Student's *t*–test confirmed the absence of significant differences between the concentrations found with the proposed method and those added to the samples. Accuracy ranged from 91.2 to 97.2% (Table 2).

3.6. Application of the method to human biofluids

Once the method was fully optimized, its suitability was tested by application to four samples of each biofluid: serum, urine, breast milk and saliva from healthy volunteers. Only Neu5Ac was detected, as show the results listed in Table 4, in all examined samples, and determined within the range 1.10– 1.82 ng mL^{-1}. The literature supports that Neu5Gc has been found in human cancer cells and in non-human mammalian

tissues [31, 32]; therefore, our results are consistent with these findings since Neu5Gc was not detected in any of the four human biofluids analyzed. It is worth pointing out that despite the low concentrations of Neu5Ac in the four human biofluids, this method is sensitive enough to detect and quantify it.

On the other hand, our results were also consistent with the concentrations of Neu5Ac metabolites reported in the literature for serum and breast milk. Table 4 also includes the concentration ranges found in the literature [20, 27, 33–35].

Table 4. Mean concentration of Neu5Ac and Neu5Gc found in serum, breast milk, urine and saliva (mean, n=3, SD in brakets) and range of concentration reported in the literature

	Main concentration (mmol L^{-1})			
Sample	**Neu5Ac** (proposed method)	**Neu5Ac** (reported in the literature)	**Neu5Gc** (proposed method)	**Neu5Gc** (reported in the literature)
Serum	1.82 (0.07)	1.36-3.40 (17)	[a]	[c]
Breast milk	1.38 (0.08)	0.2-2.28 (28,29)	[a]	[c]
Urine	1.31(0.05)	1.82-4.24 (24)[b]	[a]	[c]
Saliva	10.23(0.05)	9.7 -22.1 (17,27)	[a]	[c]

[a] *Below the LOD.* [b]*Values non-reported for healthy volunteers.*

4. Conclusions

The proposed method is a competitive approach for analysis of SIAs in biological fluids by comparison to other methods reported in the literature. The optimum conditions to liberate SIAs by the proposed method clearly improved those reported by Hurum *et al*. [29] and Spichtig *et al*. [30]. The optimum hydrolysis conditions required 12 mM HCl at 40 ºC for 20 min *versus* Spitchtig *et al*. who used 0.5 M formic acid at 80 ºC for 2 h, and Hurum *et al*. who used 50 mM sulfuric acid at 80 ºC for 1 h.

Regarding sample cleanup and preconcentration, this is the first time that a semi-automatic method based on LOV is developed with this purpose. In dealing with the derivatization step, both Hurum *et al.* and Spichtig *et al.* performed derivatization of

SIAs at 60 and 80 ºC, respectively, for 2.5 h; the method here reported only needs 20 min at 40 ºC.

In short, the total time for sample preparation in the methods proposed here was 1 h as compared to the previous studies [29, 30] which required approximately 4 h. Apart from that, analyst intervention was also reduce and precision and accuracy were improved. In addition to semi-automation, the proposed approach provides a semi-closed system, thus improving protection against degradation and decreasing the number of sample transfer steps. Additionally, a sample can be prepared in the designed instrumental configuration while running the chromatographic step of the previous one, so that continuous analysis can be done, thus providing a throughput of 1.6 samples h^{-1}.

Acknowledgements

The Spanish Ministerio de Ciencia e Innovación (MICINN) and European FEDER program are thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

References

[1] R. Schauer, Zoology 107 (2004) 49-64.

[2] H. Higashi, Y. Hirabayashi, Y. Fukui, M. Naiki, M. Matsumoto, S. Ueda, S. Kato, Cancer Res. 45 (1985) 3796-3802.

[3] S. Inoue, C. Sato, K. Kitajima, Glycobiology 20 (2010) 752-762.

[4] S. L., Acta Soc. Med. Ups 61 (1956) 74-85.

[5] O. Koldovský J. Herbst, Biol. Neonate 17 (1971) 1-9.

[6] B. Wang J. Brand-Miller, Eur. J. Clin. Nutr. 57 (0000) 1351-1369.

[7] C. E. Bertram M. A. Hanson, Brit. Med. Bull. 60 (2001) 103-121.

[8] Sillanaukee, Pönniö, Jääskeläinen, Eur. J. Clin. Invest. 29 (1999) 413-425.

[9] L. Chrostek, B. Cylwik, A. Krawiec, W. Korcz, M. Szmitkowski, Alcohol. Alcoholism. 42 (2007) 588-592.

[10] J. S. Rohrer, Anal. Biochem. 283 (2000) 3-9.

[11] M. Ito, K. Ikeda, Y. Suzuki, K. Tanaka, M. Saito, Anal. Biochem. 300 (2002) 260-266.

[12] J. Salcedo, R. Lacomba, A. Alegría, R. Barbera, E. Matencio, M. J. Lagarda, Food Chem. 127 (2011) 1905-1910.

[13] P. Allevi, E. A. Femia, M. L. Costa, R. Cazzola, M. Anastasia, J. Chromatogr. A. 1212 (2008) 98-105.

[14] N. Hashii, N. Kawasaki, Y. Nakajima, M. Toyoda, Y. Katagiri, S. Itoh, A. Harazono, A. Umezawa, T. Yamaguchi, J. Chromatogr. A. 1160 (2007) 263-269.

[15] A. Pons, C. Richet, C. Robbe, A. Herrmann, P. Timmerman, G. Huet, Y. Leroy, I. Carlstedt, C. Capon, J. P. Zanetta, Biochemistry 42 (2003) 8342-8353.

[16] S. A. M. Marzouk, S. S. Ashraf, K. A. Al Tayyari, Anal. Chem. 79 (2007) 1668-1674.

[17] P. A. Siskos M. H. E. Spyridaki, J. Chromatogr. B. 724 (1999) 205-212.

[18] D. C. A. Neville, V. Coquard, D. A. Priestman, D. J. M. te Vruchte, D. J. Sillence, R. A. Dwek, F. M. Platt, T. D. Butters, Anal. Biochem. 331 (2004) 275-282.

[19] L. Ruhaak, A. Deelder, M. Wuhrer, Anal. Bioanal. Chem. 394 (2009) 163-174.

[20] P. A. Siskos M.-H. E. Spyridaki, J. Chromatogr. B. 724 (1999) 205-212.

[21] C. J. Shaw, H. Chao, B. Xiao, J. Chromatogr. A. 913 (2001) 365-370.

[22] M. Martín, E. Vázquez, R. Rueda, Anal. Bioanal. Chem. 387 (2007) 2943-2949.

[23] S. Hara, M. Yamaguchi, Y. Takemori, K. Furuhata, H. Ogura, M. Nakamura, Anal. Biochem. 179 (1989) 162-166.

[24] E. M. Hawes, Drug Metabolism and Disposition 26 (1998) 830-837.

[25] B. Alvarez-Sanchez, F. Priego-Capote, M. D. Luque de Castro, Analyst 134 (2009) 1416-1422.

[26] Z. León-González, C. Ferreiro-Vera, F. Priego-Capote, M. D. Luque de Castro, J. Chromatogr. A. 1218 (2011) 3013-3021.

[27] F. Valianpour, N. G. G. M. Abeling, M. Duran, J. G. M. Huijmans, W. Kulik, Clin. Chem. 50 (2004) 403-409.

[28] T. Hayama, Y. Sakaguchi, H. Yoshida, M. Itoyama, K. Todoroki, M. Yamaguchi, H. Nohta, Rapid Commun. Mass Sp. 24 (2010) 2868-2874.

[29] D. C. Hurum J. S. Rohrer, Journal of Dairy Science 95 (2012) 1152-1161.

[30] V. Spichtig, J. Michaud, S. Austin, Anal. Biochem. 405 (2010) 28-40.

[31] H. Higashi, M. Naiki, S. Matuo, K. Okouchi, Biochem. Bioph. Res. Co. 79 (1977) 388-395.

[32] Y. N. Malykh, R. Schauer, L. Shaw, Biochimie 83 (2001) 623-634.

[33] S. D'Alessandro, H. M. Curbelo, O. R. Tumilasci, J. A. Tessler, A. B. Houssay, Arch. Oral Biol. 34 (1989) 829-831.

[34] S. E. Carlson, Am. J. Clin. Nutr. 41 (1985) 720-726.

[35] B. Wang, J. Brand-Miller, P. McVeagh, P. Petocz, Am. J. Clin. Nutr. 74 (2001) 510-515.

CAPÍTULO 10

Método automatizado para la determinación y el análisis confirmatorio de los ácidos N-acetilneuramínico y N-glicolilneuramínico en suero y orina mediante extracción en fase sólida acoplada en línea con cromatografía de líquidos y espectrometría de masas en tándem

Automated method for determination and confirmatory analysis of N-acetylneuraminic and N-glycolylneuraminic acids in serum and urine by solid-phase extraction on-line coupled to liquid chromatography–tandem mass spectrometry

M.I. Orozco-Solano[a,b], F. Priego-Capote[a,b], M.D. Luque de Castro[a,b*]*

[a]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain

[b]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain

Enviado a Journal of Chromatography A

Automated method for determination and confirmatory analysis of N-acetylneuraminic and N-glycolylneuraminic acids in serum and urine by solid-phase extraction on-line coupled to liquid chromatography–tandem mass spectrometry

M.I. Orozco-Solano[a,b], F. Priego-Capote[a,b*], M.D. Luque de Castro[a,b*]

ABSTRACT

N-acetylneuraminic acid (Neu5Ac) and N-acetylglycolyneuraminic acid, two acidic nine-carbon sialic acids, are involved in numerous biological processes as modulators of glycoconjugates functions. A fully automated method is here presented for determination of sialic acids in two human biofluids such as serum and urine. For this purpose, a solid-phase extraction (SPE) workstation was on-line connected to an LC–MS/MS with a triple quadrupole mass detector. Hydrolysis to release sialic acids bound to glycoconjugates and a derivatization step were implemented as treatments prior to SPE–LC–MS/MS analysis. Following thorough optimization of the SPE and LC–MS/MS conditions, the analytical procedure was validated and the standard addition calibration method used for the quantitative correction of matrix effects. The proposed method affords detection limits for the target analytes of 0.3 pg/mL and 0.5 pg/mL for acetylneuraminic and glycolylneuraminic acids, respectively. The precision (expressed as relative standard deviation) ranged between 1.7and 2.5 for within-day variability, and between 4.8 and 5.2% for within-days variability. Recovery factors estimated with a dual-cartridge configuration were above 99.54%. The total analysis time was 16 min. The method has been applied to human serum and urine of healthy volunteers. The proposed method is reliable and has a suited potential for high-throughput utilization in both clinical and research laboratories. The automation of sample preparation enables to scale-down this step and improves precision by minimization of human intervention.

Keywords: Acetylneuraminic acid, Glycolyneuraminic acid, Methyl ester acetylneuraminic acid, Serum, LC–MS/MS, On-line SPE.

1. Introduction

Sialic acids (SIAs) belong to the family of acylated derivatives of 9-C carboxylated monosaccharides and are mostly found in terminal position of glycoconjugates. The various substituents present on carbons 4, 5, 7, 8, and 9 generate a family of more than 40 different members. The substituent on carbon 5 can be an amino, an acetamido, a glycolylamido, or a hydroxyl group and defines the four major types of sialic acids: neuraminic acid (Neu), N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and ketodeoxy-nonulosonic acid (KDN), respectively. Sialic acids are involved in numerous biological functions of glycoconjugates, some of which are modulated by modifications of the SIA general structures. For example, O-acetylation of SIAs can alter the sensitivity to neuraminidases [1] and mask the ligands of *influenza A and B hemagglutinins* viruses [2]. These modifications have shown to be tissue-specific [3], and their expression is regulated [4]. However, only a few examples of their significance have been published to date. This is due to: (1) the lack of sensitive, specific analytical methods to investigate SIA substitutions; (2) their chemical lability during isolation and purification of the glycoconjugates to which they are attached; and (3) the presence of esterases in most biological materials [4]. Furthermore, even if modified SIAs survive isolation and purification protocols, defining the position of substituents in the backbone is not a straightforward matter.

The high expression of SIAs on outer cell membranes (*e.g.* more than 10 million molecules per human erythrocyte), in the interior of lysosomal membranes and in secreted glycoproteins (such as blood proteins and mucins) suggests the rol of these acids in the stabilization of molecules and membranes as well as in modulating interactions with the environment. Some biological functions such as binding and transporting ions and drugs, stabilizing the conformation of proteins including enzymes, and enhancing the viscosity of mucins arise from the relatively strong negative charge of SIAs. These acids can also protect molecules and cells from the activity of proteases or glycosidases, extending their lifetime and function. Sialic

acids are also involved in rolling and extravasations of leukocytes during inflammation due to selectins recognition [5, 6].

Detection of SIAs metabolic changes has also been involved in degenerative diseases such as atherosclerosis and diabetes as well as in neurological disorders such as Alzheimer's disease. Several human genetic disorders are associated to deregulation of SIAs levels such as hereditary inclusion body myopathy, sialuria, Salla disease (a defect in the lysosomal SIA transporter sialin), and galactosialidosis (galactosidase-peptidase-sialidase complex deficiency). Apart from them, many of the congenital disorders of glycosylation may also lead to altered sialylation [7], but little is known regarding the molecular basis of phenotypic consequences.

Concerning methods for analysis of SIAs, they can be divided into those intending to characterize glycoconjugates and those involving cleavage of glycosidic linkages for determination of the released SIAs [8]. Once the SIAs have been released a purification step is necessary. Several methodologies developed to isolate and release sialic acids from biological samples (biological fluids and tissue are the most studied), as well as dairy products have been described by Lamari and Karamanos [9] and Karamanos *et al.* [10]. In sample preparation, two treatments — enzymatic and acid hydrolysis— for releasing the non-free form of SIAs have been used, the latter being the most widely used treatment. Mild acid conditions under heating (between 70 ºC and 90 ºC) with an oven or block heater are used. Sulfuric and hydrochloric acids at concentrations 25–100 mM are the acids most commonly used in this step [11, 12]. Other studies have used auxiliary energies such as ultrasound or microwaves to decrease the hydrolysis time [13-15].

Glycoconjugates characterization has been addressed by a variety of analytical techniques including NMR [16] and mass spectrometry (MS) with different ionization techniques such as liquid secondary ionization (LSI), fast atom bombardment (FAB), electrospray ionization (ESI), or matrix-assisted laser desorption/ionization (MALDI) [17-20]. The main limitation of the approaches for analysis of glycoconjugates is identification of these complex structures. Several studies have involved derivatization methods due to the high selectivity and sensitivity this step is endowed with [21]. Furthermore, if MS methods are used, the

results are better, because additional information can be obtained on the SIA moiety —though data treatment is more complex.

Among the derivatization reagents evaluated for the determination of Neu5Ac and Neu5Gc in human serum and urine 1,2-diamino-4,6-dimethoxybenzene (DDB) and particularly 1,2-diamino-4,5-methylenedioxybenzene (DMB) —a reagent with superior selectivity and fewer interferences— have been used for analysis of SIAs by LC–MS and LC–laser-induced fluorescence (LIF) [19]. Likewise, other reagents such as ptoluenesulfonylchloride (Tos-Cl), benzoyl derivatives and *o*-phenylenediamine (OPD) have been reported for SIAs derivatization [18]. Derivatization with OPD is faster than with DMB, and the determination can be carried out either by UV absorption or fluorescence —the latter being preferable because the highest sensitivity of its product.

Regarding qualitative and quantitative analysis of SIAs, selected reaction monitoring (SRM) has been the mass detection mode more used thanks to its high selection capability, specificity and sensitivity, which have gradually attracted more attention of researchers. Ikeda *et al.* developed an effective method for the targeted analysis of theoretically expected ganglioside molecular species, which are composed of different numbers of SIAs residues, by HPLC/ESI-quadrupole linear ion trap hybrid mass spectrometer in combination with SRM. They found that the SRM detection specific for SA enabled to analyze ganglioside standards at the pmol to ftomol levels [22]. Hashi *et al.* have also reported limits of detection (LOD) in the order of fmol by a method based on nano liquid chromatography–Fourier transformation MS (FTMS) (8.6 fmol for Neu5Gc and 5.6 fmol for Neu5Ac), *versus* pmoles in the MS/MS method [19]. This method has the advantage, *versus* the fluorimetric method, of lower LODs and identification of both sialic acid species using small amounts of solvents and reagents. These features make LC–MS/MS the preferred choice for development of SIA methods as compared to LC–LIF or capillary electrophoresis (CE) coupled to either LIF or MS [20, 23, 24].

The primary aim of this work was to develop and validate an on-line SPE–LC–MS/MS analytical method to determine Neu5Ac and Neu5Gc in human serum and urine with analytical characteristics suitable for application to intervention

clinical studies. Appropriate tools to achieve this purpose were considered, (i) ultrasonic assisted derivatization to shorten the processing time; (ii) an automatic solid-phase extraction (SPE) system for efficient cleanup of the sample matrix and preconcentration of the target analytes without contact with the atmosphere, thus avoiding degradation of SIA derivatives; (iii) on-line connection of SPE with the chromatographic step to allow elution of the target analytes from the cartridge by the chromatographic mobile phase for total insertion of the eluate into the chromatographic column; thus achieving maximum sensitivity; (iv) a triple quadrupole (QqQ) mass detector in the SRM mode for selective and sensitive analysis of the target metabolites.

2. Experimental

2.1. Chemicals

LC–MS grade methanol and acetronitrile, ammonium acetate and formic acid were from Scharlab (Barcelona, Spain). Deionized water (18 mΩ·cm) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) was used for preparation of all aqueous solutions. *N*-Acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and *N*-acetylneuraminic acid methyl ester (Neu5Ac-methyl ester) were from Sigma–Aldrich (St. Louis MO, USA). Stock standard solutions at 1000 ng mL^{-1} of Neu5Ac and Neu5Gc were prepared in deionized water. Neu5Ac-methyl ester, used as internal standard (IS), was dissolved at 100 ng mL^{-1} in methanol. Multistandard solutions were prepared by diluting and mixing the stock solutions in water. All the above solutions were stored at –80 °C in glass vials until use. All chemicals were LC grade and used without further purification.

Hydrochloric acid (12 mM) from Panreac was used to hydrolyze the samples. A 4 mM 1,2-diamino-4,5-methylene dioxybenzene (DMB, Sigma–Aldrich) solution was prepared by dissolving solid DMB in the following mixture: 1.5 M acetic acid from Panreac (Barcelona, Spain), 0.8 M 2-mercaptoethanol (Merck, Darmstadt, Germany) and 14 mM sodium hydrosulfite (Panreac) in deionized water. This

solution was used for ultrasound assisted derivatization. The DMB solution was daily prepared, then stored at –20 °C in the dark until use owing to its sensitivity to light.

2.2. Subjects and samples

Blood samples for validation of the method belonged to three healthy subjects from the Blood Donors Unit at University Hospital Reina Sofía (Córdoba, Spain). All subjects gave their informed consent for blood extraction. Serum was obtained from blood samples processed within 1 h after collection and centrifuged at 4000 × *g* for 10 min; then placed in plastic tubes and stored at –80 ºC until analysis.

Urine samples were kindly donated by healthy female volunteers. No restrictions about diet or age were taken into account but none of the subjects was taking medication, and all of them were non smokers. The urine samples were centrifuged for 5 min at 5 ºC to separate solid particles. Pools of the two biofluids used for optimization of the method were prepared by mixing aliquots from the different individuals. The internal standard (0.5 ng mL^{-1}) was added to the samples after hydrolysis of conjugated SIAs.

2.3. Equipment

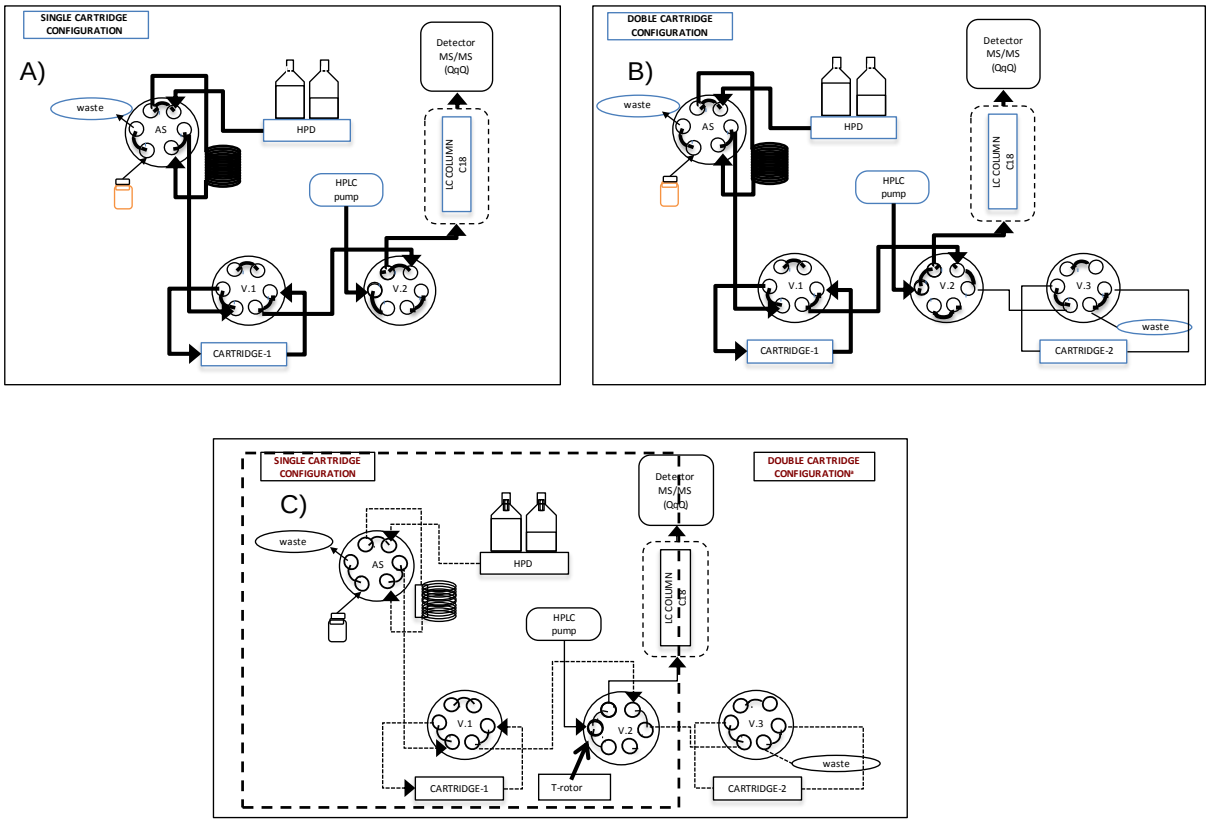
2.3.1. Ultrasound device

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450W) equipped with a cylindrical titanium–alloy probe (12.70 mm diameter), which was immersed into a lab-made stainless-steel container with eight compartments to place test tubes [25].

2.3.2. The SPE–LC–MS/MS approach

A Midas autosampler (AS) furnished with an 100 µL sample loop connected to a Prospekt-2 system (Spark Holland, Emmen, The Netherlands) was used to automate solid-phase extraction (SPE) (Supplementary Figure 1). The Prospekt system consists of an automatic cartridge exchanger and a high pressure dispenser (HPD) that enables fully automated performance of sample preparation controlled by Sparklink version 2.10 software. The automatic cartridge exchanger possesses two clamp positions in the loop of valves 1 and 3 (V1 and V3, respectively) for on-line coupling, if required, of two SPE cartridges. Hysphere resin GP (polymeric

polydivinylbenzene phase) cartridges (8 µm, 10 × 2.0 mm, Spark Holland) were used for the SPE step. Other Hysphere cartridges (10 × 2 mm) were assayed for optimization of the solid-phase extraction step —*viz.*, C8 (end-capped silica-based octyl phase), C18 (end-capped silica-based octadecyl phase) and mixed-mode (MM) polymeric anionic cartridges. The Prospekt-2 system was on-line connected to an Agilent (Palo Alto, CA, USA) 1200 Series LC system. The eluate from the chromatographic column was directly introduced into an Agilent 6410 QqQ mass spectrometer furnished with an ESI source. Agilent MassHunter Workstation was the software for data acquisition and treatment with qualitative and quantitative purposes. Chromatographic separation was performed at 25 °C using a Zorbax Eclipse XDB-C18 reversed phase analytical column (Mediterranea, 150 mm × 0.46 mm i.d., 5 µm particle size) from Agilent (Barcelona, Spain).



Supplementary Figure 1. *Scheme of the device based on an SPE workstation coupled to LC–MS/MS; single configuration (A), dual configuration (B), and single and dual configuration using a T-rotor in valve 2 (V.2).*

2.4. Proposed method

The proposed method was divided into four steps: (1) ultrasound-assisted hydrolysis of SIAs in the target sample; (2) derivatization of the target analytes to yield quinoxalinone derivatives; (3) purification of the target metabolites by automated SPE; (4) individual separation of the derivatives and determination by LC–MS.

2.4.1. Ultrasound-assisted hydrolysis

Cleavage of glycosidic bonds to release of SIAs was carried out by the protocol proposed by Orozco-Solano *et al.* [14] by placing 50 µL of the target sample in an Eppendorf tube to which 1 mL of 12 mM hydrochloric acid was added. The tube was placed in the stainless-steel container, which was filled with water at 40 ºC and where ultrasonic energy under the optimal working conditions —duty cycle 50% (*viz*. ultrasound application 0.5 s s^{-1}), output amplitude 40% of the converter (180 W) and irradiation time for 20 min— was applied. The sample contaning the hydrolyzed metabolites was centrifuged for 5 min at 2000 × *g* to separate solid particles, and the supernatant phase was collected in a 2 mL vial and used for the SPE step.

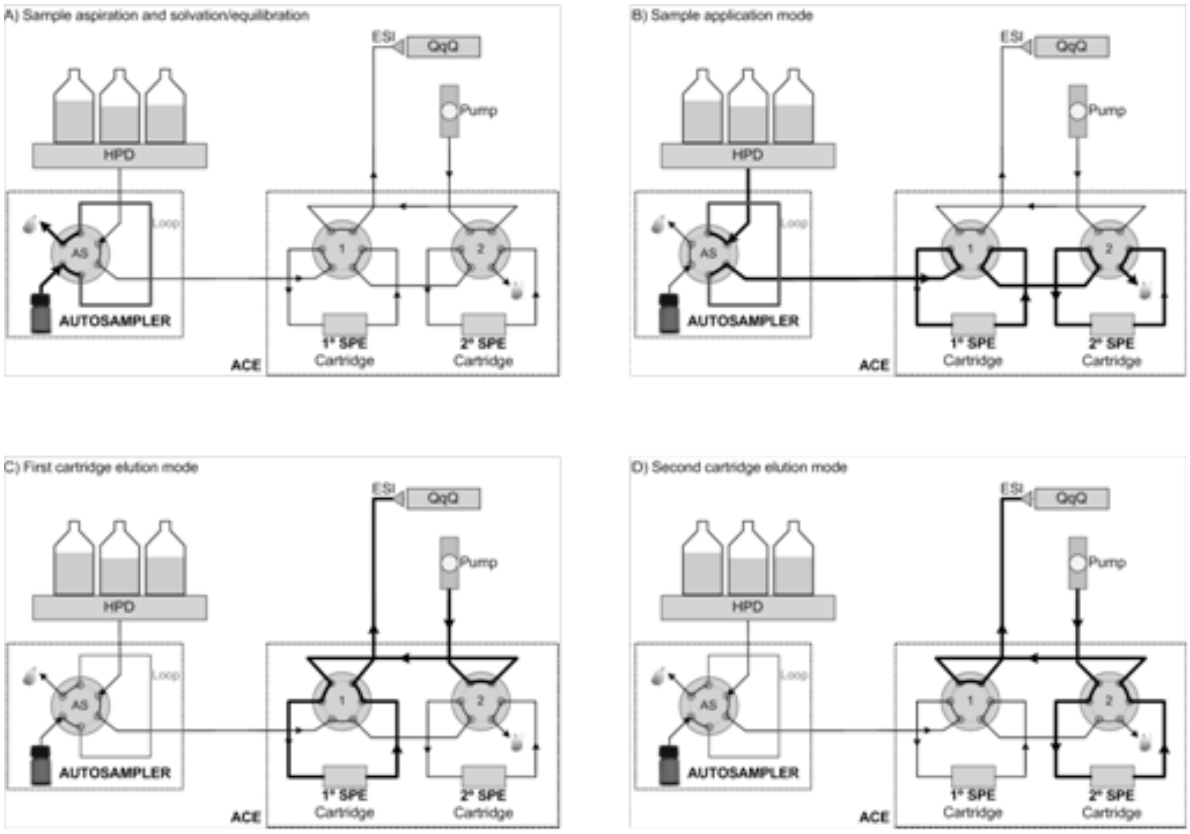
The protocol proposed here was compared with conventional hydrolysis without ultrasonic assistance as described by Shaw *et al.* [26], which is based on heating for 1 h at 80 ºC.

2.4.2. Ultrasound-assisted derivatization

Samples or standards were derivatized by adding 1 mL of the derivatization reagent to 1 mL of the supernatant resulting from hydrolysis in a 2 mL screw-cap microcentrifuge vial and transferring it to a container with eight compartments. Up to eight samples and/or standards can be derivatized simultaneously as the eight positions were subjected to equal ultrasonic irradiation (20 min at duty cycle 50%, output amplitude 60% of the converter and 40 ºC) by a probe plunged in the water where also the tubes were located for acceleration of the labeling reaction. Then, the DMB-labeled analytes were collected in an Eppendorf vial and analyzed by SPE–LC–MS/MS within 24 h.

2.4.3. Purification of the target metabolites by automated SPE and analytical protocol for their determination in serum and urine

Once the SIA quinoxalinones were formed, the analytical samples were placed in an amber injection vial in the autosampler tray. The entire procedure was automatically performed in the sequence of steps schemed in Supplementary Figure 2 (A)-to-(C), and summarized as follows:



Supplementary Figure. 2. *Scheme of the device based on an SPE workstation coupled to an LC–MS/MS system. Automatic Cartridge Exchange (ACE), ElectroSpray Ionization module (ESI), High Pressure Dispenser (HPD), Triple Quadrupole Mass Detector (QqQ).*

(A) Solvation and equilibration of the SPE sorbent: the resin GP cartridge in the ACE unit was clamped in the loop of V1, solvated and equilibrated by the HPD

as follows: 2 mL methanol at 5 mL min^{-1} for solvation (step 1) and 2 mL water at 1.8 mL min^{-1} to equilibrate the cartridge (step 2).

(B) Sample loading: the valve of the autosampler was switched to initiate the sample loading. For this purpose, 1.2 mL of loading solution (water) was circulated and passed through the sample loop to cartridge at 1.8 mL min^{-1}.

(C) Elution and chromatographic analysis: no rinsing step was necessary; therefore, once the sample loading step was finalized, an input/output signal was established between the SPE workstation and the LC unit to trigger the chromatographic run. The latter started by switching valve V1 for elution of the target metabolites. The LC pump initiated the chromatographic gradient at 1 mL min^{-1} with 85:7:8 (v/v) water–methanol–acetonitrile containing 0.1% formic acid as mobile phase. The chromatographic gradient was as follows: the initial mobile phase was maintained for 0.1 min; then, a linear gradient was programmed to obtain 50% of (6:4 v/v) acetonitrile-methanol in 10 min followed by a short linear gradient at 100% of (6:4 v/v) acetonitrile-methanol in 0.5 min maintained for 3.5 min until the end of the chromatographic step. The temperature of the column compartment was set at 25 ºC.

The ESI source was operated in the positive and negative ionization modes, using the following settings: capillary and fragmentor voltage ±3.5 kV and 175 V, respectively; N_2 nebulizer gas pressure 40 psi; and N_2 drying gas flow rate and temperature 10 L min^{-1} and 350 °C, respectively. The dwell time was set at 100 ms.

(D) Cleaning of the SPE sorbent: once the chromatographic run was finished (step 4), a step for cleaning cartridges with 4 mL water (step 5) and 4 mL methanol (step 6) started. The SPE cartridge was thus ready for being reused twice. The entire analytical process was completed in 21 min, which enabled to synchronize the SPE protocol with the chromatographic step and overlap the chromatographic step of one sample with the SPE of the next sample.

2.4.4. SRM-based quantitation of Neu5Ac and Neu5Gc

Calibration curves were built by both multistandard solutions and spiked aliquots of the serum and urine pool with different levels of standard solutions (6

levels) subjected to the SPE–LC–MS/MS process. The concentrations of standards at the lowest and highest levels of each SIA were 0.1 and 500 ng mL^{-1}. Methyl ester acetylneuraminic acid (at 0.5 μg mL^{-1}) was used as IS to obtain the calibration model for Neu5Ac and Neu5Gc. Three concentration levels (high, medium and low values, within the calibration range) were injected in triplicate to set confidence levels. The calibration plots of SIAs were used for their quantitation.

3. Results and discussion

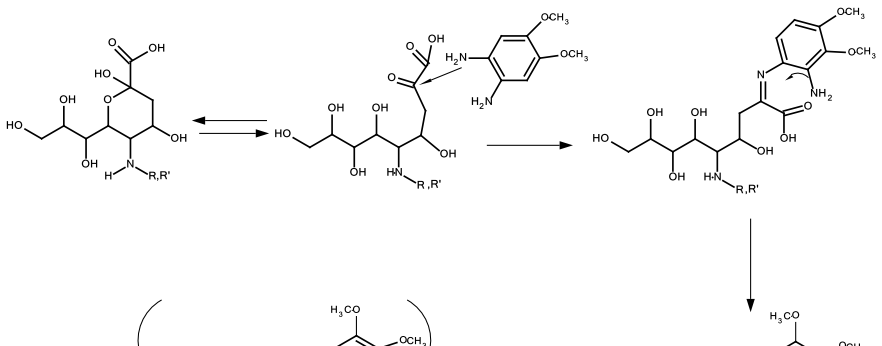
Two steps were involved in the optimization sequence: the first was focused on the best separation–determination of Neu5Ac and Neu5Gc using LC–MS/MS, and the second was aimed at the most efficient preconcentration–cleanup of the target compounds by the automatic SPE system. Optimization of the different steps was performed with multistandard solutions, with serum and urine pools prepared from the samples from healthy donors to ensure comparability of tests, all of which were performed in triplicate. After optimization a validation study was planned to assess the main analytical features of the method.

3.1. Tandem mass spectrometry detection

According to previous studies reported in the literature [26-30] Neu5Ac and Neu5Gc are the main SIAs metabolites present in biological tissues and fluids. For this reason, they were selected as target analytes in this study. The optimization study was carried out by injection of standard solutions at 1 μg mL^{-1} in water in positive and negative ionization modes using two ionization agents (0.1% v/v formic acid and 5 mM ammonium formate). All tests were duplicated for derivatized and underivatized SIAs to compare sensitivity and chromatographic resolution. The first study involved monitoring tentative adducts such as $[M–H]^-$, $[M+Cl]^-$ and $[M+HCOOH]^-$ in negative ionization mode, while $[M+Na]^+$, $[M+K]^+$ and $[M+H]^+$ adducts were monitored in positive mode. Apart from that, collision induced activation was applied for development of MS/MS methods. For underivatized Neu5Ac and Neu5Gc, negative ionization mode was favored by formation of $[M−H]^-$ adducts as precursor ions. These were *m/z* 308.2 and 324.1 for Neu5Ac and

Neu5Gc, respectively. The chromatographic signals for both SIAs were not resolved using a C18 analytical column; nevertheless, this was not a limitation since SRM transitions in MS/MS mode were different for both metabolites. Thus, the transition for quantitation of Neu5Ac was from *m/z* 308.2 to *m/z* 290.0, which fits with the loss of one molecule of water by dehydration. The behavior for Neu5Gc was different and presented a characteristic transition from *m/z* 324.1 to *m/z* 186, justified by intermolecular rearrangement. Therefore, the nil chromatographic resolution should not be a problem with a suited optimization of the dwell time.

These results suggested the comparison with a protocol based on the derivatization of the target compounds with DMB, the reagent most frequently used. In fact, previous studies had reported chromatographic separation of Neu5Ac and Neu5Gc DMB-derivatives with fluorescent detection of quinoxalinones [31]. Supplementary Figure 3 shows the scheme of the derivatization reaction which involves the addition of 116 mass units. Positive ionization mode revealed the best mode for LC–MS analysis of derivatized compounds by formation of $[M+H]^+$ adducts, which were *m/z* 426. and 442.0 for Neu5Ac and Neu5Gc, respectively. In both cases, these ions were the most intense pseudo-molecular ions, while dehydrated adducts $[M+H–H_2O]^+$ presented a relative intensity below 5% of that observed in $[M+H]^+$ adducts. The MS/MS behavior of the derivatized analytes was different to that previously exposed. The MS/MS spectrum of SIAs derivatives reported the maximum information profile when 80 eV was used as collision energy (Figure 1); this activation energy of the precursor ion corresponding to Neu5Ac led to product ions at *m/z* 408, obtained by a first dehydration step, and ion at *m/z* 313. A tentative interpretation of the fragmentation of Neu5Ac derivative is given in Figure 2.A. The loss of a molecule of water occurred by condensation of the hydroxyl groups located at C4 and C8 since the formation of a 6-member ring anhydro form is thermodynamically favored. This is supported on the fact that 4-substituted molecules fail to give this complex pattern (see below). Fragment ion at *m/z* 313 could tentatively be formed by cleavage of the acetamide group and loss of two molecules of water leading to the aromatization of the six-member ring.



Supplementary Figure. 3. *Formation of fluorescent products from Neu5Ac and Nu5Gc by derivatization with DMB.*

In the case of Neu5Gc, the precursor ion (*m/z* 442) allowed two main transitions by formation of ions at *m/z* 424 and 313 (Figure 1). The first product ion (*m/z* 424) was similarly formed by loss of a water molecule while the product ion at *m/z* 313 corresponded to the same structure formed for Neu5Gc (Figure 2.B) by cleavage of the acetamide group [3]. Table 1 lists the main parameters of the MS/MS method based on SRM mode with information of quantitative and qualitative transitions. Only one transition was selected to monitor each analyte with quantification purposes owing to the low sensitivity of secondary transitions which were used for identification. As chromatographic resolution was thus achieved, the dwell time was set at 100 ms for each SRM transition.

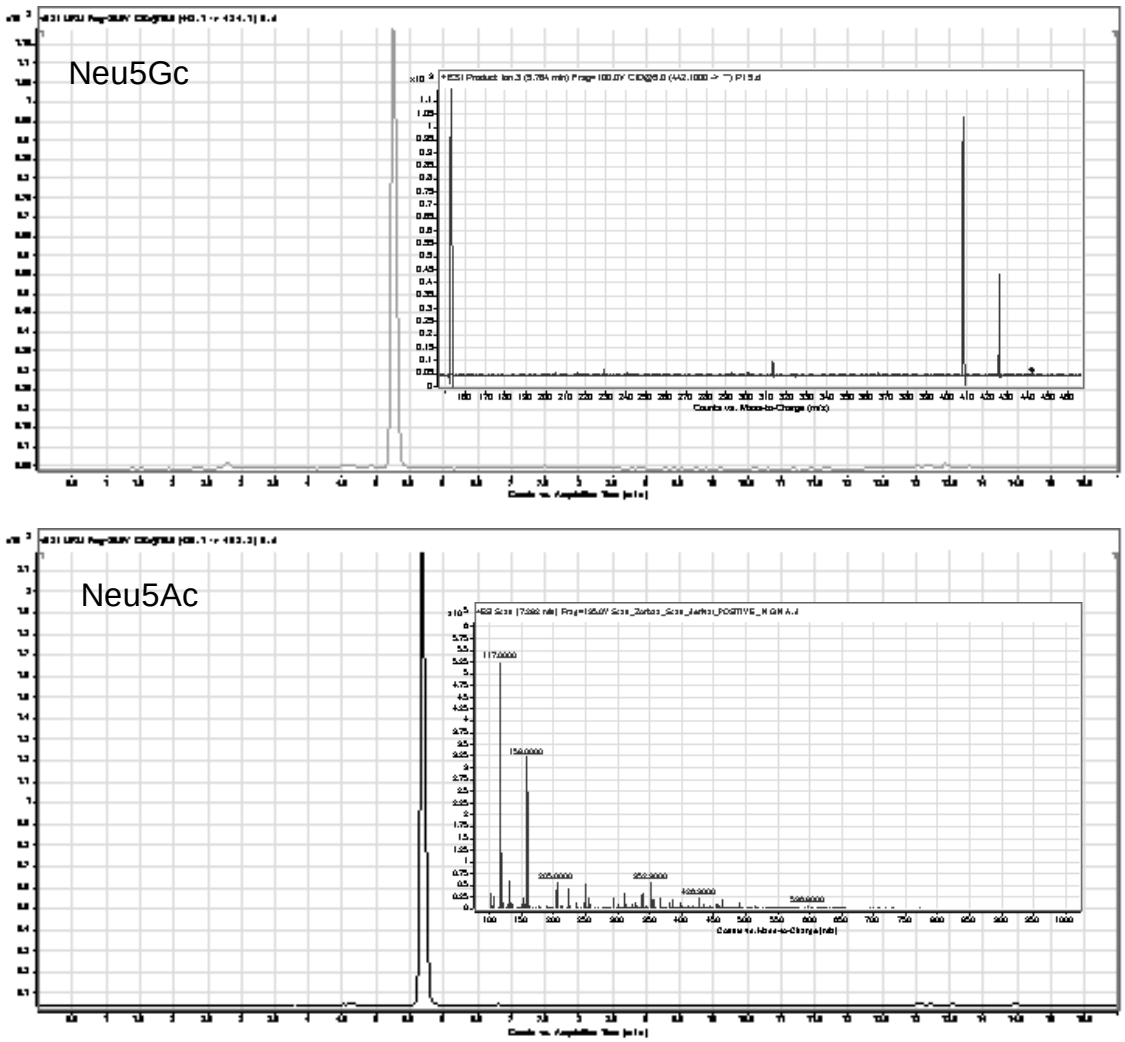


Figure 1. *Mass spectra (product ions) for Neu5Ac and Neu5Gc quinoxalinones from a serum sample spiked with the target analytes at 1 µg mL^{-1}.*

Table 1. Tandem mass spectrometry parameters and retention times for qualitative and quantitative determination of Neu5Ac and Neu5Gc.

Analyte	**Retention time (min)**	**Precursor ion (*m/z*)**	**Product ions (*m/z*)**	**Voltage MS1 (V)**	**Collision energy (eV)**	**Quantitation transition**
Neu5Gc	5.22	442.1	313.1 424.1	100	10	442.1→113.1
Neu5Ac	5.71	426.1	313.1 408.2	80	10	426.1→408.2
Methyl ester-Neu5Ac[a]	2.60	153.1	106.1 136.1 123.0	80	40	153.1→136.0

[a]Internal standard

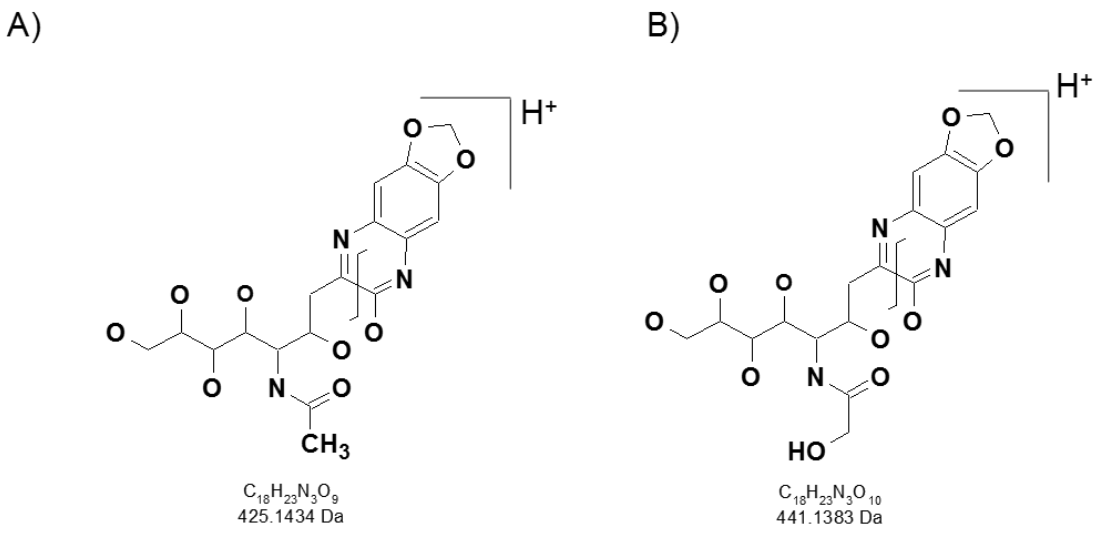
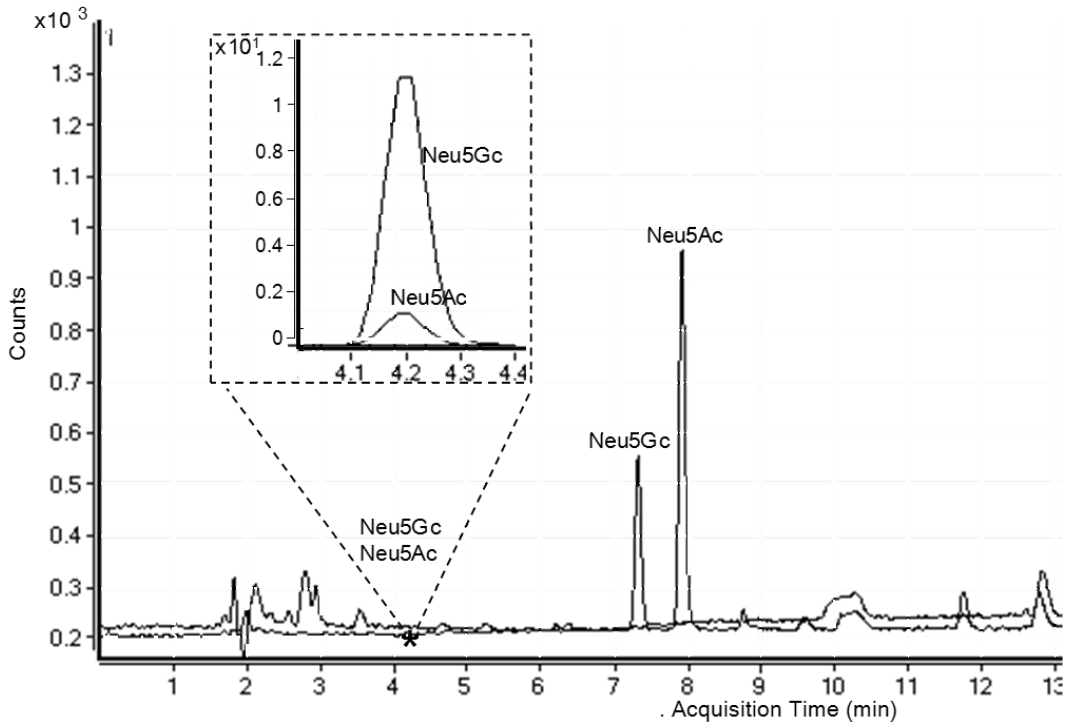


Figure 2. *Fragmentation of DMB-Neu5Ac (A) and DMB-Nue5Gc (B).*

As shows Supplementary Figure 4, the sensitivity of LC–MS/MS determination in SRM mode was clearly higher for derivatized SIAs than for direct analysis of the target metabolites. Apart from this increase of sensitivity, chromatographic resolution was considerably improved by baseline separation of both signals. Supplementary Figure 4 also shows an SRM chromatogram revealing clear separation of the signals for both SIAs. Therefore, it is worth mentioning that DMB derivatization of SIAs with subsequent reverse phase LC separation improved both sensitivity and selectivity by enhancing chromatographic resolution of O-acetylated derivatives.

Due to the incidence of the ESI process on the sensitivity of LC–MS/MS detection, variables such as ionization temperature, pressure and nebulizer voltage were studied by a multivariate response surface design with 16 experiments and 2 central points. In this case, the precursor ions of Neu5Ac and Neu5Gc were monitored in single ion monitoring mode (SIM). The optimum values of temperature, pressure and capillary voltage were 350 ºC, 40 psi, and 3500 V, respectively.



Supplementary Figure. 4. *Total ion chromatogram (TIC) in the positive ionization mode for Neu5Ac and Nu5Gc obtained after derivatization (---) and without derivatization (___). The experimental conditions are described under Materials and methods. The zoomed the peaks show the overlapping of the non-derivaized metabolites.*

3.2. Optimization of the automated solid-phase extraction step

Biological samples are characterized by a complex matrix which makes difficult targeted analysis of metabolites. Apart from that, SIAs usually constitute a part of protein, glucide or lipid structures. For these reasons, determination of SIAs cannot be carried out without a previous clean-up step. In this research, SPE was on-line coupled to LC–MS/MS for direct analysis of two biological fluids thus avoiding protein precipitation, liquid–liquid extraction as well as time-consuming protocols for concentration of target analytes. Maximum extraction efficiency was the parameter considered in the optimization of sample preparation. The SPE sorbent, sample volume, loading solvent (type, volume and flow rate) and elution flow rate were studied by a univariate approach because of the number of non-continuous variables involved (*i.e.* SPE sorbent, loading and washing solvents). Both a multistandard solution at 0.1 μg mL^{-1} of the target analytes and pools of both

biological fluids were used for optimization of the SPE process to compare retention/elution efficiency and matrix effects. The pool was prepared by mixing aliquots of the donor individuals, then spiked with a multistandard solution containing Neu5Ac and NeuGc standards at 0.1 μg mL^{-1}. Each experiment was made in triplicate to evaluate the variability of the test.

3.2.1. Selection of the SPE sorbent

An anion exchange column is the most common option for purification of samples prior to determination of SIAs. In this study, three types of SPE sorbents (described in section 2.3.2) including medium and non-polar materials (C8 and C18) as well as resin polymeric phases with mixed retention mechanisms (resin GP and MM anionic sorbents) were assayed for optimization of the SPE step. The sorbents were tested by the standard protocol for each specific phase (Table 2) based on sample loading with pure water and subsequent elution with the corresponding chromatographic mobile phase. The instrumental configuration used in this case is included in Supplementary Figure 1.A under "Single cartride configuration". Only in the case of the MM anionic sorbent a different protocol was tested due to composition differences between the required elution solution and the optimized chromatographic phase. This aspect, specified in Table 2, involved the instrumental configuration illustrated in Supplementary Figure 1.C under "Focusing elution configuration". This elution mode is based on mixing the chromatographic mobile phase (delivered by the LC pump) with an additional solution (pumped by HPD of the Prospekt system) in a suited proportion to favor the controlled elution of the target analytes in a short interval (usually below 1 min). Both solutions are merged with the aid of a T-shaped rotor located in valve 2 (V2), and subsequently mixed in the mix-tubing located before the SPE cartridge. The elution solution was 90:10 (v/v) methanol–water at pH 3.5 adjusted with 1 M formic acid. Both the mobile phase and elution solution were pumped at 0.5 mL min^{-1} for 1 min. Then, V2 was switched allowing passage to the chromatographic column of only the programmed mobile phase at 1 mL min^{-1}.

Table 2. Solid-phase extraction standard protocols used for optimization of the sorbent

Step	Reversed phase	Anionic protocol
Solvatation	1 mL ACN	1 mL ACN
Equilibration	1 mL water	1 mL 20% ACN 2% ammonia
Sample loading	1 mL water	1 mL 20% ACN 2% ammonia
Elution	Mobile phase with formic acid	Mixed mobile phase (formic acid and 90% methanol) Elution time rotor T:1 min, flow: 0.5 mL min^{-1}

The results obtained with this study are in Figure 3 that shows SRM chromatograms of Neu5Ac and Neu5Gc quinoxalinones obtained from a spiked serum pool subjected to SPE by the different sorbents. The retention capability of Neu5Ac and Neu5Gc in the sorbents was similar and resulted in the following sequential retention order: Resin GP > MM anionic > C18 > C8, which proved the high retention capability of mixed-mode polymeric materials over C8 and C18 sorbents.

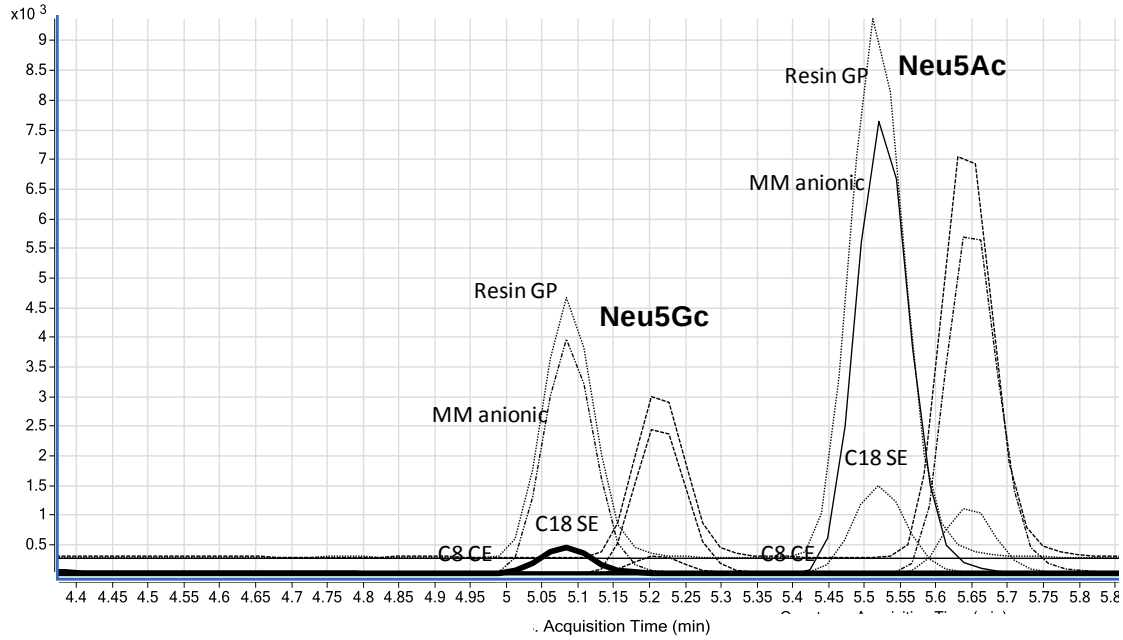


Figure 3. Evaluation of the retention capability of SPE sorbents by comparison of SRM chromatograms for Neu5Ac quinoxalinones: C8(EC) (end-capped silica-based octyl phase), C18(EC) (end-capped silica-based octadecyl phase), Resin GP

(polymeric polydivinylbenzene phase) and MM anion (polymer mixed-mode ion exchangers).

3.2.2. Optimization of the step for sample loading

Different methanol–water and ACN–water compositions were tested as loading solvent to favor the retention of the target metabolites. Nevertheless, the optimum loading solvent was pure water since small concentrations of organic solvents promoted partial elution of the target metabolites, even with a 5% methanol or ACN. The loading volume was studied from 0.5 to 4 mL by setting the minimum value as twice the sum of the sample loop, transport tubing and internal cartridge volume. 1.2 mL was selected for further experiments because higher volumes did not favor retention of the analytes. Assayed loading flow rates ranged from 0.5 to 4 mL min^{-1}, selecting 1.8 mL min^{-1} as the highest flow rate without losses of analytes.

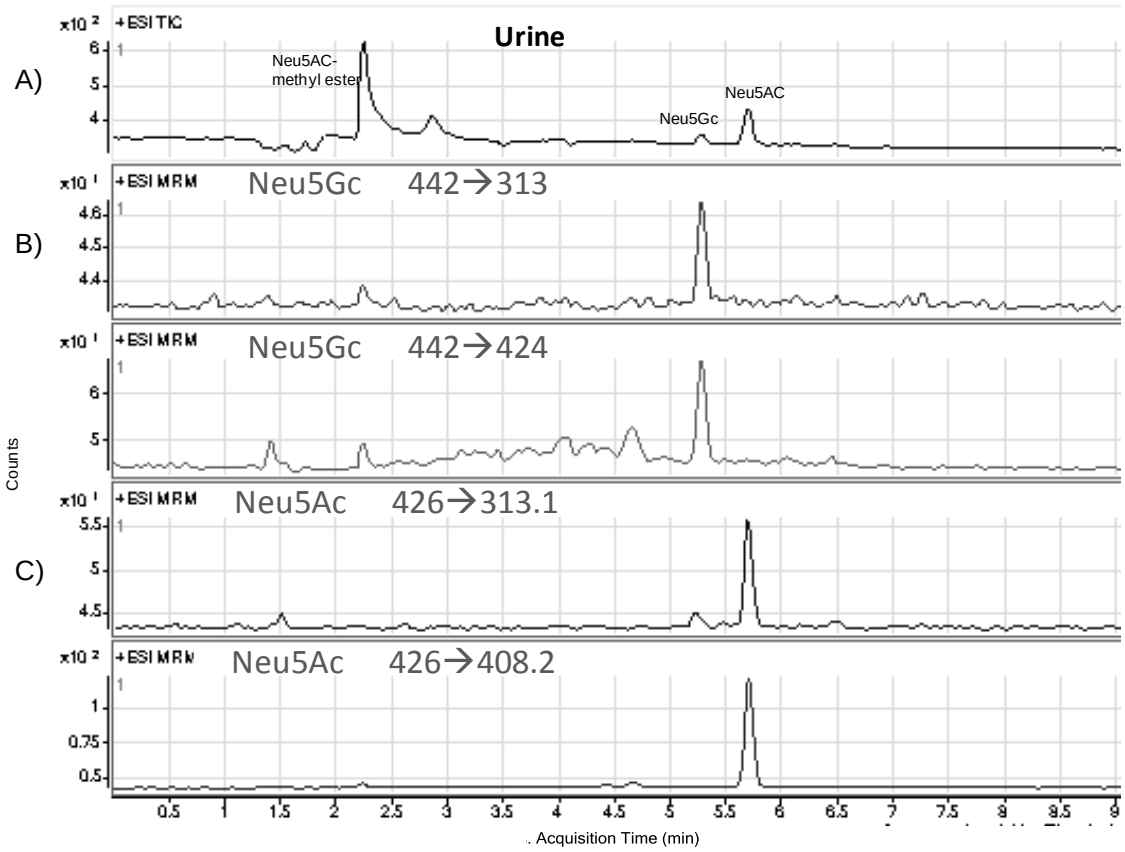
3.2.3. Optimization of the washing step

Deionized water solutions containing methanol within the range 0–30% (v/v) were evaluated as washing solutions of the SPE cartridge. Volumes within 0–4 mL were tested to remove retained salts and other compounds from the biofluids. However, the washing step with any solvent composition led to partial elution of the retained analytes and, for this reason, this step was discarded, after omission of which no differences were found between the SPE protocols for analysis of serum and urine.

3.2.4. Mode and time for elution of the target metabolites

The eluting solution was the chromatographic mobile phase (85:7:8 (v/v) water–methanol–acetonitrile containing 0.1% formic acid) to initiate the separation process. The elution time, expressed as the period during which the V1 valve is switched to the LC analytical column, was the only variable studied. The optimization of this variable is of particular interest to preserve the capability of the guard and analytical columns, but also to avoid peak broadening effects. The elution time was studied to ensure complete elution of derivatized Neu5Ac and NeuGc without eluting matrix interferents. This was achieved in 1 min due to the content of organic solvents in the initial mobile phase, 15% of methanol–acetonitrile, which avoids elution of lipid metabolites. After that, the valve was switched again to

continue with the chromatographic separation with a flow rate of 1 mL min^{-1}. Longer times led to peak band-broadening effects and caused elution of less polar interferents such as phospholipids with potential ionization suppression in the electrospray unit. Figure 4 shows SRM chromatograms by monitoring selected transitions of target metabolites in spiked and non-spiked serum by the resulting protocol.



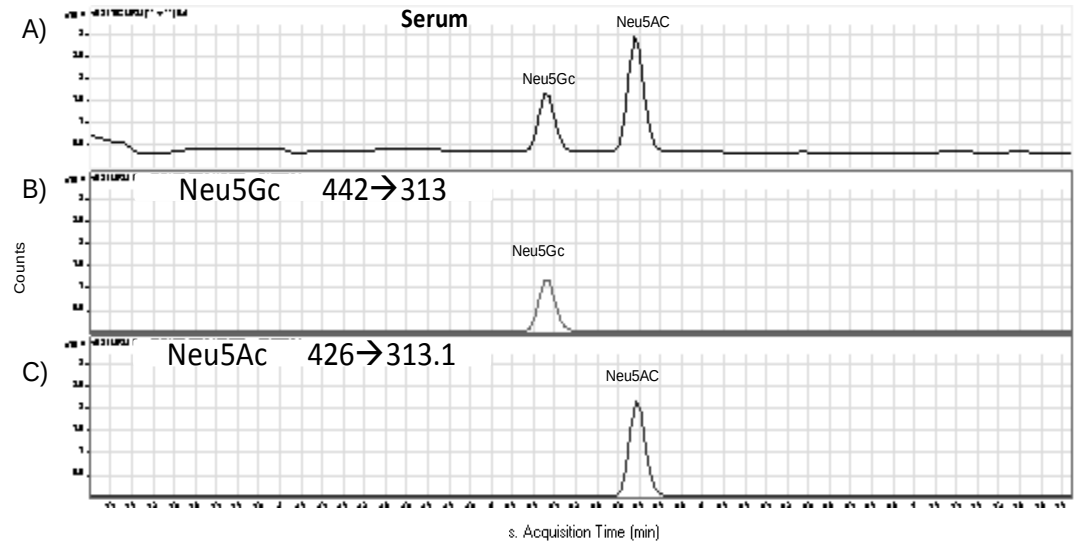


Figure 4. *Total ion chromatograms (TICs) in the positive ionization mode for Neu5Ac and Neu5Gc quinoxalinones (A); and SRM chromatograms for each target compound (B and C) as obtained by analyzing urine and serum samples spiked at 10 ng mL^{-1}.*

3.3. Analytical features of the method

The influence of matrix effects associated to both biofluids on the efficiency of the SPE–LC–MS/MS determination of Neu5Ac and Neu5Gc was evaluated using three calibration models to compare the slopes of each calibration equation. A calibration set was prepared by a pool of each biofluid spiked with the target analytes at concentrations ranging from 1 to 100 ng mL^{-1}. These two models were compared with a third one obtained with standard solutions. Statistical comparison revealed significant differences between the slopes of the calibration models prepared for both SIAs with biofluids as compared to that obtained with standard solutions (p = 0.05). For this reason, the standard addition method was selected for quantitative analysis, which allowed matrix interferences to be corrected. Calibration curves (n = 5) exhibited excellent linearity for Neu5Ac and NeuGc, with regression coefficients equal or above 0.997 within the dynamic range. The LOD and quantitation (LOQ) were determined according to the International Conference on Harmonization (ICH) guidelines [29]. Table 3 lists the calibration equations, regression coefficients and LOD and LOQ values, which ranged between 0.03 and 0.16 ng mL^{-1} for Neu5Ac (3 and 16 pg on-column, respectively) and 0.04 and 0.17 ng mL^{-1} for Neu5Gc (4 and 17 pg on-column, respectively).

Table 3. Estimation of the accuracy estimation of the method for determination of Neu5Ac and Neu5Gc in serum and urine

Analyte	Parameter	Urine	Serum
Neu5Ac	Calibration curve[a]	0.147 + 2.076	1.231 + 2.34
	Intercept	0.0000 $\pm$ 0.0001	0.0000 $\pm$ 0.0001
	R^{2} [b]	0.998	0.997
	On column LOD (ng mL^{-1})	0.03	0.05
	On column LOQ (ng mL^{-1})	0.11	0.17

Neu5Gc	Calibration curve[a]	0.185 + 1.568	1.855 + 0.02
	Intercept	0.01 ± 0.007	0.0002 ± 0.0006
	R^{2} [b]	0.995	0.998
	On column LOD (ng mL^{-1})	0.05	0.04
	On column LOQ (ng mL^{-1})	0.16	0.12

[a]Number of points: n = 7.
[b]Regression coefficient: R

The accuracy of the method was evaluated by analysis of aliquots of both biofluids spiked at three concentration levels (1, 20 and 50 ng mL^{-1}) with the target SIAs. Non-spiked aliquots were also analyzed to estimate the background content of Neu5Ac and Neu5Gc. The results for each sample, and their standard deviations, are included in Table 4. The standard deviation was calculated as the variability of the concentrations extrapolated in the calibration curve. Student's *t*-test confirmed the absence of significant differences between the concentrations found by the proposed method and the concentrations added to the samples, which testifies the accuracy of the proposed method. Analyte recoveries ranged from 90 to 105%, which supports the correction of matrix effects ascribed to each biofluid. Recovery factors were additionally estimated by analysis of both spiked biofluids (1, 20 and 50 ng mL^{-1}) using the dual-cartridge on-line configuration illustrated in Supplementary Figure 1.B. With this configuration, two cartridges (in this method resin GP sorbents) are put in serial to compare the amount of analyte retained in each cartridge and, therefore, this approach enables to estimating recovery factors and breakthrough concentration [32]. Recovery factors were calculated as the concentration retained in cartridge 1/[concentration retained in cartridge 1+ concentration retained in cartridge 2]. Recoveries in this test ranged from 99.5 to 99.7% in human urine and from 99.1 to 99.45% in serum.

Finally, the precision of the method was evaluated by calculation of the within-day variability (repeatability) by in-triplicate analysis of three samples of each biofluid from volunteers. They were spiked with the target analytes at 20 ng mL^{-1}. This parameter ranged from 1.7 to 2.5% expressed as relative standard deviation (RSD). On the other hand, between-days variability (reproducibility) was determined by in-triplicate analysis of three samples of each biofluid (also spiked with the target

analytes at 20 ng mL^{-1}) from volunteers over three consecutive days. Between-days variability ranged from 4.8 to 5.2%, expressed as RSD.

A comparison between methods based on LC–LIF and LC–MS for analysis of SIAs in biological samples such as urine and serum allows to check the lowest LOD and LOQ of the LC–MS method (0.88 and 2.66 ng mL^{-1}, respectively) [30] *versus* those of the LC–LIF method (of 5.8 and 19 ng mL^{-1}, respectively) [20]. The improvement in sensitivity is higher when LC–MS/MS is used, as the detection and quantification limits are at the picomolar levels in this case [31]. These are also the levels found for LOD and LOQ by the method here reported, which, in addition, allows fully automation thanks to the on line coupling of SPE with the LC–MS/MS step. In this way, the determination of Neu5Ac and Neu5Gc in both urine and serum could be for the analysis of pathological tissues, cancers and nervous system diseases, among others.

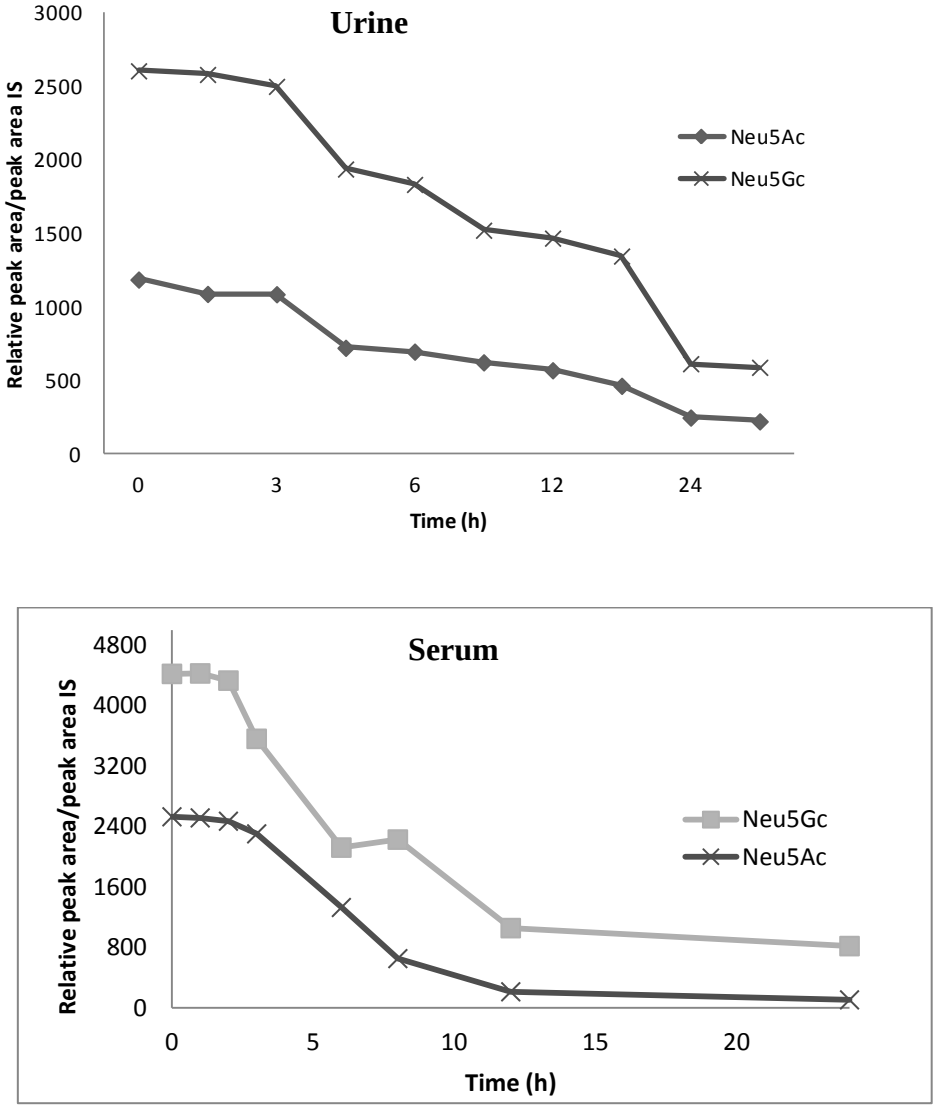
Table 4. Intra-day and inter-day variability for each analyte expressed as percentage

	Analyte	Within-day variability, RSD (%)[a]	Between-day variability, RSD (%)[a]
Serum	Neu5Ac	4.6	7.2
	Neu5Gc	3.8	6.7
Urine	Neu5Ac	2.5	4.8
	Neu5Gc	1.7	5.2

[a] *RSD (%) relative standard deviation (n = 5).*

3.4. Stability considerations

The stability of SIA quinoxalinones has not been previously studied. For this purpose, a stability study at room temperature was planned by analysis of SIA derivatives at 0, 1, 2, 3, 4, 6 12, 18 and 24 h after derivatization. The results, listed in Supplementary Figure 5, show that the quinoxalinone derivatives remained stable for at least 3 h under the studied conditions. Beyond that time, a significant instability of derivatized SIAs in biofluids was detected. Therefore, analysis should be performed within 3 h after derivation



Supplementary Figure. 5. *Stability kinetics of quinoxalinone derivatives formed after derivatization with DMB.*

3.5. Application of the method to human biological samples

The suitability of the proposed SPE–LC–MS/MS method was tested by in triplicate application to human serum and urine samples from volunteers. As show the results listed in Table 5, only Neu5Ac could be quantified in all examined samples, in concentrations ranging between 1.7 and 5.3 mM. The literature supports that Neu5Gc has been found in human cancer cells and in non-human mammalian tissues [33, 34]. It is worth pointing out that despite the low concentrations of Neu5Ac in the two human biofluids, this method is sensitive enough to detect and

quantify it. Additionally, levels presented for Neu5Ac were consistent with the values reported in the literature which are also listed in Table 5 [30, 35-38].

Table 5. Levels of Neu5Ac and Neu5Gc in serum and urine from volunteers. The results are expressed in absolute excreted mass by considering the corresponding dilution factor and the total volume of sample collected

Neu5Ac (mM)		Neu5Gc (mM)		Neu5Ac (reported in the literature, mM)
Serum	Urine	Serum	Urine	
0.5 (0.2)	0.82 (0.1)	N.D.	N.D.	1.36-3.40 (Serum) [29]
1.7 (0.3)	5.31 (0.4) (0.5).	N.D.	N.D.	1.82-4-24 (Urine) [8]

N.D. Non detectable

4. Conclusion

A fully automated on-line SPE–LC–MS/MS method for the determination of the main SIAs present in human biofluids, Neu5Ac and Neu5Gc, has been developed. The proposed method requires minimal human intervention, thus avoiding errors associated to the analyst. The analytical features of the proposed method are better than those methods previously reported in the literature. A complete validation study has been developed to evaluate the capability of the method to be implemented in clinical laboratories. Tandem mass spectrometry by SRM of the derivatized analytes was optimized for confirmatory analysis of both SIAs. The ensuing method was used to determine the target compounds in human biofluids.

Acknowledgments

The Spanish MEC and European FEDER program are thanked for financial support through project CTQ2012-37428. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

References

[1] Q. Zhou, K. L. Moore, D. F. Smith, A. Varki, R. P. McEver, R. D. Cummings, The Journal of Cell Biology 115 (1991) 557-564.

[2] T. Angata A. Varki, Chemical Reviews 102 (2002) 439-470.

[3] R. Klein, S. Crafts-Brandner, M. Salvucci, Planta 190 (1993) 498-510.

[4] A. Varki, Glycobiology 3 (1993) 97-130.

[5] C. Strell F. Entschladen, Cell Communication and Signaling 6 (2008) 10.

[6] Y.-C. Chang, S. Uchiyama, A. Varki, V. Nizet, mBio 3 (2012)

[7] H. H. Freeze, Glycobiology 11 (2001) 129R-143R.

[8] E. M. Hawes, Drug Metabolism and Disposition 26 (1998) 830-837.

[9] F. N. Lamari N. K. Karamanos, J. Chromatogr. B. 781 (2002) 3-19.

[10] N. K. Karamanos, A. J. Aletras, C. A. Antonopoulos, A. Hjerpe, C. P. Tsiganos, European Journal of Biochemistry 192 (1990) 33-38.

[11] M. Martín, M. Ramirez, E. Vázquez, R. Rueda, Anal. Bioanal. Chem. 404 (2012) 919-919.

[12] K. Ortner W. Buchberger, Electrophoresis 29 (2008) 2233-2237.

[13] K. R. Anumula P. B. Taylor, Anal. Biochem. 203 (1992) 101-108.

[14] M. I. P.-C. Orozco-Solano, F.; Luque de Castro M.D, Submite to Analytica Chimica Acta (September 2012)

[15] J.-P. Zanetta, A. Pons, M. Iwersen, C. Mariller, Y. Leroy, P. Timmerman, R. Schauer, Glycobiology 11 (2001) 663-676.

[16] L. W. Jaques, B. F. Riesco, W. Weltner Jr, Carbohydrate Research 83 (1980) 21-32.

[17] S. P. Galuska, R. Geyer, M. Mühlenhoff, H. Geyer, Anal. Chem. 79 (2007) 7161-7169.

[18] X. Luo, H. Yang, C. Liang, S. Jin, Toxicon 55 (2010) 421-429.

[19] N. Hashii, N. Kawasaki, Y. Nakajima, M. Toyoda, Y. Katagiri, S. Itoh, A. Harazono, A. Umezawa, T. Yamaguchi, J. Chromatogr. A. 1160 (2007) 263-269.

[20] H. Nie, Y. Li, X.-L. Sun, Journal of Proteomics 75 (2012) 3098-3112.

[21] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Biochem. 164 (1987) 138-145.

[22] W. Chai, V. E. Piskarev, B. Mulloy, Y. Liu, P. G. Evans, H. M. I. Osborn, A. M. Lawson, Anal. Chem. 78 (2006) 1581-1592.

[23] K. Iwatsuka, S.-i. Yasueda, E. Bando, H. Fujii, T. Terada, H. Okubo, H. Iwamoto, M. Kinoshita, K. Kakehi, J. Chromatogr. B. 879 (2011) 2866-2870.

[24] R. Lacomba, J. Salcedo, A. Alegría, M. Jesús Lagarda, R. Barberá, E. Matencio, Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 346-357.

[25] O. F. Mijangos Ricárdez, J. Ruiz-Jiménez, L. Lagunez-Rivera, M. D. Luque de Castro, Phytochem. Analysis 22 (2011) 484-491.

[26] C. J. Shaw, H. Chao, B. Xiao, J. Chromatogr. A. 913 (2001) 365-370.

[27] L. Warren, Journal of Biological Chemistry 234 (1959) 1971-1975.

[28] K. Engelmann, C. L. Kinlough, S. Müller, H. Razawi, S. E. Baldus, R. P. Hughey, F.-G. Hanisch, Glycobiology 15 (2005) 1111-1124.

[29] L. A. Hammad, M. M. Saleh, M. V. Novotny, Y. Mechref, Journal of the American Society for Mass Spectrometry 20 (2009) 1224-1234.

[30] F. Valianpour, N. G. G. M. Abeling, M. Duran, J. G. M. Huijmans, W. Kulik, Clin. Chem. 50 (2004) 403-409.

[31] S. Hara, M. Yamaguchi, Y. Takemori, K. Furuhata, H. Ogura, M. Nakamura, Anal. Biochem. 179 (1989) 162-166.

[32] M. Calderón-Santiago, F. Priego-Capote, J. G. Galache-Osuna, M. D. Luque de Castro, Journal of Pharmaceutical and Biomedical Analysis 70 (2012) 476-484.

[33] H. Higashi, M. Naiki, S. Matuo, K. Okouchi, Biochem. Bioph. Res. Co. 79 (1977) 388-395.

[34] Y. N. Malykh, R. Schauer, L. Shaw, Biochimie 83 (2001) 623-634.

[35] S. D'Alessandro, H. M. Curbelo, O. R. Tumilasci, J. A. Tessler, A. B. Houssay, Arch. Oral Biol. 34 (1989) 829-831.

[36] S. E. Carlson, Am. J. Clin. Nutr. 41 (1985) 720-726.

[37] P. A. Siskos M.-H. E. Spyridaki, J. Chromatogr. B. 724 (1999) 205-212.

[38] B. Wang, J. Brand-Miller, P. McVeagh, P. Petocz, Am. J. Clin. Nutr. 74 (2001) 510-515.

PARTE IV

Desarrollo y aplicación de plataformas de análisis global en nutrimetabolómica

La Parte IV y última de esta Memoria está dedicada al desarrollo de una plataforma analítica global y su aplicación en el área de la nutrimetabolómica. Se culmina así la investigación realizada en el área de la nutrimetabolómica al trabajar con muestras pertenecientes a un estudio europeo sobre nutrición (LIPGENE) de amplia repercusión. Las muestras utilizadas (un total de 300) correspondían a plasma de 76 individuos con síndrome metabólico que se habían sometido a cuatro dietas de intervención isoenergéticas durante 12 semanas con diferencias en el contenido graso y en la calidad de la misma.

La plataforma diseñada fue basada en LC–TOF/MS de alta resolución y se aplicó al estudio de las fracciones polar y no polar de las muestras de plasma. Un exhaustivo tratatamiento quimiométrico con las herramientas apropiadas en cada caso condujo a la construcción de modelos de clasificación y de predicción y a la identificación tentativa de los metabolitos con capacidad para la discriminación entre las diferentes clases de los individuos en estudio. La investigación global se ha dividido en dos partes: La orientada a la comparación de los perfiles metabolómicos del plasma de los individuos en estudio (Capítulo 11) y a la evaluación del período de lipemia postprandial de cada una de las dietas en los individuos que las ingirieron (Capítulo 12).

CAPÍTULO 11

Estudio comparativo nutrimetabolómico de la influencia de dietas de intervención con un determinado contenido graso en el perfil metabólico del plasma mediante LC–TOF/MS

Comparative nutrimetabolomic study of the influence of fat intervention diets on plasma metabolic profile by LC–TOF/MS

M.I. Orozco-Solano[a,b], F. Priego-Capote[a,b], M.D. Luque de Castro[a,b*]*

[a]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain

[b]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain

Enviado a Journal of Nutritional Biochemistry

ELSEVIER

Comparative nutrimetabolomic study of the influence of fat intervention diets on plasma metabolic profile by LC–TOF/MS

M.I. Orozco-Solano[a,b], F. Priego-Capote[a,b*], M.D. Luque de Castro[a,b*]

ABSTRACT

A global nutrimetabolomic study is here presented to compare the metabolic profiles of plasma from metabolic syndrome patients subjected to four isoenergetic intervention diets with a different fat content. The diets (12 weeks) were divided into two groups according to the fat content: 38% and 28% fat content. The two high-fat content diets were characterized by a high proportion of saturated fatty acids (16% SFA) or monounsaturated fatty acids (20% MUFA). On the other hand, the two low-fat content diets were described as high-complex carbohydrate diets (8% SFA, 11% MUFA, 6% PUFA) supplemented with 1 g/d high oleic acid sunflower oil or with 1.24 g/d very long chain *n-3* PUFA supplement. Plasma metabolic profiles of 76 volunteers before and after the intervention study were obtained after sample fractionation with 1:2 methanol:chloroform with subsequent LC–TOF/MS analysis in positive and negative ionization modes. Statistical analysis allowed discrimination of the metabolic profiles associated to each diet by PCA and PLS-DA multivariate tools, except for the methanolic fraction corresponding to the low-fat diet supplemented with n-3 PUFA. Differentiation was also attained for each intervention state *versus* the preintervention state with tentative identification of those metabolites with high discrimination capability. In a final step, tentative identification of those metabolites contributing to explain the variability occasioned by each intervention diet was carried out. The important role of certain families of lipids such as glycerophospholipids, diglycerides, triglycerides and eicosanoids as well as other changes affecting the metabolism of sugars, amino acids and vitamins was described.

Keywords: Nutrimetabolomics, Global profiling, Human plasma, LC–TOF/MS, nutritional studies.

1. Introduction

Nutritional studies play a key role in the identification of essential nutrients needed for human growth and health. Nowadays, nutritional scientists are challenged to find new ways to treat or prevent diseases brought on by nutritional oversufficiency such as obesity, diabetes, chronic inflammation and/or cardiovascular diseases. One of the main approaches to face up these studies is nutrimetabolomics, which is aimed at: (1) food component analysis; (2) food quality/authenticity detection and; (3) biomarkers of consumption; and (4) elucidation and detection of effect and pathology-risk biomarkers in nutritional and epidemiological studies. The last approach has been used to assess metabolic changes caused by administration of certain nutrients with an impact on human health. The starting hypothesis is that the majority of metabolism alterations derived from these treatments can be effectively monitored through metabolic changes in biological fluids such as serum/plasma or urine. One of the most common analytical strategies to detect and discriminate metabolic changes is metabolomic fingerprinting, aimed at developing classification models based on snapshots or fingerprints obtained by analysis of samples with suited detection techniques [1]. However, data quality in these approaches is limited since low discrimination among metabolites is attained. Data quality is considerably enhanced by application of a global metabolomic approach focused on detection and identification of those metabolites with the highest contribution to explain the observed variability. This strategy seems to be specially suited to detect and discriminate metabolic changes caused by nutritional states.

Considering the chemical diversity and number of metabolites in human biofluids (a conservative estimation of human serum reported 4229 metabolites and wide range of concentrations up to 11 orders of magnitude observed in human serum [2–4]), different analytical platforms need to be combined to approach full coverage of the entire metabolome. In fact, a variety of techniques can be employed to develop proper coverage in each case, with nuclear magnetic resonance (NMR) and mass spectrometry (MS) being the most used. Unique features of NMR (capability for identification, absolute quantification, good reproducibility, easy sample preparation and relatively high sample throughput) make this technique a suited tool for multicomponent analysis of body fluids [8], which can be enhanced by coupling to liquid chromatography (LC) [4, 9-11]. Coupling of MS to LC (LC–MS) or gas chromatography (GC–MS) in various configurations has been widely used to study metabolic changes associated with

pathological states and nutritional studies [5-7]. The choice of a separation technique prior to MS detection is primarily dictated by analyte properties, with GC–MS as more suitable for volatile and semi-volatile analytes, LC–MS as the most suitable for polar, thermolabile or non-volatile analytes and capillary electrophoresis (CE) as an alternative for charged analytes [8].

Comparing the use of LC–MS to other instrumental approaches for metabolic analysis studies [7, 13, 14], sensitivity is significantly higher than that characteristic of NMR, thus providing the possibility to monitor metabolic changes that remained unnoticed when working with NMR. LC–MS allows analysis of a wide range of metabolite classes. The widespread availability of robust LC–MS methods in many research laboratories has increased enormously its use for metabolic profiling over the past few years. In fact, many studies have provided higher metabolome coverage (approximately 55–70% of total metabolites detected using multiple platforms) by a single experiment using LC–MS than by GC–MS or CE–MS [4] (an estimated 10–15% overlap is usual between LC–MS and GC–MS platforms [8]). Therefore, LC–MS can provide comprehensive metabolite coverage of untargeted analyses on a single platform [15]. LC–MS metabolomic studies generally involve differential comparison of several subjects or treatment groups (*e.g.* normal *versus* treated individuals, healthy *versus* ill individuals) without *a priori* identification of all metabolites detected.

The LIPGENE project is a large European, multi-centre project which includes a human intervention trial (486 volunteers at baseline) conducted to compare the impact of different types and amounts of dietary fatty acids on insulin sensitivity. This human intervention study is supported on a food-exchange model involving four diets for alteration of dietary fat quantity and quality in the participants. The key aspects of this strategy for correlation and discrimination of metabolic patterns with the supplied diets were: (i) diets differ significantly in overall fat intake but remain isoenergetic; (ii) the two high-fat diets are significantly higher in the content of saturated fatty acids (SFAs) or monounsaturated fatty acids (MUFAs) as compared with the two low-fat diets; (iii) the effect of long-chain *n-3* polyunsaturated fatty acids (PUFAs) supplementation was tested in a low-fat background diet. Supported on this study, an LC–TOF/MS platform has been applied to obtain metabolic profiles of plasma from individuals subjected to the four isoenergetic intervention diets under controlled conditions for three months. The aim was to detect and evaluate plasma metabolic differences according to the administered diets.

For this purpose, samples in basal and post-basal state were analyzed by LC–MS in high-resolution mode and the resulting data sets were treated by multivariate statistical analysis for identification of the main variability sources.

2. Experimental

2.1. Chemicals

LC–MS grade methanol, chloroform and acetonitrile (Scharlab, Barcelona, Spain) were used for sample treatment and preparation of chromatographic mobile phases. MS-grade formic acid (Scharlab) was used as ionization agent in LC–MS analyses, while deionized water (18 MΩ·cm) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare the chromatographic aqueous phases.

2.2. Instruments and apparatus

Centrifugation was carried out by a thermostated centrifuge Thermo Sorvall Legend Micro 21 R from Thermo (Thermo Fisher Scientific, Bremen, Germany). A Vacufuge centrifugal vacuum concentrator from Eppendorf (Eppendorf, Inc., Hamburg, Germany) was used to evaporate samples to dryness.

All samples were analyzed by an 1200 Series LC system (Agilent Technologies, Waldbronn, Germany), which was coupled to an Agilent 6530 TOF mass spectrometer equipped with a dual electrospray ionization source. MassHunter Workstation software (Agilent Technologies B.05.00) was used for data acquisition and qualitative analysis while Mass Profiler Professional (Agilent Technologies 2.2), was used for processing raw MS data, including feature extraction, data filtering, statistical analysis, molecular formula generation for detected ions and database searching. Compound identification was performed using the METLIN Personal Metabolite Database and the Molecular Formula Generation algorithm (Agilent Technologies). The Human Metabolome Database (HMDB) was used to confirm and extend identification.

2.3. Meals composition

Four isoenergetic diets that differed in fat quantity and quality were planned for this study. Two diets were designed to provide 38% energy (E) from fat and the other two with 28% E from fat with the following asigned names and compositions:

(1) HSFA: high-fat content (38% energy) with high proportion of saturated fatty acids (16% SFA), while monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs, respectively) were present at 12% and 6%, respectively.

(2) HMUFA: high-fat content (38% energy) with high proportion of MUFAs (20%), while SFA and PUFAs were 8% and 6%, respectively.

(3) LFHCC: low-fat content (28% energy) with high-complex carbohydrate diet (8% SFA, 11% MUFA, 6% PUFA), supplemented with 1 g/d high oleic acid sunflower oil supplement.

(4) LFHCC (n-3): low-fat (28% energy), high-complex carbohydrate diet (8% SFA, 11% MUFA, 6% PUFA), supplemented with 1.24 g/d very long chain *n-3* PUFA supplement.

2.4. Subjects and samples

This research was conducted within the framework of the LIPGENE integrated project (diet, genomics and metabolic syndrome (MetS): an integrated nutrition, agro-food, social and economic analysis). A total of 76 patients with MetS from the LIPGENE cohort were accepted to participate in this study aimed at evaluating metabolic alterations by LC–MS/TOF. All participants gave written informed consent and underwent a comprehensive medical history, physical examination and clinical chemistry analysis before enrolment. None of the subjects was taking medication or supplementary vitamins with influential effect on plasma metabolome.

Patients were randomly divided into four groups to receive one of the four dietary interventions for 12 weeks under controlled conditions. Both the design of the intervention study and intervention protocol have been described in detail by Shaw *et al.* [16]. Plasma control samples were obtained in basal state prior to the intervention diets as control samples (PRE). Patients arrived at clinical centers after 12 weeks intervention study following a 12-h fast refrained from smoking during the fasting period and

abstained from alcohol intake during the preceding 7 d. In the laboratory and after cannulation a fasting blood sample was taken for each individual (POS).

Sampling was performed following the Recommendations on Biobanking Procedures for plasma processing and management recently published by the European Consensus Expert Group [17]. In the serum/plasma processing and management the following aspects were taken into account: (i) use of EDTA as anticoagulant; (ii) sample storage at a temperature of –80 ºC, (iii) recording of the time from collection through processing; (iv) experimental definition of time limits appropriate for analytes measurement. Venous blood samples were collected using tubes containing 1 g/L of EDTA, which were stored in containers with ice in the dark. Special care was taken to avoid exposure to air, light and ambient temperature. Plasma was separated from whole blood by low speed centrifugation at 1500 × g for 15 min at 4 °C within 1 h after extraction. The plasma samples were stored at –80 °C until analysis. During the sampling period the volunteers did not consume any food.

2.5. Sample treatment

Plasma samples (100 μL) immersed in a bath ice were treated with 300 μL of 1:2 methanol–chloroform. The mixture was shaken for 2 min and stabilized for 3 min. The organic phase was separated after centrifugation for 5 min at 4 ºC and 13800 × g. Both phases (aqueous and organic) were collected in different vials, then placed in the LC autosampler for subsequent analysis.

2.6. LC–TOF/MS analysis

Chromatographic separation was performed using a Teknokroma Mediterranean Sea C18 analytical column (100 mm × 0.46 mm i.d., 3 μm particle size, from Barcelona, Spain) kept at 25 ºC. The mobile phases consisted of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in ACN. The elution program was as follows: 0–2 min, 3% B; 2–30 min, 100% B. A post-run of 5 min was included to equilibrate the column. The flow rate was maintained at 0.8 mL/min. The volume of injected sample was 10 μL and the injector needle was washed 10 times with 70% methanol between injections. Therefore, the needle seat back was flushed for 15 s at a flow rate of 4 mL/min with 70% methanol to avoid cross contamination. Both the methanol and chloroform fractions were analyzed in negative and positive ionization modes. The operating conditions in negative and

positive ionization modes were as follows: gas temperature, 325 °C; drying gas, nitrogen at 8 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, nitrogen at 11 L/min; capillary voltage, 4000 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 spectra s^{-1}; mass range 60–1100 *m/z*) was governed via the Agilent Mass Hunter Workstation software. The instrument gave typical resolution 15000 FWHM (Full Width at Half Maximum) at *m/z* 112.9856 and 30000 FWHM at *m/z* 1033.9881. To assure the desired mass accuracy of recorded ions continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3Htetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. In negative ion mode, ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 1033.988109 adduct of HP-921 were used. The instrument was calibrated and tuned according to the procedures recommended by the manufacturer.

2.7. Data Processing and Statistical Analysis.

MassHunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing all data obtained by LC–TOF/MS in full scan MS mode. Treatment of raw data file was initiated by extraction of potential molecular features (MFs). Molecular features extraction (MFE) was based on the extraction algorithm that locates and groups all ions related to the same neutral molecule. This relation is referred as to the covariance of peaks within the same chromatographic retention time, the charge-state envelope, isotopic distribution, and/or the presence of adducts and dimmers. The MFE took into account all ions exceeding 5000 counts, with charge state limited to a maximum of two and a peak spacing tolerance of 0.0025 *m/z* (plus 50 ppm). Each feature was given by a minimum of two ions. The extraction algorithm was based on a common organic model with chromatographic separation. The allowed positive ions were protonated species and sodium adducts (+H, +Na, +K), and the negative ions formed by formate adducts and proton losses (-H, -Cl, +HCOO). The neutral losses by dehydration and the lost of phosphate or a methyl group were also included to identify features corresponding to the same molecule. Therefore, some ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by retention time (RT), intensity in the apex of chromatography peaks and accurate mass. Background contribution was removed by subtraction of MFs linked to plasticizers,

solvent impurities, and other contaminants after analysis of blank samples (methanol and chloroform) under identical instrument operation conditions. Hence, data files were created in compound exchange format (.cef files) for each sample and exported into the Mass Profiler Profesional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing. Figure 1 shows the base peak chromatograms in negative ionization mode from an individual prior to the intervention study (PRE), and from individuals after the four intervention diets (POS).

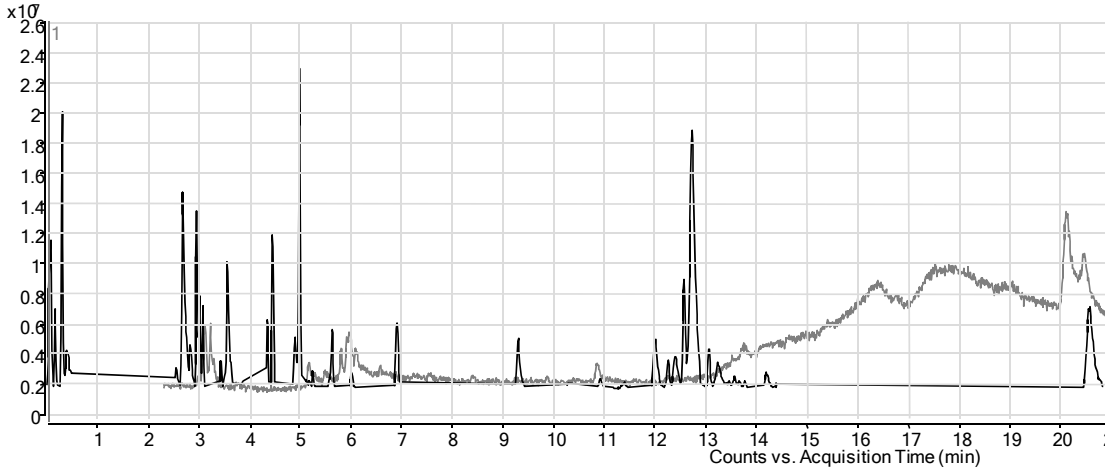


Figure 1.Base peak chromatogram in negative ionization mode from a control sample (black line), and from a sample obtained after intake (grey line).

Once the .cef files were exported into MPP, the data were preprocessed by alignment of retention times and *m/z* values across the data matrix using a tolerance window of 1.0 min and 30 ppm mass accuracy. An export for recursion file was obtained in this step and used for the data re-alignment by using in this case a tolerance window of 0.2 min and 30 ppm mass accuracy. Data pretreatment was based on baselining to remove background noise and normalization by logarithmic transformation to reduce relatively large differences among the respective MF abundances. Stepwise reduction of MFs number was performed based on frequency occurrence, abundance of the respective MFs in classes and results of one-way analysis of variance (ANOVA). MPP software also allowed unsupervised and supervised analysis by PCA and PSL-DA of the data. In the case of PCA, data scaling by mean centering was used as pretreatment. Auto-scaling was selected in the case of PLS-DA. The validation model selected was N-Fold, by which the classes in the input data were randomly divided into N equal times; N-1 parts were used

for training, and the remaining one part was used for testing. The process is repeated N times, with a different part used for testing in each iterative step. Then, repetitions and a fold number of 3 were selected for all validations.

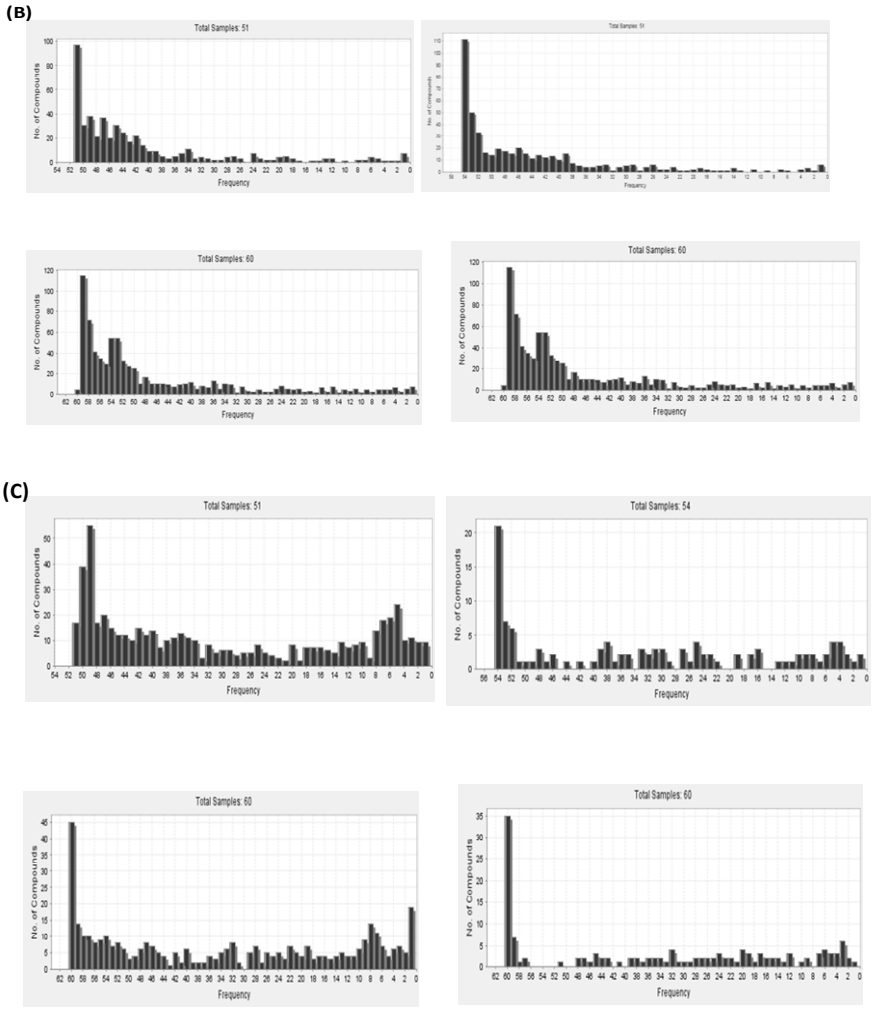
3. Results and discussion

A common factor associated to metabolomic analysis in clinical and nutritional studies dealing with humans is the biological variability among individuals, frequently higher than variability associated to the effect caused by internal factors (diseases, metabolic disorders) or external factors (diet, lifestyle). For this reason, other variability sources can be masked by the variability among individuals. In this research, data acquisition was carried out to observe the biological variability not ascribed to diets intake as compared the variability after diets intake. For this purpose, the chromatograms acquired in basal-state (PRE) were compared with those acquired after postprandial lipemia (POS) by consumption of the prepared meal.

3.1. Data mining and pre-treatment

The goal in this preliminary step was to obtain a global profile of metabolites present in polar and non-polar aqueous phases representative of individuals subjected to every diet. The comparison among the different profiles would allow evaluation of similarity/dissimilarity patterns according to the administered diets. A critical step for comparison of metabolite profiles was the alignment of chromatograms, which was carried out in a two-step process to improve efficiency. This process was carried out for all samples pertaining to each intervention diet, and also for samples corresponding to the basal state. Attending to this preclassification, frequency plots of molecular features were obtained for each diet as a result of the second alignment step. Supplementary Figure 1.A-to-C shows the frequency histogram that represents the number of molecular features detected as a function of the number of samples for each group. As can be seen in Supplementary Figure 1.A, the frequency profiles define samples in basal state with a homogeneous metabolic composition since at least 73 samples out of75 treated samples contained a 70% of total molecular features detected with similar behavior for both positive and negative ionization modes. Supplementary Figure 1.B and C illustrates the frequency histograms of post-basal states from the four tested diets both in positive and

negative ionization modes. As can be seen, the number of extracted compounds in the negative ionization mode was twice that obtained in the positive ionization mode. Hence, the number of MFs aligned across the sample set was significantly influenced by the ionization mode as well as by the intensity threshold setting used during the data mining procedure [18]. Differences were found among the four diets in the positive ionization mode: diets HSFA and LFHCC presented a smaller number of compounds than diets HMUFA and LFHCC (n-3).



Supplementary Figure 1. Frequency diagrams obtained in basal-state samples (A), post basal-state sample in negative ionization-mode (B), and positive ionization-mode (C). Diet 1: rich in SFA, Diet 2: rich in MUFA, Diet 3: rich in CH , and Diet 4 rich in CH and enrichment with PUFA-n3

Taken into account the complexity of the data obtained and the presence of signal redundancy, a strategy was designed to simplify the dimensionality of the multivariate matrix. Both positive and negative ions with different *m/z* values corresponding to adducts, isotopes or multiply charged species gave an identical elution profile promoting the presence of several MFs for the same tentative metabolite. To solve this problem a filter by frequency involving the elimination of those MFs not present in at least 75% of all samples belonging to one of the four classes was tested. One of the characteristic

Ionization mode	Intervention study (diet)	Number of MFs across the sample set				
		Initial[a]		Filtering by frequency[b]		Filtering by fold change[c]
ESI(-)		Pre	Pos0	Pre	Pos	

aspects of this approach is the significant influence on metabolism of factors such as nutrition and some anthropometric parameters that influence variability. The purpose of this filter was to reduce the data matrix only to MFs representative of the four predefined diets classes. As the number of obtained MFs aligned across was significantly influenced by the ionization mode, Table 1 shows the difference in the number of MFs achieved by using both filter classes and for each ionization mode. This filter affected significantly to the positive ionization mode, while its influence was quite lower for the negative ionization mode.

	1	737	543	441	353	518
	2	744	565	406	317	514
	3	749	1107	378	440	671
	4	746	822	442	513	746
	1	155	539	56	263	580
ESI(+)	2	147	134	52	47	157
	3	148	372	57	156	353
	4	149	152	51	54	39

It is worth mentioning that the final group of MFs which passed filtration was automatically re-extracted from the raw data files of all samples (basal and post-basal states) using recursive feature extraction. Extracted ion chromatograms (EICs) for each ion in MF composite mass spectra were obtained in this way. A careful inspection of resultant EICs from the recursive analysis was performed to avoid any false positive MFs. With regards to the relatively high number of MFs, even after the filtration procedures and the results of PCA (discussed below) recursion and EIC inspection was performed for MFs from both ionization modes. One false positive (the same MF) was removed from each of the filtered MF groups. On the other hand, false negatives (*i.e.* MFs potentially missed during data mining) were not detected.

Table 1. The overview of MFs number extracted applying two filtering steps, filters by frequency and fold change.

[a] *After removal of MFs extracted from blank samples.*
[b] *Filtering criterion was the presence of an MF in at least 75% of samples at least in one group.*
[c] *The criterion to pass the filter was FC≥2.0*

3.2. Unsupervised analysis by principal component analysis

PCA is a frequently employed unsupervised pattern recognition approach enabling data dimensionality reduction, while retaining maximum variability of the data. Following the above data pre-treatment, PCA was employed in the first stage of chemometric analysis for both positive and negative data to evaluate sample clustering according to the studied diets and to find the most suitable group of MFs for discrimination among diets. For this study, classification between PRE and POS states of the individuals was maintained. The POS period for 12 weeks is sufficient to find metabolism patterns in human plasma after intake of the different diets. Figure 2 illustrates the PCA scores plots obtained in both ionization modes, positive and negative, for the polar and non-polar fractions. As can be seen, the PRE state was clearly discriminated from all post-basal states corresponding to the different diets only for the lipidic phase (Fig. 2.A). Apart from that, the different POS

states were separated except for the two diets with high fat content, those with high proportion of SFA and MUFA. Although diet number 3, LFHCC, was close to diets based on high fat content, these were completely separated. The PCA distribution was different for the polar fraction, as shows Fig. 2.B. As can be seen, the basal state was overlapped completely with the POS state corresponding to LFHCC (n-3) diet based on supplementation with PUFAs. On the other hand, the other three POS states were clearly differentiated. This first study allowed detecting metabolic differences in plasma after the different intervention diets depending on the fraction analyzed, lipidic or non-lipidic.

A) B)

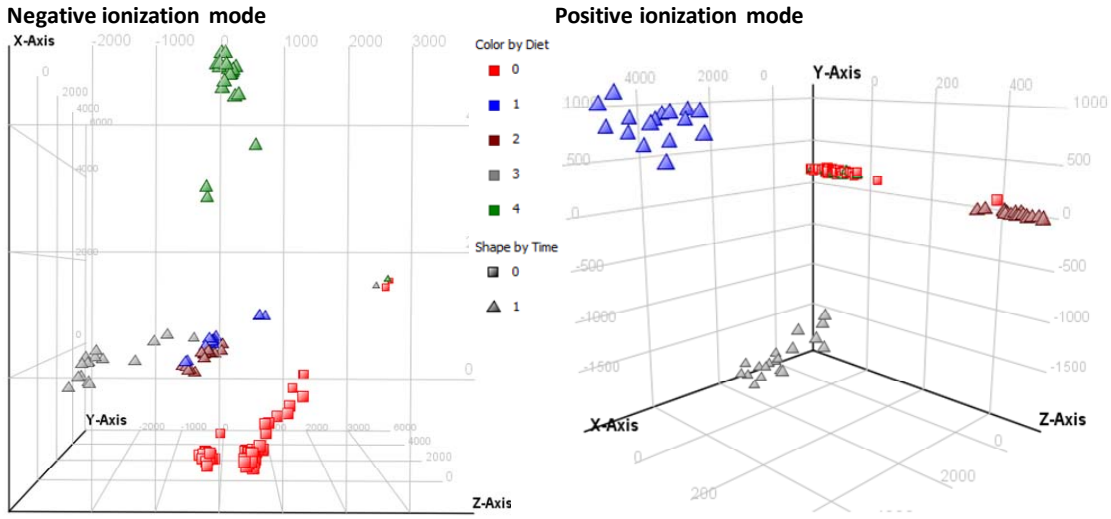


Figure 2. *PCA scores plots with 5 variables (0=basal state, post-basal state 1=Diet1, 2= Diet2, 3= Diet3, and 4=diet4) after application of each intervention diet by both negative and positive ionization-mode.*

This study was complemented with independent PCAs for each intervention diet, in which each POS state was compared with the PRE state considering the same individuals group. The goal in this case was to confirm the results provided by the overall PCA including all intervention diets. Figures 3 and 4 show the score plots for each diet in negative and positive ionization modes, respectively. As can be seen, the POS states were clearly discriminated from PRE states in negative ionization mode in three-dimensional plots (explaining around 35% of variability), which is in agreement with the results presented in Figure 2. On the other hand, the positive ionization mode for analysis of the non-lipidic fraction revealed discrimination for all POS states excepting for that

corresponding to LFHCC (n-3) diet, which was not separated from individuals in PRE state. Therefore, this study confirms the results obtained in the overall PCA dealing with all the intervention diets.

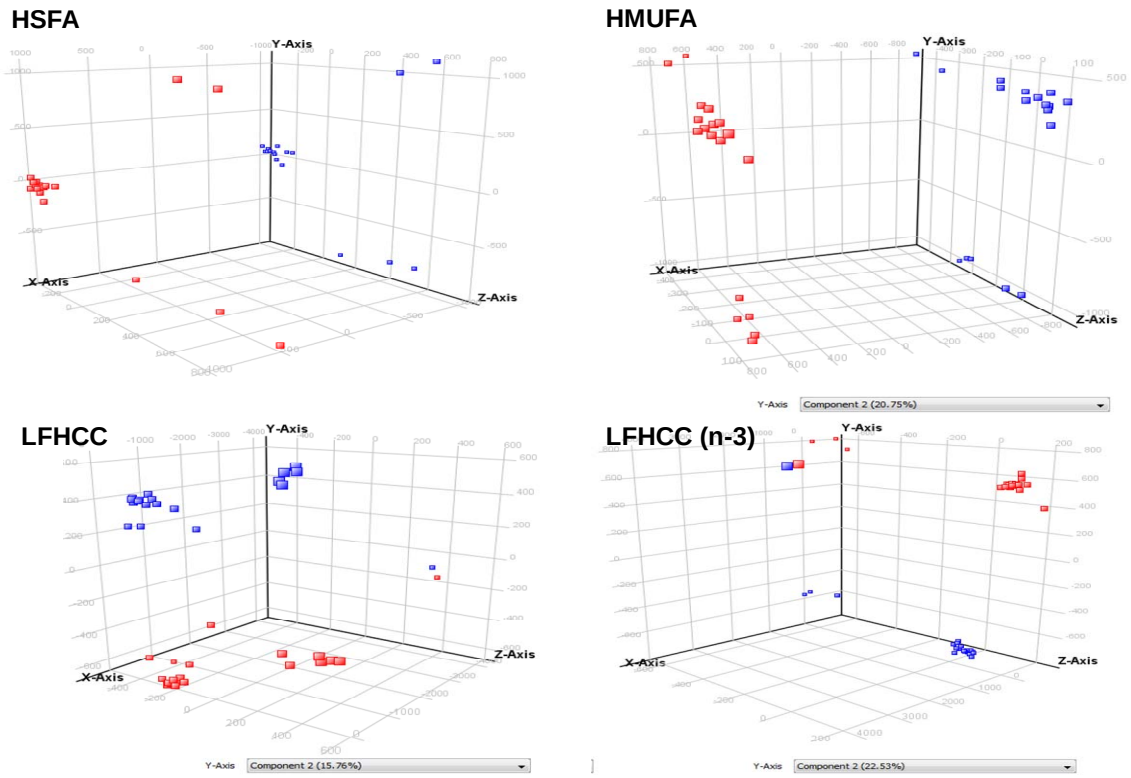


Figure 3. *PCA scores plots of the comparative study of classes (0=basal-state and 1=post-basal) for each diet (1=Diet1, 2= Diet2, 3= Diet3, and 4=diet4) in negative ionization-mode.*

3.3. Supervised analysis by partial least squares discriminant analysis

The observed variability in the metabolite profiles of serum according to the intervention diet were attempted to be modelled by PLS-DA, a widely used supervised pattern recognition method with capability for sample classification and prediction. For this purpose, data from samples in PRE and POS states were used to look for discrimination patterns with independent evaluation for training and validation steps.

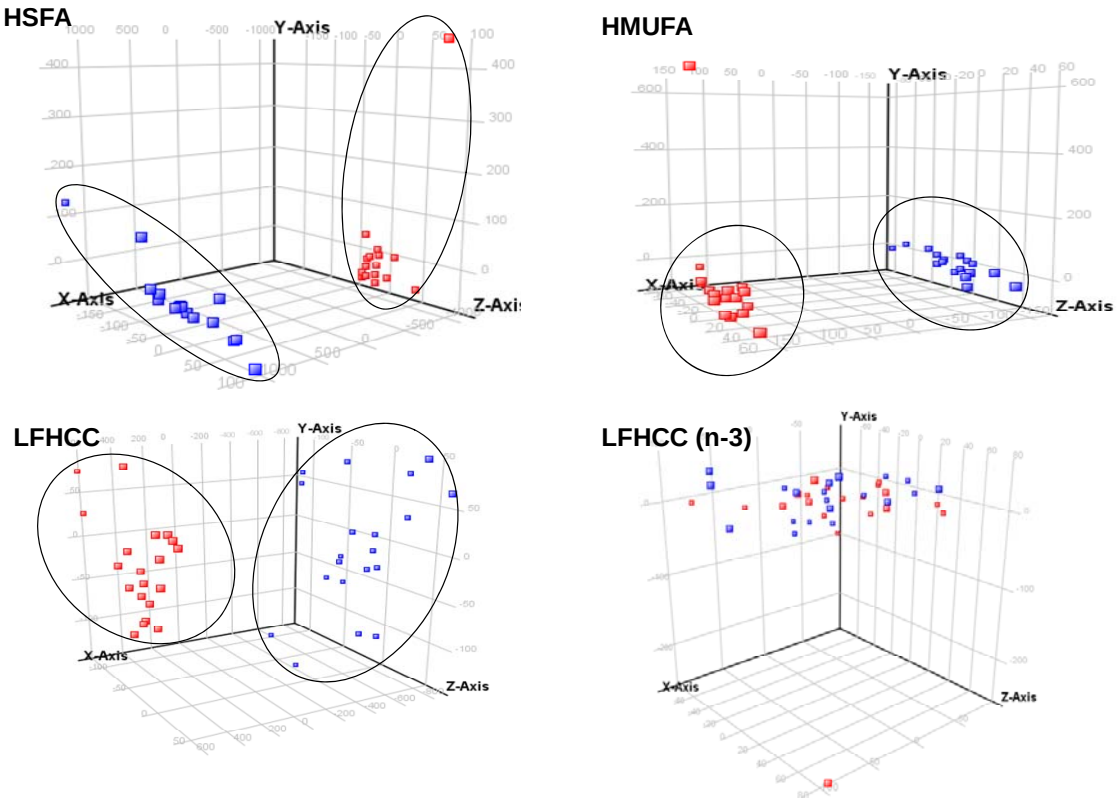


Figure 4. PCA scores plots of the comparative study of classes (0=basal-state and 1=post-basal) for each diet (1=Diet1, 2= Diet2, 3= Diet3, and 4=diet4) in positive ionization-mode.

The study was split attending to the lipidic and non-lipidic fractions to compare the classification capability for both phases. The scores plots obtained with the three first latent variables are shown in Figure 5. A clear clustering of individuals attending to the intervention diet can be visualized. In the case of the negative ionization mode, the different classes were correctly separated. Thus, the PRE and POS states corresponding to low-fat diets were clearly discriminated, while samples representing the two high-fat diets were close. Anyway, two sub-clusters were identified despite the fact that serum metabolic profiles detected after these two intervention diets were quite similar. Concerning the analysis of the non-lipidic fraction, the PRE state was completely overlapped with the LFHCC (n-3) diet based on PUFAs supplementation. The other three intervention diets were perfectly separated. Statistical evaluation of both classification models was supported on the prediction accuracy, which expresses the percentage of correctly classified samples in a given class. The results of samples classification are presented in terms of accuracy for the training and validation steps (by cross-validation)

both describing the prediction capability of the model. The prediction accuracy is represented as a confusion matrix in Tables 2 and 3, with the true class in rows and the predicted class in columns.

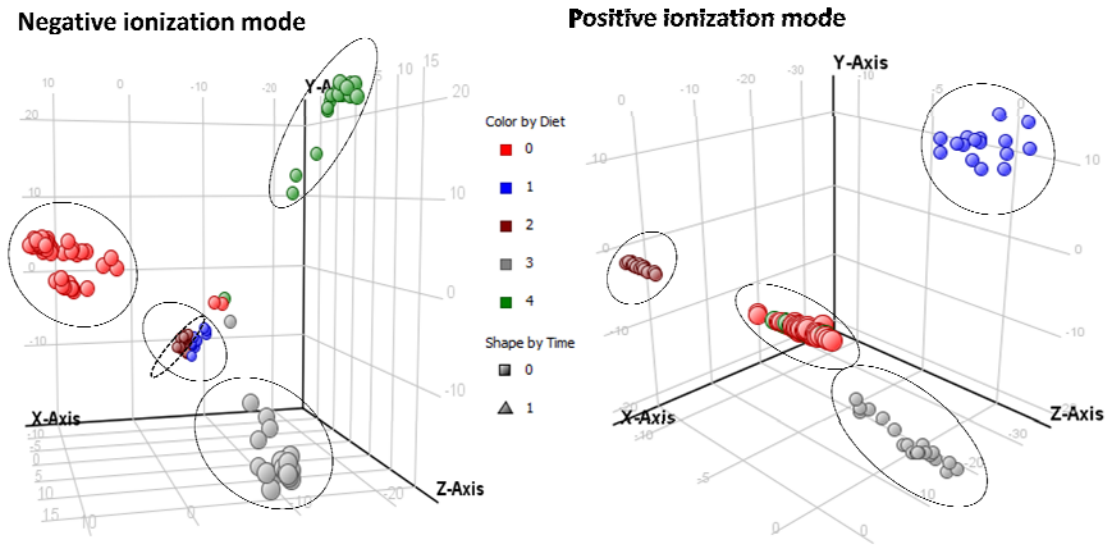


Figure 5. *PLS-DA scores plots with 5 variables (0=basal state, post-basal state 1=Diet1, 2= Diet2, 3= Diet3, and 4=diet4) after application of each intervention diet by both negative and positive ionization-mode.*

In the negative ionization mode the prediction accuracy in the training step was 100% for the four groups corresponding to intervention diets, while this was 98.6% for the PRE state. Validation was quite similar since the prediction capability was above 98.7% for the samples obtained after intervention diets, while individuals in basal state were classified with 73% accuracy. Concerning the positive ionization mode, the situation was different for LFHCC (n-3) diet since this class was not well predicted both in training and validation steps. As deduced from PLS plots, samples corresponding to this class were mostly predicted as PRE state. This result could be justified by the low number of molecular features detected in this class of samples. However, the intervention diet based on high-fat content and MUFAs also led to detection of few number of entities as compared to the resting diets but this was perfectly classified.

The PLS-DA studies confirmed the preliminary results obtained with PCA. According to both studies, the metabolic profile associated to the lipidic fraction was significantly affected after the four intervention diets as compared to the PRE state. On

the other hand, the metabolic profile associated to the non-lipidic fraction was affected for the high-fat diets (HSFA and HMUFA) and the low-fat diet LFHCC (supplemented with high oleic sunflower oil), while the low-fat LFHCC (n-3) diet did not alter significantly the metabolic composition of plasma.

3.4. Qualitative comparison of plasma metabolome profiles associated to intervention diets versus basal state

Metabolic changes in plasma from individuals after intervention diets were assessed by qualitative comparison of molecular features extracted from LC–MS analysis of samples corresponding to PRE and POS states. Comparison was carried out by Venn diagrams to estimate the impact of the diet on the metabolic composition of plasma. Figure 6 shows the corresponding diagram for each intervention study both in positive and negative ionization, in which the number of MFs detected exclusively in PRE and POS states can be compared with those detected prior to and after the intervention study. The results can be interpreted independently according to the ionization mode. In negative ionization mode, the distribution of MFs was quite similar as a function of the fat content. Thus, HSFA and HMUFA reported similar distributions of MFs with most entities detected both in basal and post-basal states. Metabolic changes were more noticeable for low-fat intervention diets since a high number of entities were exclusively detected in POS state. The positive ionization mode reported different results since only HSFA and LFHCC intervention diets allowed detecting significant metabolic changes in POS state. On the other hand, the HMUFA and LFHCC (n-3) intervention diets did not provide any significant alteration as deduced from the number of entities detected only in plasma from individuals after the 12-weeks intervention diet.

This qualitative comparison allows stating that intervention diets promoted metabolic changes as compared to the basal state. Different effects were observed as a function of the fraction analyzed: lipidic or non-lipidic. Thus, the lipidic fraction, which was analyzed by negative ionization mode, enabled to reveal significant metabolic alterations in plasma from individuals after the intervention diets based on LFHCC and LFHCC(n-3), both with low-fat content. On the other hand, the non-lipidic fraction only allowed detecting differences for HSFA and LFHCC intervention diets.

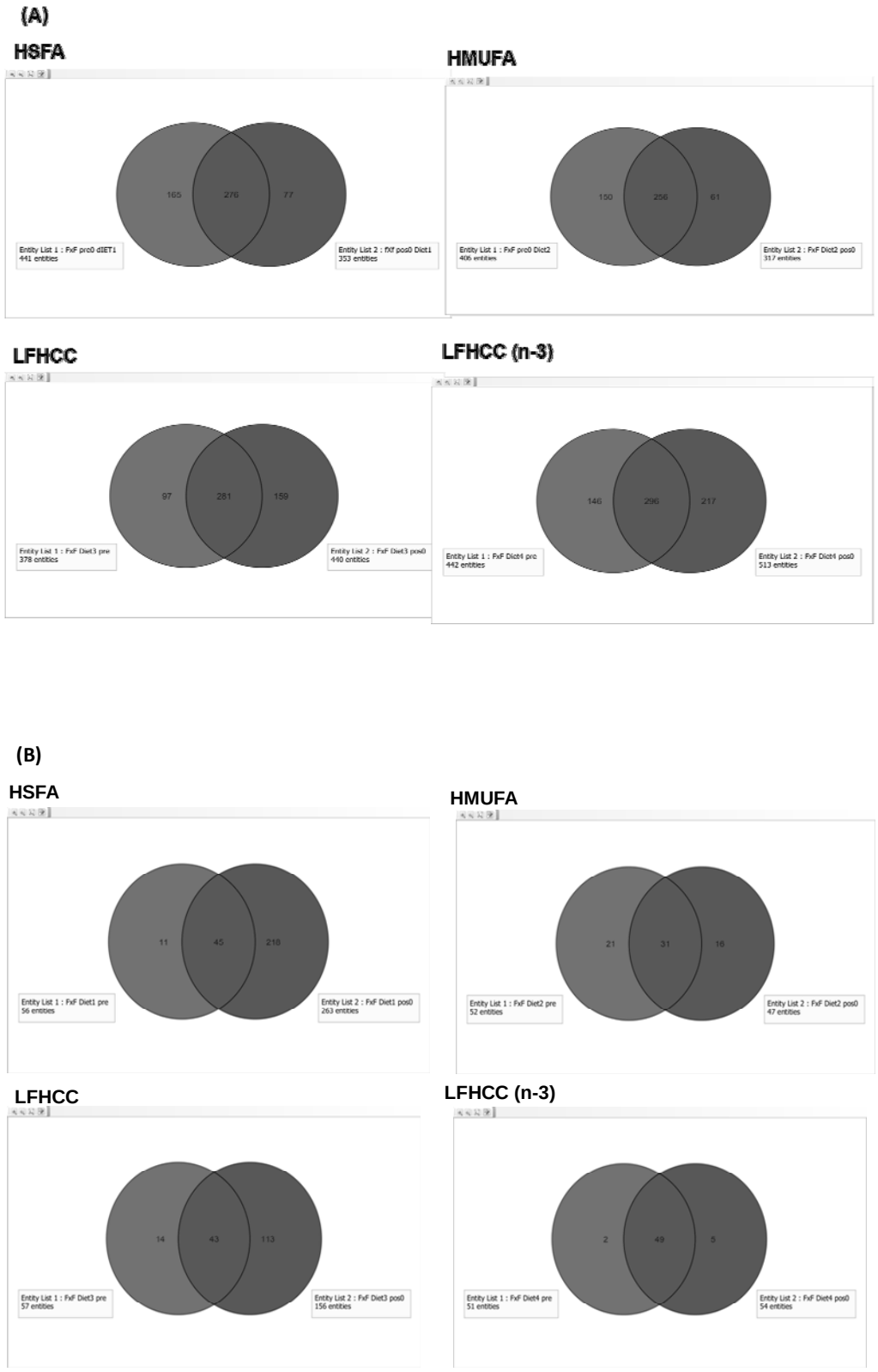


Figure 6. .Venn diagrams for the intervention diets.pos0:after intake; pre: before intake. Negative ionization-mode (A) and positive ionization-mode (B).

3.5. Tentative identification of significant metabolites for intervention diets

Once the PLS models were explored those MFs that contributed to discrimination of individuals after each intervention diet *versus* the basal state were filtered. For this purpose, the significance of each molecular entity present in the data sets was estimated according to a Volcano test. The algorithm supporting this test was used to compare two metabolic conditions and detect the most relevant changes. A Volcano test combines an ANOVA test (by setting a *p*-value as significance cut-off) and a fold change analysis to detect those features (tentative metabolites) that display large-magnitude changes in relative concentration from a statistical point of view. The statistical requirements were: significant change between metabolic states at 99% of confidence level and with a relative concentration change of at least 50% (fold change ratio > ±2.0). Hence, the Volcano test enabled to detect a range from 157 to 274 molecular entities with a significant change between PRE and POS states for the four diets in the negative ionization mode (Table 1). In the positive ionization mode, a range from 26 to 212 molecular entities was obtained for the three intervention diets which were discriminated from the PRE state. As previously mentioned, no discrimination was observed between PRE- *versus* POS state corresponding to the LFHCC (n-3) intervention diet in positive ionization mode.

After this significant evaluation, a final identification step was carried out for those MFs reported by the Volcano test. Identification was supported on automated searching of *m/z* experimental values in the HMDB and METLIN databases. The main adducts considered in the negative ionization mode were $[M–H]^–$, $[M+Cl]^–$ and $[M+HCOOH–H]^–$, while $[M+H]^+$, $[M–H_2O+H]^+$, $[M+Na]^+$ and $[M+K]^+$ were taken into account for the positive ionization mode. Double and triple charge states were also considered for $[M–H]^–$ and $[M+H]^+$ adducts.

3.5.1 Tentative identification of metabolites present in the non-lipidic phase

Identifications according to specified databases revealed the presence of a high proportion of exogenous metabolites. These identifications are of interest to evaluate the incidence of intervention diets and the metabolism of exogenous substances which are dependent on the variety of consumed foods. Nevertheless, the aim of this research was to focus on those metabolic changes involving endogenous metabolites caused by intervention diets.

The analysis of the non-lipidic fraction reported less identified metabolites than that for the lipidic fraction. Table 2 lists the tentative identifications for each intervention diet. Although the plasma fractionation step favor the extraction of lipids to the chloroform, a great variety of phospholipids, eicosanoids or glycerides were detected in the methanolic phase. These compounds were not considered in this section since they were better characterized in the chloroform fraction. However, despite the fractionation step, some lipids such as ceramides, sphingomyelins and cardiolipins, which were altered in relative concentration terms in individuals subjected to high-fat diets, were better characterized in this fraction.

Two vitamin E metabolites (13-hydroxy-γ-tocotrienol and α-CEHC) were identified in plasma from individuals who consumed the two intervention diets based on a high-fat content. One other metabolite which was altered in individuals after the two high-fat diets was farnesol, a signalling molecule intermediate in the biosynthesis of isoprenoids and cholesterol. One other common alteration associated to individuals after the two high-fat diets was the presence of significant change in the concentration of CDP-glucose, involved in the metabolism of amino sugars and nucleotide sugars. All these metabolic changes revealed a similar pattern for the two high-fat diets (HSFA and HMUFA). Nevertheless, specific changes associated to each of these two diets were also found. Thus, numerous dipeptides were detected in plasma from individuals after HSFA and HMUFA diets. The presence of all these peptides is indicative of proteolytic degradation of larger proteins and, therefore, these diets reported an effect on metabolism of proteins. Creatinine levels were also altered as a result of the HSFA diet, which could support a short-term effect in the renal function of these individuals. The concentration of bilirubin in plasma was significantly changed in individuals after HSFA and LHFCC diets but not in those subjected to HMUFA diet, which is other discriminating metabolic change. Similar to bilirubin, carnitine derivatives contributed to explain the metabolic variability of individuals for HSFA and LHFCC diets.

Changes in the metabolism of amino acids were also detected after the three particular diets that reported metabolic differences *versus* the PRE state. One example corresponded to dityrosine, which is frequently linked to situations of oxidative stress, was one of the most remarkable changes in individuals after HMUFA and LHFCC diets.

3.5.2 Tentative identification of metabolites present in the lipidic phase

Similar to the non-lipidic phase, identifications could be split into exogenous and endogenous metabolites. Most exogenous metabolites were related with nutrients associated to the intervention diets. Thus, some examples of exogenous metabolites were: 5-phenyl-1-pentanol or octyl octanoate, which are frequently used as flavoring ingredients; 1-O-beta-D-glucopyranosyl-2,3-di-O-palmitoylglycerol, found in fruits; and (E,E)-2,6-octadienal, present in fats and oils. These exogenous metabolites were not considered in this elucidation study since this research was aimed at identifying tentatively endogenous metabolites which were altered in terms of relative concentration due to the intervention diets. Focused on endogenous metabolites, most significant changes in relative concentration occurred for common families of metabolites such as lysophospholipids and phospholipids, diglycerides, triglycerides and arachidonic acid metabolites produced by enzymatic oxidation. Table 3 lists the significant molecular features detected in this fraction for each intervention diet and tentative metabolites identified with their *m/z* values. The first clear difference is the number of diglycerides and triglycerides discriminating individuals subjected to particular diets. Thus, the highest number of significant triglycerides was detected in individuals subjected to HSFA and HMUFA intervention diets, while in the case of diglycerides corresponded to HSFA, HMUFA and LFHCC intervention diets. It seems quite clear that metabolic changes affecting to the glyceride profile were not significant to discriminate individuals controlled with the LFHCC (n-3), which was supplemented with EPA. It is worth mentioning that monoglycerides were less affected by intervention diets since only the monigluceride (22:0) contributed to explain the variability of individuals after the HSFA diet. It is worth mentioning that fatty acids forming part of glycerides were marked by the intervention diets. Thus, a high proportion of SFA and MUFA was detected in these lipidic structures in individuals subjected to HSFA and HMUFA diets, respectively. On the other hand, the proportion of PUFAs in significant glycerides included in Table 3 was higher for individuals controlled with the LFHCC and LFHCC (n-3). The metabolism of fatty acids was partially affected by all intervention diets as shows the presence of fatty acids and derivatives such as oxidation products in Table 3. Hydroxylated forms were detected in the four diets. An intermediate in the biosynthesis of fatty acids such as 3-oxohexadecanoic acids was affected by three intervention diets.

It should also be emphasized the role of PUFA metabolites such as eicosanoids, which are well-known because of their involvement in biological functions such as the inflammation cascade. The highest variety of eicosanoids contributing to explain the

variability associated to intervention diets was found in individuals subjected to LFHCC (n-3) diet despite the fact that this diet was supplemented with n-3 PUFAs and the detected eicosanoids are derivatives of arachidonic acid (n-6 PUFA). The panel of significant eicosanoids was mainly formed by hydroxy fatty acids (HETEs) and epoxy derivatives (EETs). The formation of these eicosanoids is frequently linked to oxidative stress and inflammation processes.

One other single metabolites contributing to discrimination of specific diets were dihydroxyvitamin D3 metabolites (1,25- or 24,25-isomers), which explained the variability caused by intake of high-fat content and low-fat content diets supplemented with high oleic sunflower oil. Also, cholic acid and other bile acids explained partially the variability in individuals who ingested HMUFA and LFHCC diets, while cholesterol sulfate aided in the discrimination of LHFCC n-3 diet.

The family of phospholipids was the main group of metabolites allowing discrimination of the four post-intervention groups. In fact, numerous compounds of each phospholipidic family were detected, which is quite logical taking into account the non-polar properties of phospholipids. Table 3 shows the variations in the *m/z* values of significant phospholipids detected in samples from each intervention diet. As can be seen, the set of significant phospholipids for each diet varies qualitatively with contribution of glycerophosphocholines, glycerophosphoethanolamine, glycerophosphatidylinositols, glycerophosphatidic acids, glycerophospholipids and phosphatidylglycerolphosphates. Concerning lysophospholipids (lysoPLs), the panel of the most concentrated metabolites of this family in serum was only identified in samples from HSFA and HMUFA individuals.

.

Table 2. Retention time, accurate mass error, identification code and ion used to identify tentative compounds present in the different diets. Methanolic fraction

Diet	HMDB ID	Common Name	Adduct	MW Difference (Da)	Compound MW (Da)	Delta	MW Difference (ppm)	Retention time (min)
HSFA	HMDB37555	Sandoricin	M+Na	611.246280	588.257062	0.004280	7.0020876	4.2
	HMDB41373	Ustiloxin B	M+NH4	663.265415	645.231592	0.003085	4.65123001	3.1
	HMDB38762	Kaempferol 3-sophoroside 7-(2-feruloylglucoside)	M+2H	475.134052	948.253552	0.002652	5.58158269	4.2
	HMDB36190	2-Heptylfuran	M+K	205.098923	166.135765	0.001903	9.2784495	6.2
	HMDB40237	Dihydro-2-methoxy-2-methyl-3(2H)-thiophenone	M+2ACN+2H	115.053898	146.040150	0.000118	1.02560628 10.4074428	7.5
	HMDB30994	5-Heptyltetrahydro-2-oxo-3-furancarboxylic acid	M+NH4	246.169982	228.136159	0.002562	0.91220272	5.8
	HMDB36190	2-Heptylfuran	M+NH4	184.169588	166.135765	0.000168	1.32120718	3.5
	HMDB36183	4-(4-Methylphenyl)-2-butanone	M+NH4	180.138288	162.104465	0.000238	2.7365141	7.2
	HMDB14401	Lymecycline	M+Na	625.248011	602.258793	0.001711	2.32524791	4.5
	HMDB14401	Lymecycline	M+K	641.221951	602.258793	0.001491	0.48306492	3.8
	HMDB40915	2-(4-Methyl-1,3-pentadienyl)anthraquinone	M+K	327.078188	288.115030	0.000158	9.79311296	2.4
	HMDB11684	N(6)-(Octanoyl)lysine	2M+3H2O+2H	572.443106	272.209993	0.005606	0.57478611	4.2
	HMDB40915	2-(4-Methyl-1,3-pentadienyl)anthraquinone	M+K	327.078188	288.115030	0.000188	1.16344269	3.2

	HMDB01388	Alpha-Linolenic acid	2M+3H2O+2H	584.472280	278.224580	0.000680	6.59352614	4.7
	HMDB35217	10-Acetoxyligustroside	M+2H	292.104704	582.194856	0.001926	5.5960934	3.4
	HMDB40241	1-(2-Thienyl)-1-heptanone	M+Na	219.081404	196.092186	0.001226	1.18299593	3.6
	HMDB34182	1,5,8-Trihydroxy-3-methyl-2-prenylxanthone	M+H+K	191.040386	342.110338	0.000226	1.76631734	3.1
	HMDB38	N-Norgramine	M+2Na	103.03924	160.100048	0.0001	7.0020876	5.8
HMUFA	HMDB38167	Maalialcohol	M+NH4	240.232188	222.198365	0.000022	0.09157807	4.3
	HMDB39532	(6Z,9Z,12Z)-6,9,12-Pentadecatrien-2-one	2M+3H2O+2H	468.388551	220.182715	0.000519	1.10805441	2.6
	HMDB40915	2-(4-Methyl-1,3-pentadienyl)anthraquinone	M+K	327.078188	288.11503	0.000068	0.20790136	4.4
	HMDB40915	2-(4-Methyl-1,3-pentadienyl)anthraquinone	M+K	327.078188	288.11503	0.000128	0.39134374	3.6
	HMDB15680	Nonoxynol-9	M+NH4	634.452471	616.418648	0.000071	0.11190752	3.7
	HMDB01388	Alpha-Linolenic acid	2M+3H2O+2H	584.47228	278.22458	0.00086	1.47141281	4.2
	HMDB38060	Triparinarin	M+H+Na	445.319467	866.64244	0.002253	5.05928927	3.1
	HMDB14488	Protriptyline	2M+NH4	544.368622	263.1674	0.000578	1.06178052	4.2
	HMDB01388	Alpha-Linolenic acid	2M+3H2O+2H	584.47228	278.22458	0.00172	2.94282562	6.2
	HMDB33648	4',5,6,7,8-Pentahydroxy-3'-	M+2H	167.033885	332.053217	0.001085	6.49568799	7.5

		methoxyflavone						
	HMDB33244	Dibutyl phthalate	M+H	279.159085	278.151809	0.001685	6.03598482	
	HMDB06088	Scyllitol	M+Na	203.052606	180.063388	0.000114	0.56143086	5.8
	HMDB34220	Levoinositol	M+Na	203.052606	180.063388	0.000114	0.56143086	3.5
LFHCC	HMDB37287	3-(2-Methylpropyl)pyridine	2M+K	309.172757	135.104799	0.000337	1.09000548	7.2
	HMDB06088	Scyllitol	M+Na	203.052606	180.063388	0.000064	0.31518926	4.5
	HMDB36158	4-Deacetylneosolaniol	2M+ACN+Na	744.320171	340.152203	0.003029	4.06948531	3.8
	HMDB36157	15-Deacetylneosolaniol	2M+ACN+Na	744.320171	340.152203	0.003029	4.06948531	2.4
	HMDB14932	Primidone	M+H+Na	121.051011	218.105528	0.000101	0.834359	4.2

Table 3. Tentative compounds present in the different diets

Nº	F.C.	HSFA	Nº	F.C.	HMUFA	Nº	F.C.	LFHCC	Nº	F.C.	LFHCC (n-3)
1	<(-16)	9-HODE 12,13-EpOME 9,10-Epoxyoctadecenoic acid 13S-hydroxyoctadecadienoic acid	1	>16	Tryptophanol	1	>16	Oleic acid	1	<(-16)	Disaccharide
			2	<(-16)	3-Hydroxydecanoic acid	2	>16	Arachidonic acid	2	-1.57	LysoPE(18:2(9Z,12Z)/0:0)
			3	<(-16)	3-Oxooctadecanoic acid	3	>16	Alpha-Pinene-oxide			
2	<(-16)	Octadecanedioic acid 12,13-DHOME 9,10-DHOME	4	<(-16)	Octadecanedioic acid 12,13-DHOME 9,10-DHOME	4	<(-16)	Disaccharide			
			5	<(-16)	Palmitoleic acid Palmitelaidic acid						
3	-1.5	LysoPE(18:1(9Z)/0:0)									
4	-1.5	LysoPE(18:1(11Z)/0:0)	6	>16	9,10-Dihydroxyoctadecanoic acid						
5	<(-16)	4,4-Dimethylcholesta-8,14,24-trienol 5-Dehydroavenasterol	7	>16	Palmitic acid						
			8	>16	5'-Methylthioadenosine						
			9	<(-16)	Eicosenoic acid						
6	<(-16)	Disaccharide	10	>16	3-Methyladipic acid Pimelic acid						
7	<(-16)	Riboflavin									
8	-1.75	LysoPE(18:2(9Z,12Z)/0:0)	11	1.5	L-Tryptophan						
			12	<(-16)	Disaccharide						
			13	-1.81	LysoPE(18:1(9Z)/0:0)						
			14	-1.81	LysoPE(18:1(11Z)/0:0)						
1	<(-16)	9-HODE 12,13-EpOME 9,10-Epoxyoctadecenoic	1	>16	L-Tyrosine	1	<(-16)	3-Hydroxydecanoic acid	1	<(-16)	9,10-Dihydroxyoctadecanoic acid

		acid 13S-Hydroxyoctadecadienoic acid							2	<(-16)	Palmitic acid
			2	<(-16)	3-Hydroxydecanoic acid	2	<(-16)	9,10-Dihydroxyoctadecanoic acid	3	<(-16)	Eicosenoic acid
						3	<(-16)	Palmitic acid	4	<(-16)	Disaccharide
			3	<(-16)	9,10-dihydroxyoctadecanoic acid	4	<(-16)	Eicosenoic acid			
2	<(-16)	Octadecanedioic acid 12,13-DHOME 9,10-DHOME	4	<(-16)	3-Oxooctadecanoic acid	5	-2.13	LysoPE(18:1(9Z)/0:0)			
			5	<(-16)	Octadecanedioic acid 12,13-DHOME 9,10-DHOME	6	-2.13	LysoPE(18:1(11Z)/0:0)			
						7	1.51	LysoPC(15:0)			
						8	<(-16)	Disaccharide			
			6	<(-16)	Palmitoleic acid Palmitelaidic acid	9	1.63	LysoPC(16:0)			
3	<(-16)	Eicosenoic acid	7	<(-16)	Palmitic acid						
4	<(-16)	Riboflavin	8	<(-16)	Eicosenoic acid						
5	<(-16)	4,4-Dimethylcholesta-8,14,24-trienol 5-Dehydroavenasterol	9	<(-16)	Disaccharide						
6	<(-16)	4alpha-Formyl-4beta-methyl-5alpha-cholesta-8,24-dien-3beta-ol									
			1	1.51	Glucosamine	1	<(-16)	Eicosenoic acid	1	<(-16)	Eicosenoic acid
									2	-2.54	Oleic acid

4. Conclusions

A global nutrimetabolomic study has been developed to compare metabolic effects caused by four intervention diets differentiated in fat quantity and quality. Plasma samples taken in the preintervention and postintervention states were fractioned with methanol–chloroform for independent analysis of the two fractions by LC–MS with a high-resolution mass spectrometer. Multivariate statistical analysis by PCA and PLS-DA has revealed: (i) discrimination among metabolic profiles of plasma representing the four postintervention states; (ii) discrimination of each postintervention state *versus* the preintervention state, except for the methanolic fraction from the LHFCC (n-3) diet. In a final step, tentative identification of those metabolites contributing to explain the variability occasioned by each intervention diet was carried out. The important role of certain families of lipids as well as other changes affecting to the metabolism of sugars, amino acids and vitamins has been described.

Acknowledgments

The Spanish Ministerio de Ciencia e Innovación (MICINN) and European FEDER program are thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

References

[1] M. Mamas, W. Dunn, L. Neyses, R. Goodacre, Archives of Toxicology 85 (2011) 5-17.

[2] S. Moco, J. Vervoort, R. J. Bino, R. C. H. De Vos, R. Bino, Tract-Trend. Anal. Chem. 26 (2007) 855-866.

[3] M. Brown, W. B. Dunn, P. Dobson, Y. Patel, C. L. Winder, S. Francis-McIntyre, P. Begley, K. Carroll, D. Broadhurst, A. Tseng, N. Swainston, I. Spasic, R. Goodacre, D. B. Kell, Analyst 134 (2009) 1322-1332.

[4] N. Psychogios, D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend, D. S. Wishart, PLoS ONE 6 (2011) e16957.

[5] K. J. Boudonck, M. W. Mitchell, L. Német, L. Keresztes, A. Nyska, D. Shinar, M. Rosenstock, Toxicologic Pathology 37 (2009) 280-292.

[6] K. A. Lawton, A. Berger, M. Mitchell, K. E. Milgram, A. M. Evans, L. Guo, R. W. Hanson, S. C. Kalhan, J. A. Ryals, M. V. Milburn, Pharmacogenomics 9 (2008) 383-397.

[7] G. A. Theodoridis, H. G. Gika, E. J. Want, I. D. Wilson, Analytica Chimica Acta 711 (2012) 7-16.

[8] D. Vuckovic, Anal. Bioanal. Chem. 403 (2012) 1523-1548.

[9] J. L. Griffin, Current Opinion in Chemical Biology 10 (2006) 309-315.

[10] J. L. Griffin R. A. Kauppinen, Journal of Proteome Research 6 (2006) 498-505.

[11] G. a. Graça, I. F. Duarte, B. J. Goodfellow, I. M. Carreira, A. B. Couceiro, M. d. R. r. Domingues, M. Spraul, L.-H. Tseng, A. M. Gil, Anal. Chem. 80 (2008) 6085-6092.

[12] R. Ramautar, G. Somsen, G. de Jong, Anal. Bioanal. Chem. 387 (2007) 293-301.

[13] A. Koulman, G. Lane, S. Harrison, D. Volmer, Anal. Bioanal. Chem. 394 (2009) 663-670.

[14] W. B. Dunn, N. J. C. Bailey, H. E. Johnson, Analyst 130 (2005) 606-625.

[15] K. Sandra, A. d. S. Pereira, G. Vanhoenacker, F. David, P. Sandra, J. Chromatogr. A. 1217 (2010) 4087-4099.

[16] D. I. Shaw, A. C. Tierney, S. McCarthy, J. Upritchard, S. Vermunt, H. L. Gulseth, C. A. Drevon, E. E. Blaak, W. H. M. Saris, B. Karlström, O. Helal, C. Defoort, R. Gallego, J. López-Miranda, D. Siedlecka, M. Malczewska-Malec, H. M. Roche, J. A. Lovegrove, British Journal of Nutrition 101 (2009) 750-759.

[17] P. Bernini, I. Bertini, C. Luchinat, P. Nincheri, S. Staderini, P. Turano, Journal of Biomolecular NMR 49 (2011) 231-243.

[18] L. Vaclavik, O. Lacina, J. Hajslova, J. Zweigenbaum, Analytica Chimica Acta 685 (2011) 45-51.

[19] L. A. Berrueta, R. M. Alonso-Salces, K. Héberger, J. Chromatogr. A. 1158 (2007) 196-214.

[20] T. S. Metkus, D. Dobrosielski, K. Stewart, Journal of the American College of Cardiology 59 (2012) E1753-E1753.

CAPÍTULO 12

Global nutrimetabolomic analysis of human plasma by LC–TOF/MS in high-resolution mode to evaluate the metabolic changes occurring during postprandial lipemia of high- and low-fat intervention diets

Comparative nutrimetabolomic study of the influence of fat intervention diets on plasma metabolic profile by LC–TOF/MS

M.I. Orozco-Solano[a,b], F. Priego-Capote[a,b], M.D. Luque de Castro[a,b*]*

[a]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain *[b]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain*

Enviado a Journal of Nutritional Biochemistry

Global nutrimetabolomic analysis of human plasma by LC–TOF/MS in high-resolution mode to evaluate the metabolic changes occurring during postprandial lipemia of high- and low-fat intervention diets

M.I. Orozco-Solano, F. Priego-Capote, M.D. Luque de Castro

Abstract

Clear assessment of the metabolic profile of subjects after consumption of specific diets is an important challenge in modern nutrition. The aim of this study was to evaluate plasma metabolic changes occurring in plasma from metabolic syndrome patients subjected to four intervention diets with different fat composition and quality —*viz.* rich in saturated fatty acids (SFA), named as HSFA; rich in monounsaturated fatty acids (MUFA), named as HMUFA; high-complex carbohydrate diets, supplemented with high oleic sunflower oil, named as LFHCC, or with long chain n-3 PUFA, named as LFHCC (n-3). For this purpose, samples obtained in postprandial lipemia at 0, 4 and 8 h after a normal food associated to each intervention diet were analyzed by a global metabolomic approach. Plasma was fractioned in methanol/chloroform phases which were analyzed independently by LC–TOF/MS in high resolution mode. Principal Component Analysis (PCA) and Partial Least Square–Discriminant Analysis (PLS–DA) were carried out to establish differences among classes (intake of four diets) and to build classification and prediction models. Prediction models based on PLS-DA

enabled to detect that metabolic changes during postprandial lipemia were higher in the chloroform fraction (lipids) than in the methanolic fraction (polar compounds). In fact, the resulting classification models developed with the lipid fraction allowed discriminating postintervention states POS4 y POS8 versus POS0, which was collected at the beginning of the postintervention study. Metabolic changes during postprandial lipemia for each diet were correlated with those potential metabolites contributing to explain the observed variability.

Keywords: Nutrimetabolomics, Global profiling, Human plasma, LC–TOF/MS, MUFA, SFA, Long-chain *n*-3 PUFA, Food exchange

1. Introduction

Nowadays, metabolomics has been proposed as a powerful tool for exploring the complex connections and feedback between nutrition and health. Nutritional metabolomics allows investigating the role of dietary components in the maintenance of health and development of risk diseases. There are three main aims to be pursued in nutrimetabolomics analysis: (1) food component analysis; (2) food quality/authenticity detection; (3) metabolic alterations caused by diet intervention studies. The most challenging and unexploited area in nutrimetabolomics is probably its use for monitoring diet interventions. This area could be applied to assess metabolic changes related to administration of certain nutrients which could have an impact on human health. In fact, metabolism alterations derived from these treatments can be effectively monitored through metabolic alterations in biological fluids such as urine or serum. Improved dietary

guidelines and mandated food supplementation with essential minerals and vitamins have been remarkably successful in treating most nutritional "deficiencies". Recently, nutritional scientists are challenged with finding new ways of treating or preventing diseases brought on by nutritional "oversufficiencies" such as obesity, diabetes, chronic inflammation and cardiovascular diseases. It should be also worthwhile to identify bioactive food components that potentially increase life expectancy, reduce weight, enhance physical or mental performance and prevent diseases such as atherosclerosis, diabetes, cancer and arthritis (4).

One other potential application of the assessment of diet interventions is focused on the discovery of new biomarkers in human nutritional research. Three main categories are suggested: (1) assessment of nutritional and dietary interventions; (2) diet exposure and food consumption monitoring; and (3) health phenotype and metabolic impact of diet. The combination of these three strategies could aid to find specific metabolic patterns associated to standard intervention diets which could be of aid for clinicians in the search for personalized diets.

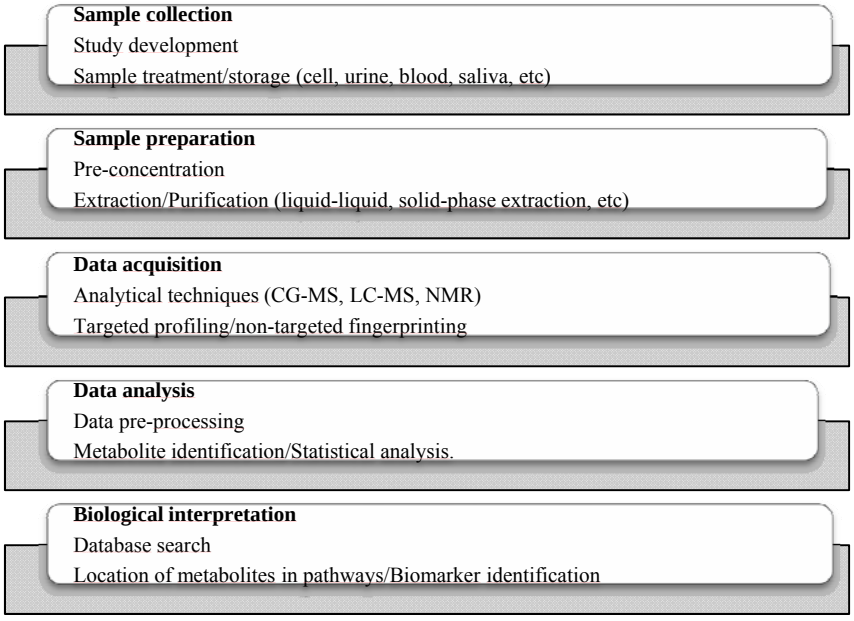
Nutrimetabolomics studies have been addressed by three approaches (2,16). The targeted approach is based on the analysis of a predefined metabolite group associated with a particular pathway and is supported on quantitative results. The non-targeted analysis enables to obtain a broad picture of the metabolome with the aim of detecting the maximum number of metabolites in a sample, including those that are unknown or poorly characterized. Global profiling can be used to study metabolite patterns in response to a disease, drug administration, environmental effects or lifestyle factors, elucidating the new roles of some metabolites. This second

metabolomic approach provides qualitative/quantitative data and is considered to be a hypothesis-generating approach, whereas the first one is regarded as a hypothesis-driven method. The third approach is metabolomic fingerprinting analysis which provides snapshots representing the metabolic state of a particular group of samples for development of classification models. The quality level of the information provided by fingerprinting methods is limited since the main purpose of them is to find discrimination patterns associated to each class.

Normally, analytical methods dealing with nutrimetabolomic studies required the following steps: sample collection, sample preparation, data acquisition, data analysis, and biological interpretation (Supplementary Figure 1). In addition, it is necessary to highlight the importance of applying a quality control pipeline in metabolomic studies. For this purpose a single or several analytical technologies can be used. Among them, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most commonly used in metabolomic analysis. Regarding to data analysis and biological interpretation different tools have been described for their handling.

The scheme followed in nontargeted nutrimetabolomics studies entails: selection of an adequate food material for diet intervention as well as the appropriate population to participate in the study. To obtain statistically representative results, sample size and even criteria for both the action and control groups have to be predefined. Aspects to be taken into account are: (i) analysis of biofluid(s) after drug or diet intake, which involves the group of patients and also untreated individuals (control group) under the same working conditions; (ii) supervised or non-supervised

statistical analysis to find diet-related differences between groups; (iii) determination of analytical features responsible of these differences; for which establishment of an appropriate sample collection time after intervention is crucial. A successful study results in the proposal of biomarkers of the given differences. This scheme is part of the discovery phase aimed at identifying biomarkers. A number between 10 and 100 samples controlled by confounding factors are required in this stage. Once the putative biomarkers are identified, the process of validation in a general population is necessary. The aim of the second phase is to evaluate the specificity and selectivity of the biomarkers using an absolute quantification by a targeted approach applied to a range of 100–1000 samples.



Supplementary Figure 1*. Metabolomics workflow*

Focused on nutrimetabolomics, the aim of the present research was to determine metabolic changes in the postprandial lipemia period associated to four different intervention diets which were administered for 12 weeks under controlled conditions. Diets were differentiated by the content and quality of fat. Plasma from individuals was sampled before the intervention diets and during a postprandial lipemia period (0, 4 and 8 h) after the 12 weeks intervention period. Plasma was fractionated according to polarity for independent analysis of the resulting fractions (aqueous/methanol and chloroform phases) by LC–TOF/MS. The cohort selected for this study was composed by metabolic syndrome patients to find differences correlated with the diet supplied.

2. Experimental

2.1. Chemicals

LC–MS grade acetonitrile (ACN) and formic acid from Scharlab (Barcelona, Spain), and deionized water (18 MΩ·cm) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) were used to prepare the chromatographic phases. LC grade methanol and chloroform from Scharlab were used for sample preparation.

2.2. Instruments and apparatus

Centrifugation was carried out by a thermostated centrifuge Thermo Sorvall Legend Micro 21 R from Thermo (Thermo Fisher Scientific, Bremen, Germany). A Vacufuge centrifugal vacuum concentrator from

Eppendorf (Eppendorf, Inc., Hamburg, Germany) was used to evaporate samples to dryness.

An 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) was used for separation of the analytes in all samples. It was coupled to an Agilent 6530 TOF high-resolution mass spectrometer equipped with a dual electrospray (ESI) source for continuous infusion of the chromatographic elution and reference compounds for mass calibration. MassHunter Workstation software (Agilent Technologies), including Data acquisition, Qualitative Analysis and Mass Profiler Professional, used for processing raw MS data, including feature extraction, data filtering, statistical analysis by ANOVA and PCA, followed by the construction of PLS prediction models, molecular formula and database searching. Compound identification was performed using the METLIN Personal Metabolite Database throughout the IDbrowser tool and the Molecular Formula Generation algorithm (Agilent Technologies). The Human Metabolome Database (HMDB) was used to confirm and extend identification.

2.3. Meals composition

Four isoenergetic diets that differed in fat quantity and quality were planned for this study. Diets were designed to provide 38 and 28% energy (E) from fat with the following asigned names and compositions:

(1) HSFA: high-fat content (38% energy) with high proportion of saturated fatty acids (16% SFA) while monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs, respectively) were present at 12% and 6%, respectively.

(2) HMUFA: high-fat content (38% energy) with high proportion of MUFAs (20%), while SFA and PUFAs were 8% and 6%, respectively.
(3) LFHCC: low-fat content (28% energy) with high-complex carbohydrate diet (8% SFA, 11% MUFA, 6% PUFA), supplemented with 1 g/d high oleic acid sunflower oil supplement.
(4) LFHCC (n-3): low-fat (28% energy), high-complex carbohydrate diet (8% SFA, 11% MUFA, 6% PUFA), supplemented with 1.24 g/d very long chain *n-3* PUFA supplement.

2.4. Subjects and samples

This research was conducted within the framework of the LIPGENE integrated project (Diet, genomics and metabolic syndrome (MetS): an integrated nutrition, agro-food, social and economic analysis). A total of 76 patients with MetS from the LIPGENE cohort were recruited to participate in this postprandial study. All participants gave written informed consent and underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrolment. Patients were randomly split into four groups in order to receive one of four intervention diets for 12 weeks. Blood samples were taken before the 12 weeks intervention study as controls (PRE samples).

Each intervention center (located in eight European countries) performed a postintervention (wk 12) postprandial challenge with the same fat composition as that consumed on the assigned dietary period. Patients arrived at clinical centers at 800 h following a 12-h fast refrained from

smoking during the fasting period and abstained from alcohol intake during the preceding 7 d. In the laboratory and after cannulation a fasting blood sample was taken before the test meal, which was then ingested within 20 min under supervision. The test meal, which was prepared in each center, reflected fatty acid composition of each subject chronic dietary intervention. Subsequent blood samples were drawn at 0, 4 and 8 h after the meal (POS samples). The intervention study design and intervention protocol, which also provided information about pre-, mid-, and post-intervention food consumption and dietary compliance at each center, have been described in detail by Shaw et al. [1].

Venous blood samples were collected using tubes containing EDTA to give a final concentration of 0.1% EDTA and stored in containers with ice in the dark. Plasma was separated by centrifugation at 1500 × g for 15 min at 4ºC. Special care was taken to avoid exposure to air, light and ambient temperature. The plasma samples were stored at–80 °C until analysis.

2.5. Sample treatment

Plasma samples (100 μL) immersed in a bath ice were treated with 300 μL of 1:2 methanol–chloroform. The mixture was shaken for 2 min and stabilized for 3 min. The mixture was centrifuged for 5 min at 4 ºC and 13800 × g. Both phases (aqueous and organic) were collected in different vials, then placed in the LC autosampler for subsequent analysis under different conditions.

2.6. LC–TOF/MS analysis

Chromatographic separation was performed using a Teknokroma Mediterranean Sea C18 analytical column (100 mm × 0.46 mm i.d., 3 μm particle size, from Barcelona, Spain) kept at 25 ºC. The mobile phases consisted of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in ACN. The elution program was as follows: 0–2 min, 3% B; 2–30 min, 100% B. A post-run of 5 min was included to equilibrate the column. The flow rate was maintained at 0.8 mL/min. The volume of injected sample was 10 μL and the injector needle was washed 10 times with 70% methanol between injections. Therefore, the needle seat back was flushed for 15 s at a flow rate of 4 mL/min with 70% methanol to avoid cross contamination. Both the methanol and chloroform fractions were analyzed in negative and positive ionization modes. The operating conditions in negative and positive ionization modes were as follows: gas temperature, 325 °C; drying gas, nitrogen at 8 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, nitrogen at 11 L/min; capillary voltage, 4000 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 spectra s^{-1}; mass range 60–1100 *m/z*) was governed via the Agilent Mass Hunter Workstation software. The instrument gave typical resolution 15000 FWHM (Full Width at Half Maximum) at *m/z* 112.9856 and 30000 FWHM at *m/z* 1033.9881. To assure the desired mass accuracy of recorded ions continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3Htetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. In negative ion mode, ions with *m/z* 119.0362 (proton abstracted purine) and

m/z 1033.988109 adduct of HP-921 were used. The instrument was calibrated and tuned according to procedures recommended by the manufacturer.

2.7. Data processing and statistical analysis

MassHunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing all data obtained by LC–TOF/MS in full scan MS mode. Treatment of raw data file was initiated by extraction of potential molecular features (MFs), which was based on the extraction algorithm (MFE) that locates and groups all ions related to the same neutral molecule. This relationship is referred as to the covariance of peaks within the same chromatographic retention time, the charge-state envelope, isotopic distribution, and/or the presence of adducts and dimmers. The MFE took into account all ions exceeding 5000 counts, with charge state limited to a maximum of two and a peak spacing tolerance of 0.0025 *m/z* (plus 50 ppm). Each feature was given by a minimum of two ions. The extraction algorithm was based on a common organic model with chromatographic separation. The allowed positive ions were protonated species and sodium adducts (+H, +Na, +K), and the negative ions formed by formate adducts and proton losses (–H, +Cl, +HCOOH). The neutral losses by dehydration and the lost of phosphate or a methyl group were also included to identify features corresponding to the same molecule. Therefore, some ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by retention time (RT), intensity in the apex of

chromatographic peaks and accurate mass. Background contribution was removed by subtraction of MFs linked to plasticizers, solvent impurities, and other contaminants after analysis of blank sample (methanol) under identical instrument operation conditions. Hence, data files were created in compound exchange format (*.cef* files) for each sample and exported into the Mass Profiler Profesional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing.

Once the *.cef* files were exported into MPP, the data were preprocessed by alignment of retention times and *m/z* values across the data matrix using a tolerance window of 1.0 min and 30 ppm mass accuracy. An export for recursion file was obtained in this step and used for the data re-alignment by using in this case a tolerance window of 2.0 min and 30 ppm mass accuracy. Data pretreatment was based on baselining to remove background noise and normalization by logarithmic transformation to reduce relatively large differences among the respective MF abundances. Stepwise reduction of MFs number was performed based on frequency occurrence, abundance of the respective MFs in classes and results of one-way analysis of variance (ANOVA). MPP software also allowed unsupervised (principal components analysis —PCA) and supervised analysis (partial least squares–discriminant analysis —PLS–DA) of the data, which were used for sample classification and pattern recognition. The former is a non-supervised approach that allows exploring data groupings or patterns among samples. Thus, in PCA, the data are transformed to a K-dimensional space (where K is equal to the number of variables) and subsequently projected into a few principal components that describe the maximum variation within the data. In the case of PCA, data

scaling by mean centering was used as pretreatment, while auto-scaling was selected in the case of PLS–DA. The validation model selected was N-Fold, by which the classes in the input data are randomly divided into N equal times; N-1 parts are used for training, and the remaining one part is used for testing. The process repeats N times, with a different part being used for testing in each iteration. Then, repetitions and a fold number of 3 were selected for all validations.

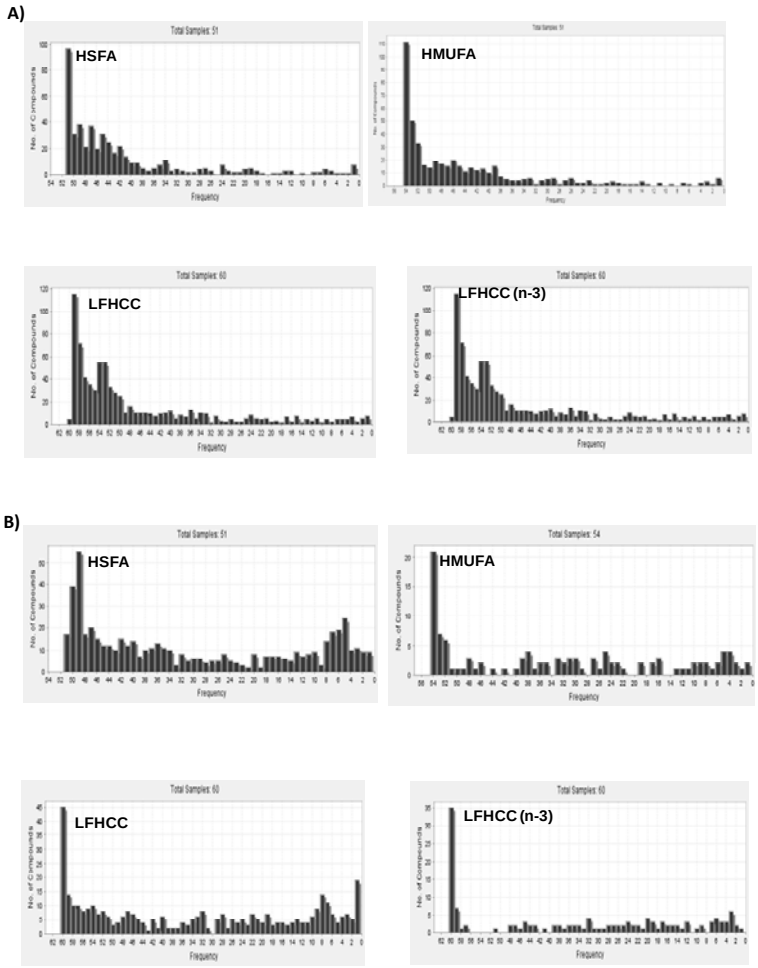
3. Results and discussion

A global approach based on non-targeted analysis of samples was selected to characterize plasma metabolome from MetS patients before the preintervention study and after the intervention study during the postprandial lipemia period. Samples were named PRE for those samples taken before the intervention study and POS for those sampled during the postprandial lipemia period. The different sampling times were differentiated as follows: POS0, POS4 and POS8 for plasma obtained at 0, 4 and 8 h . This period has been previously defined to find metabolism patterns in human plasma after intake of the different diets [2].

3.1. Data preprocessing

Data sets were prepared with MFs extracted from LC–TOF/MS analysis for each diet and ionization mode: (4 postintervention states + 1 preintervention state) × 2 ionization modes = 10 data sets. Each data set was aligned using the protocol described in section 2.7 to obtain frequency histogram plots representing the number of MFs detected in a particular number of samples. Supplementary Figure 2 shows the frequency

histograms for each diet in both ionization modes, which represent the product of the number of MFs detected and the number of samples for each diet: HSFA, HMUFA, LFHCC and LFHCC (n-3). Most of the MFs contained in each data set was present in a high proportion of samples. Thus, frequency plots allowed estimating the number of MFs detected in a representative number of samples (75%). Nevertheless, there is a high contribution of entities which were detected in a low number of samples and, therefore, could introduce in the data sets sources of variability associated to reduced number of individuals. For this reason, the next objective was to eliminate these less representative MFs to avoid redundant information in the development of statistical models. A frequency filter was applied to each data set in order to keep all those MFs defining a high proportion of samples pertaining to each class. A cut-off value of 75% of the number of samples was set in this frequency filter. As shows Table 1, the data set corresponding to the preintervention state was initially formed by 2976 MFs in negative ionization mode after alignment, whereas the sets corresponding to HSFA, HMFA, LFHCC and LFHCC (n-3) intervention diets contained 2354, 2976, 3428 and 3265 MFs, respectively. The application of the frequency filter enabled to reduce the number of features up to 641 for the preintervention state and up to 441,406, 378 and 442 for the four classes corresponding to postintervention states. Therefore, there was a relevant simplification of the data set with elimination of MFs present in reduced number of individuals. Table 1 also shows similar results for the positive ionization mode.



Supplementary Figure 2. Frequency diagrams obtained in post-state samples using negative ionization-mode (A), and positive ionization-mode (B).

3.2. Metabolomic discrimination of individuals after the intake of intervention diets with different quantity and quality of dietary fat

Diet metabolic effects at different biological levels have been proved to persist during postprandial lipemia. In the nutrimetabolomics

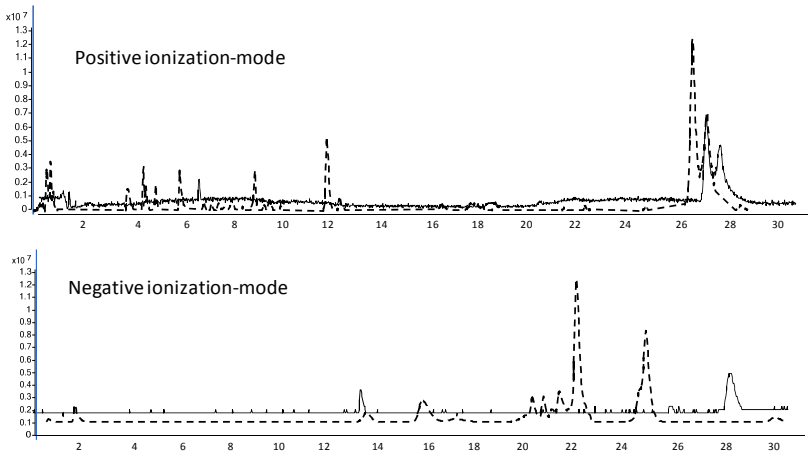
context, the base peak chromatograms (BPCs) reported by LC–TOF/MS analysis of plasma from individuals in the preintervention and postintervention states (PRE and POS, respectively) revealed significant changes which may be associated with each intervention diet and with the postprandial lipemia according to each diet. Supplementary Figure 3 illustrates BPCs in both ionization modes for one individual subjected to HSFA diet. The main differences were observed between the BPCs corresponding to the PRE state and those provided by POS states, while subtle visual differences were observed among BPCs obtained for each POS state.

Table 1. Number of MFs extracted by applying filters by frequency and fold change.

Ionization mode	**Intervention study (diet)**	**Number of MFs across the sample set**				
		Initial[a]		Filter by frequency[b]		Filter by fold change[c]
		Pre	Pos	Pre	Pos	
ESI(-)		2976		641		
	1		2354		441	518
	2		2976		406	514
	3		3428		378	671
	4		3265		442	746
ESI(+)		594		63		
	1		2156		263	580
	2		536		47	157
	3		1478		156	353
	4		596		54	39

An extensive study was planned by statistical analysis using PCA and PLS-DA to evaluate the main variability source for each intervention diet. The first study dealt with the comparison of plasma metabolome

profiles of individuals after intake of each intervention diet with those obtained before the intervention diets. Figure 1 shows the scores plots obtained in PCA in both ionization modes for each predefined intervention diet. As can be seen, PCA plots revealed a clear discrimination of the PRE state *versus* the different POS states for all diets in both ionization modes except for that corresponding to the analysis of LFHCC (n-3) diet in positive ionization mode. This diet only reported discrimination between PRE and POS states in negative ionization mode. For the other three diets, discrimination was observed both for positive and negative ionization modes, which fit with the analysis of methanolic and chloroform fractions, respectively. Apart from that, it is worth mentioning that no dicrimination was detected among the different POS states. Therefore, unsupervised analysis by PCA allowed discriminating the main cause of variability in those data sets, which was supported on metabolic differences ascribed to the 12 weeks intervention diets.



Supplementary Figure 3. *BPCs in both ionization modes for one individual subjected to HSFA diet, positive ionization mode (A) and, negative ionization mode (B).*

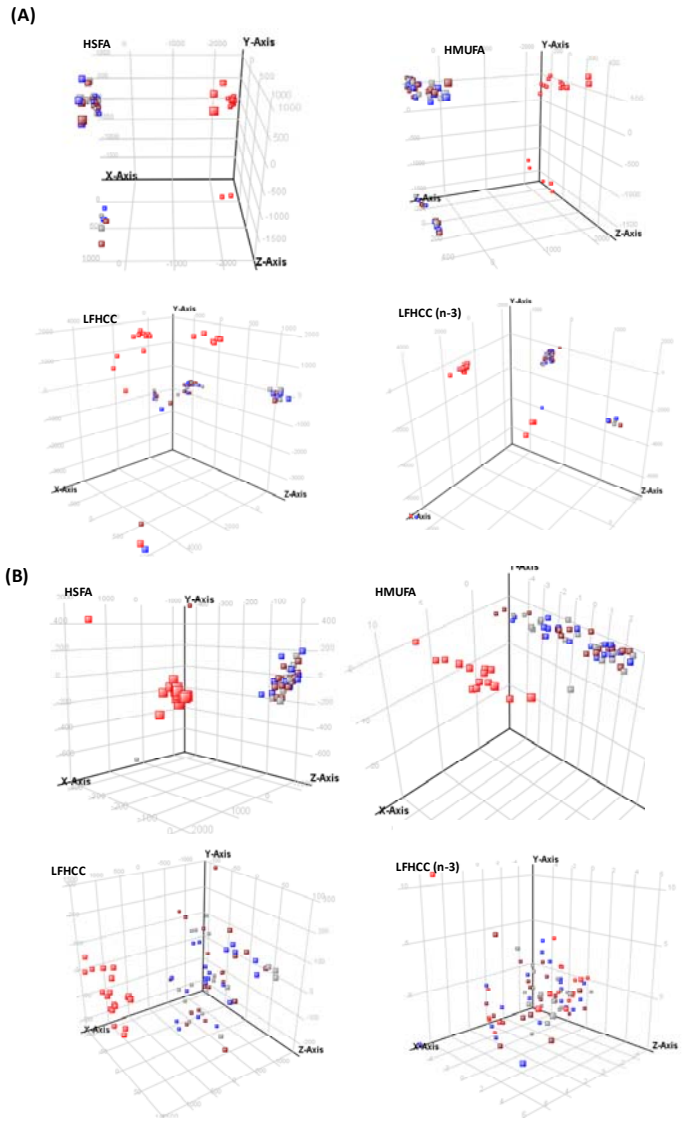


Figure 1. *PCAs after application of each intervention diet (red color represents the sampling in preintervention state, blue, brown and gray sampling times t1, t4 and t8, respectively). (A) negative and (B) positive ionization modes*

After this preliminary evaluation, supervised analysis with PLS-DA was carried out separately for each diet at both ionization modes. The PLS

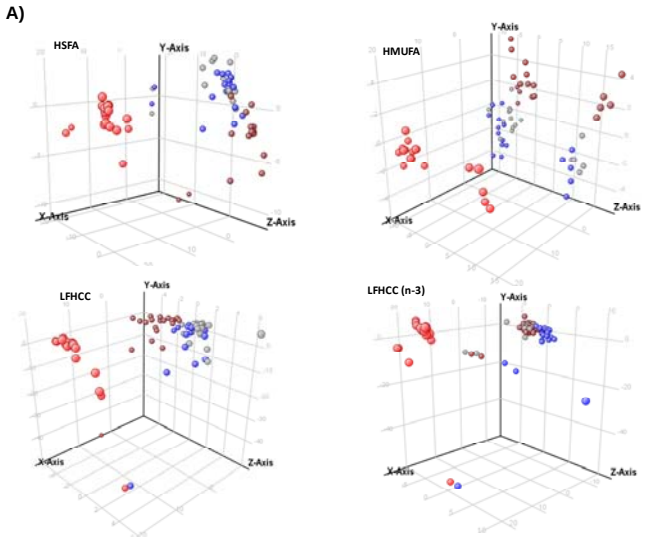
scores plots allowed specific patterns to be visualized. Partial discrimination was observed for certain situations such as the analysis of the chloroform phase (negative ionization mode) for HMUFA diet, which showed a partial discrimination among POS states. No clear discrimination among the three POS states was observed for the resting diets, which could be ascribed to the high variability provided by the 12 weeks intervention study (POS states *versus* the PRE state).

3.3. Metabolomics discrimination of different postprandial lipemia states for each intervention diet

Taken into account the results found in the first multivariate analysis, the variability associated to intervention diets masked the variability due to the postprandial lipemia time-course. For this reason, new PLS-DA studies were developed with samples corresponding to postprandial lipemia states (POS0, POS4 and POS8). Figure 2 shows the PLS-DA plots for each intervention diet revealing discrimination among POS states, if present. In the analysis of the methanolic fraction (Figure 2.A) incomplete discrimination was observed for LFHCC (n-3) diet while complete separation was observed between POS0 and POS4/POS8 states for HSFA and LFHCC diets. This result would be indicative of noticeable metabolic changes on the course of the postprandial lipemia for both diets. On the other hand, the HMUFA diet led to almost complete discrimination among the three POS states.

The chloroform phase (negative ionization mode) also provided a behavior depending on the diets. Thus, HSFA and LFHCC (n-3) diets

allowed discriminating between POS0 and POS4/POS8, demonstrating a postprandial lipemia effect, while HMUFA and LFHCC diets led to almost complete discrimination among the three POS states. With these premises, metabolic changes occurring during postprandial lipemia for each intervention diet were patent. In the case of HSFA intervention diet no discrimination was attained between postintervention states POS4 y POS8. Complete differentiation among the three postprandial states was observed for HMUFA in both analyzed fractions, which would define metabolic changes along the postprandial lipemia period. Concerning the two low-fat diets, the LFHCC (n-3) only enabled to differentiate POS4 and POS8 from POS0 for the lipidic fraction, while LFHCC led to the same separation in the methanolic fraction while the lipidic profile pointed out significant alterations during the postprandial lipemia period.



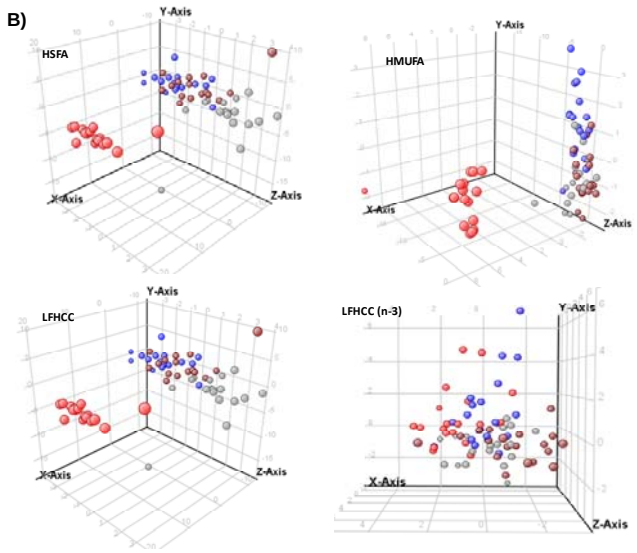


Figure 2*. PLSs after application of each intervention diet (red color represents the -sampling time t0-posintervention state, blue, brown and gray sampling times t1, t4 and t8, respectively). (A) negative and (B) positive ionization modes.*

3.4. Qualitative comparison of metabolite profiles obtained during postprandial lipemia for each intervention diet

Metabolic profiles associated to different sampling times of the postprandial lipemia period were compared at qualitative level for each intervention diet. For this purpose, Venn diagrams were represented for each diet at both ionization modes in order to set differences for each plasma fraction: non-lipidic and lipidic phases. The analyses corresponding to the three postintervention states (POS0, POS4 and POS8) were included in the Venn diagrams. Figure 3.A and B shows the Venn diagrams for the four diets distinguishing between positive and negative ionization modes, respectively. It is worth mentioning that this type of comparison is

exclusively qualitative and it is referred to those alterations caused by MFs which are only detected in postintervention states POS4 and POS8, but also to potential metabolites only detected in POS0 state. As can be seen, most molecular entities were detected in the three postintervention states. However, the total number of entities in both ionization modes depended on the diet. Concerning the aqueous phase (Figure 3.A), the highest number of entities discriminating POS4 and POS8 states from POS0 were obtained in HSFA and LFHCC diets, which were 45 and 32, respectively. On the other hand, this number was quite limited in HMUFA and LFHCC (n-3) diets, which reported 7 and 9 entities, respectively. Therefore, this comparison focused on the methanolic fraction (polar fraction) enabled to support higher metabolic alterations at qualitative level during postprandial lipemia in individuals subjected to HSFA and LFHCC diets. On the other hand, qualitative metabolic changes affecting to this fraction of metabolites were limited for HMUFA and LFHCC (n-3) diets.

The Venn diagrams for the lipid phase (Figure 3.B) revealed a more similar behaviour for all diets since relevant numbers of MFs were exclusively detected in the analysis of POS4/POS8 states or in POS0 state. This value representing metabolic changes ranged from 19 in HSFA diet to 79 in LFHCC diet (50 and 42 entities for HMUFA and LFHCC (n-3) diets, respectively). Therefore, the lipid fraction enabled to identify lower qualitative alterations in HSFA diet than in the resting diets. Attending to this study, it seems that metabolic alterations were more sensitive by analysis of the lipidic fraction, which could be logical taking into account that diets are isoenergetic but they are differentiated by the fat content and quality.

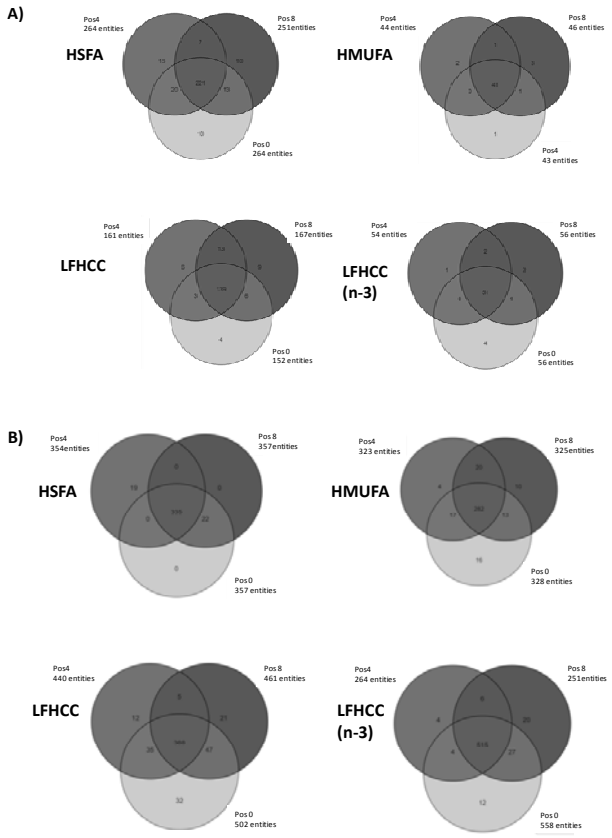


Figure 3. *.Venn diagrams for the intervention diets after intake. Negative ionization-mode (A) and positive ionization-mode (B).*

3.5. Development of PLS-DA prediction models for discrimination among postintervention states during postprandial lipemia

The last statistical analysis was focused on the development of prediction models based on supervised PLS-DA analysis for discrimination of postintervention states examined along the postprandial lipemia study. Thus, six PLS-DA models were developed for each diet at both ionization

modes (three models per ionization mode) intended to classify samples as follows: POS0 *versus* POS4, POS0 *versus* POS8 and POS4 *versus* POS8. Crossvalidation was used as validation approach for each model due to the reduced number of samples for the different diets (19 samples × 2 states = 38 samples). Table 2 and 3 shows the values of prediction capability obtained for all diets in positive and negative ionization modes, respectively. As can be seen, the prediction capability values were excellent for the training step but the validation step revealed fails in these statistical models. Concerning the methanolic phase (Table 2) analyzed in positive ionization mode, prediction of POS0 *versus* POS4 or POS8 ranged from 94.1 to 100% for all diets except for LFHCC (n-3) diet, which reported discrimination efficiency below 65%. Therefore, this study confirms previous results obtained with other statistical tests that described low metabolic changes in the plasma methanol fraction from individuals controlled with the LFHCC (n-3) diet.

The lipid phase provided similar results (Table 3) but POS4 and POS8 states were well predicted for the four diets. Thus, the prediction values were always above 95%, which involves a high capability to recognize the diet followed by individuals forming the studied cohort. If both plasma fractions are compared, the lipid phase possesses higher discrimination potential than methanol phase to classify individuals according to the diet or predict the intervention diet followed by them.

The discrimination between postintervention states POS4 and POS8 was not possible in both plasma fractions. Prediction capability values ranged from 38 to 60% for the methanol phase, while in the chloroform phase they were from 36.1 to 52.9%.

Table 2. Confusion matrix corresponding to each PLS–DA for the intervention diets to create classification and prediction models (negative ionization mode)

			Validation			**Training**		
			Predicted		Accuracy	Predicted		Accuracy
		Diet	(t0)	(t4)	(%)	(t0)	(t4)	(%)
t0 vs t4	true (t0)	HSFA	17	0	100	17	0	100
		HMUFA	18	0	100	18	0	100
		LFHCC	19	1	95.00	20	0	100
		LFHCC (n-3)	20	0	100	20	0	100
	true (t4)	HSFA	0	17	100	0	17	100
		HMUFA	0	18	100	0	18	100
		LFHCC	1	19	95.00	0	20	100
		LFHCC (n-3)	0	20	100	1	19	95.00
	overall Accuracy	HSFA			100			100
		HMUFA			100			100
		LFHCC			95			100
		LFHCC (n-3)			100			97.50
			Predicted		Accuracy	Predicted		Accuracy
			(t4)	(t8)	(%)	(t4)	(t8)	(%)
t4 vs t8	true (t4)	HSFA	8	9	47.05	15	2	88.23
		HMUFA	3	15	16.67	14	4	77.78
		LFHCC	6	14	30.00	20	0	100
		LFHCC (n-3)	5	15	25.00	18	2	90.00
	true (t8)	HSFA	7	10	58.82	2	15	88.23
		HMUFA	8	10	55.56	4	14	77.78
		LFHCC	5	15	75.00	1	19	95.00
		LFHCC (n-3)	8	12	60.00	2	18	90.00
	overall Accuracy	HSFA			52.94			88.23
		HMUFA			36.11			77.78
		LFHCC			52.50			97.50
		LFHCC (n-3)			42.50			90.00
			Predicted		Accuracy	Predicted		Accuracy
			(t0)	(t8)	(%)	(t0)	(t8)	(%)
t0 vs t8	true (t0)	HSFA	17	0	100	17	0	100
		HMUFA	18	0	100	18	0	100
		LFHCC	20	0	100	20	0	100
		LFHCC (n-3)	20	0	100	20	0	100
	true (t8)	HSFA	0	17	100	0	17	100
		HMUFA	0	18	100	0	18	100
		LFHCC	0	20	100	0	20	100
		LFHCC (n-3)	0	20	100	0	20	100
	overall Accuracy	HSFA			100			100
		HMUFA			100			100
		LFHCC			100			100
		LFHCC (n-3)			100			100

Table 3. Confusion matrix corresponding to each PLS–DA (shown in Figure 1) for the intervention diets to create classification and prediction models (positive ionization mode)

				Validation			**Training**		
				Predicted		Accuracy	Predicted		Accuracy
			Diet	(t0)	(t4)	(%)	(t0)	(t4)	(%)
t0 vs t4		true (t0)	HSFA	17	0	100	17	0	100
			HMUFA	16	1	94.12	17	0	100
			LFHCC	20	0	100	20	0	100
			LFHCC (n-3)	11	9	55.00	19	1	100
		true (t4)	HSFA	0	16	100	0	16	100
			HMUFA	0	18	100	0	17	100
			LFHCC	0	20	100	0	20	100
			LFHCC (n-3)	7	13	65.00	0	20	100
		overall Accuracy	HSFA			100			100
			HMUFA			97.14			100
			LFHCC			100			100
			LFHCC (n-3)			65.00			97.50
				Predicted		Accuracy (%)	Predicted		Accuracy (%)
				(t4)	(t8)		(t4)	(t8)	
t4 vs t8		true (t4)	HSFA	7	10	41.18	17	0	100
			HMUFA	10	8	55.56	17	1	94.44
			LFHCC	6	14	30.00	20	0	100
			LFHCC (n-3)	11	9	55.00	19	1	95.0
		true (t8)	HSFA	11	6	38.23	0	17	100
			HMUFA	7	11	58.33	0	18	100
			LFHCC	10	10	40.00	0	20	100
			LFHCC (n-3)	7	13	60.00	1	19	95.00
		overall Accuracy	HSFA			38.23			100
			HMUFA			58.33			100
			LFHCC			40.00			95.00
			LFHCC (n-3)			60.00			95.00
				Predicted		Accuracy	Predicted		Accuracy
				(t0)	(t8)	(%)	(t0)	(t8)	(%)
t0 vs t8		true (t0)	HSFA	17	0	100	17	0	100
			HMUFA	16	1	94.11	16	1	94.11
			LFHCC	20	0	100	20	0	100
			LFHCC (n-3)	11	9	55.00	19	1	95.00
		true (t8)	HSFA	0	17	100	0	17	100
			HMUFA	0	18	100	0	28	100
			LFHCC	0	20	100	0	20	100
			LFHCC (n-3)	7	13	65.00	0	20	100
		overall Accuracy	HSFA			100			100
			HMUFA			97.14			97.12
			LFHCC			100			100
			LFHCC (n-3)			60.00			97.50

3.6. Tentative identification of metabolic changes occurring during the postprandial period associated to the four intervention diets

Once the PLS models were explored those MFs that contributed to discrimination of individuals during postprandial lipemia of each intervention diet (POS0 *versus* POS4/8) were filtered. For this purpose, the significance of each molecular entity present in the data sets was estimated according to a Volcano test. The algorithm supporting this test was used to compare two metabolic conditions and detect the most relevant changes. A Volcano test combines an ANOVA test (by setting a *p*-value as significance cut-off) and a fold change analysis to detect those features (tentative metabolites) that display large-magnitude changes in relative concentration from a statistical point of view. The statistical requirements were: significant change between metabolic states at 99% of confidence level and with a relative concentration change of at least 50% (fold change ratio > ±2.0). Hence, the Volcano test enabled to detect a range from XXX to YYY molecular entities with a significant change for the four diets in the negative ionization mode (Table 1). In the positive ionization mode, a range from CCC to BBB molecular entities was obtained.

After this significant evaluation, a final identification step was carried out for those MFs reported by the Volcano test. Identification was supported on automated searching of *m/z* experimental values in the HMDB and METLIN databases. The main adducts considered in the negative ionization mode were $[M–H]^-$, $[M+Cl]^-$ and $[M+HCOOH–H]^-$, while $[M+H]^+$, $[M–H_2O+H]^+$, $[M+Na]^+$ and $[M+K]^+$ were taken into account for the positive ionization mode. Double and triple charge states were also

considered for $[M–H]^-$ and $[M+H]^+$ adducts. Table 4 lists those metabolites which were tentatively identified in this study.

Tentative identification of metabolites present in the methanol phase was mainly focused on glycerophospholipids and, particularly, glycerophosphocholines. In the case of HSFA glycerophosphatidylinositol phosphate, glycerophosphatidylinositol biphosphate and phosphatidylglycerol metabolites were also contributed to explain the variability observed during postprandial lipemia. The presence of these phospholipids varieties in the methanol fraction could be attributed to the fact that they are ionized more easily in positive ionization mode. Apart from phospholipids other metabolites such as ganglioside, diglycerides and bile acid metabolites were also detected significant during the postprandial lipemia of individuals subjected to HSFA, LFHCC and LFHCC (n-3) diets.

In the negative ionization mode, tentative identification provided marked differences between the four groups of individuals. Thus, individuals subjected to HSFA diets were preferentially discriminated by triglycerides and an important phospholipid such lysoPE(18:0). The latter also contributed to differentiate the postprandial lipemia states of individuals subjected to HMUFA together with glycerophosphatidyl inositol metabolites. For low fat diets, lysoPE(18:1) represented the family of lysophospholipids instead of the saturated form. Apart from that, three metabolites such as adenine, α-carotene and neuraminic acid were significantly detected during postprandial lipemia of individuals subjected to LFHCC (n-3) diet. Finally, a wide range of metabolites changed relatively their concentration in the postprandial period for the LFHCC diet. They included gangliosides, diglycerides and triglycerides as well as oxidized metabolites of fatty acids such as eicosanoids. In fact, eicosanoids

were only detected as significant metabolites in individuals controlled by this diet.

Table 2. Accurate mass error, identification code and ion used to identify tentative compounds present in the different diets. Methanolic fraction

Compound	Name	Adduct	Adduct MW (Da)	Compound MW (Da)	Delta	ppm
HMDB00328	12-Ketodeoxycholic acid	M+K	429.240168	390.27701	0.000468	1.09029871
HMDB00467	Nutriacholic acid	M+K	429.240168	390.27701	0.000468	1.09029871
HMDB03180	Cortol	M+H+K	204.113354	368.256274	0.000374	1.83231519
HMDB00374	17-Hydroxyprogesterone	M+H+K	185.094964	330.219495	0.000906	4.89478471
HMDB08039	PC(18:0/18:2(9Z,12Z))	M+H	786.600731	785.593455	0.004969	6.3170549
HMDB07920	PC(14:1(9Z)/22:1(13Z))	M+H	786.600731	785.593455	0.004969	6.3170549
HMDB08071	PC(18:1(11Z)/18:1(9Z))	M+H	786.600731	785.593455	0.004969	6.3170549
HMDB08559	PC(22:1(13Z)/14:1(9Z))	M+H	786.600731	785.593455	0.004969	6.3170549
HMDB08135	PC(18:2(9Z,12Z)/18:0)	M+H	786.600731	785.593455	0.004969	6.3170549
HMDB07888	PC(14:0/22:2(13Z,16Z))	M+H	786.600731	785.593455	0.004969	6.3170549
HMDB08039	PC(18:0/18:2(9Z,12Z))	M+H	786.600731	785.593455	0.000931	1.18357378
HMDB07920	PC(14:1(9Z)/22:1(13Z))	M+H	786.600731	785.593455	0.000931	1.18357378

HMDB08071	PC(18:1(11Z)/18:1(9Z))	M+H	786.600731	785.593455	0.000931	1.18357378
HMDB08559	PC(22:1(13Z)/14:1(9Z))	M+H	786.600731	785.593455	0.000931	1.18357378
HMDB08135	PC(18:2(9Z,12Z)/18:0)	M+H	786.600731	785.593455	0.000931	1.18357378
HMDB07888	PC(14:0/22:2(13Z,16Z))	M+H	786.600731	785.593455	0.000931	1.18357378
HMDB08623	PC(22:4(7Z,10Z,13Z,16Z)/14:0)	M+Na	804.551373	781.562155	0.001473	1.83083399
HMDB08169	PC(18:3(6Z,9Z,12Z)/18:1(11Z))	M+Na	804.551373	781.562155	0.001473	1.83083399
HMDB08202	PC(18:3(9Z,12Z,15Z)/18:1(11Z))	M+Na	804.551373	781.562155	0.001473	1.83083399
HMDB08107	PC(18:1(9Z)/18:3(9Z,12Z,15Z))	M+Na	804.551373	781.562155	0.001473	1.83083399
HMDB08074	PC(18:1(11Z)/18:3(9Z,12Z,15Z))	M+Na	804.551373	781.562155	0.001473	1.83083399
HMDB08138	PC(18:2(9Z,12Z)/18:2(9Z,12Z))	M+Na	804.551373	781.562155	0.001473	1.83083399
HMDB08214	PC(18:3(9Z,12Z,15Z)/20:4(8Z,11Z,14Z,17Z))	M+H	804.553781	803.546505	0.003881	4.82379189
HMDB08468	PC(20:4(8Z,11Z,14Z,17Z)/18:3(6Z,9Z,12Z))	M+H	804.553781	803.546505	0.003881	4.82379189
HMDB08023	PC(16:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	M+H	804.553781	803.546505	0.003881	4.82379189
HMDB08039	PC(18:0/18:2(9Z,12Z))	M+H	786.600731	785.593455	0.000391	0.49707556
HMDB07920	PC(14:1(9Z)/22:1(13Z))	M+H	786.600731	785.593455	0.000391	0.49707556
HMDB08071	PC(18:1(11Z)/18:1(9Z))	M+H	786.600731	785.593455	0.000391	0.49707556
HMDB08559	PC(22:1(13Z)/14:1(9Z))	M+H	786.600731	785.593455	0.000391	0.49707556
HMDB08135	PC(18:2(9Z,12Z)/18:0)	M+H	786.600731	785.593455	0.000391	0.49707556
HMDB07888	PC(14:0/22:2(13Z,16Z))	M+H	786.600731	785.593455	0.000391	0.49707556
HMDB08039	PC(18:0/18:2(9Z,12Z))	M+H	786.600731	785.593455	0.000131	0.16653938
HMDB07920	PC(14:1(9Z)/22:1(13Z))	M+H	786.600731	785.593455	0.000131	0.16653938
HMDB08	PC(18:1(11Z)/18:1(9Z))	M+	786.600	785.593	0.0001	0.16653

071		H	731	455	31	938
HMDB08559	PC(22:1(13Z)/14:1(9Z))	M+H	786.600731	785.593455	0.0001 31	0.16653938

HMDB08135	PC(18:2(9Z,12Z)/18:0)	M+H	786.600731	785.593455	0.000131	0.16653938
HMDB07888	PC(14:0/22:2(13Z,16Z))	M+H	786.600731	785.593455	0.000131	0.16653938
HMDB00830	Neuraminic acid	M-H	266.088141	267.095417	0.001359	5.10733021
HMDB00034	Adenine	M-H	134.047219	135.054495	0.000431	3.21528491
HMDB03993	Alpha-Carotene	M-2H	267.211825	536.438202	0.002345	8.77580923
HMDB11475	LysoPE(18:1(9Z))	M-H	478.293913	479.301189	0.003907	8.16861744
HMDB07045	DG(14:1(9Z)/18:2(9Z,12Z)/0:0)	M+FA-H	607.457926	562.459725	0.005474	9.01132369
HMDB09825	PI(18:1(11Z)/18:1(9Z))	M+FA-H	907.55533	862.557129	0.00727	8.01053088
HMDB09847	PI(18:2(9Z,12Z)/18:0)	M+FA-H	907.55533	862.557129	0.00727	8.01053088
HMDB07540	DG(20:4(8Z,11Z,14Z,17Z)/18:3(9Z,12Z,15Z)/0:0)	M+NH4	656.524848	638.491025	0.006152	9.37055165
HMDB07286	DG(18:3(6Z,9Z,12Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	M+NH4	656.524848	638.491025	0.006152	9.37055165
HMDB29095	Tryptophyl-Tyrosine	M+H	368.160482	367.153206	0.000112	0.30421516
HMDB08623	PC(22:4(7Z,10Z,13Z,16Z)/14:0)	M+Na	804.551373	781.562155	0.004173	5.18674151
HMDB08169	PC(18:3(6Z,9Z,12Z)/18:1(11Z))	M+Na	804.551373	781.562155	0.004173	5.18674151
HMDB08202	PC(18:3(9Z,12Z,15Z)/18:1(11Z))	M+Na	804.551373	781.562155	0.004173	5.18674151
HMDB08107	PC(18:1(9Z)/18:3(9Z,12Z,15Z))	M+Na	804.551373	781.562155	0.004173	5.18674151
HMDB08074	PC(18:1(11Z)/18:3(9Z,12Z,15Z))	M+Na	804.551373	781.562155	0.004173	5.18674151
HMDB08138	PC(18:2(9Z,12Z)/18:2(9Z,12Z))	M+Na	804.551373	781.562155	0.004173	5.18674151
HMDB08214	PC(18:3(9Z,12Z,15Z)/20:4(8Z,11Z,14Z,17Z))	M+H	804.553781	803.546505	0.006581	8.17968936
HMDB08468	PC(20:4(8Z,11Z,14Z,17Z)/18:3(6Z,9Z,12Z))	M+H	804.553781	803.546505	0.006581	8.17968936

HMDB08023	PC(16:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	M+H	804.553781	803.546505	0.006581	8.17968936
HMDB13019	Neuromedin C 1-8	M+NH4	893.448891	875.415068	0.005991	6.70547589
HMDB00034	Adenine	M-H	134.047219	135.054495	0.000919	6.85579311
HMDB01018	UDP-D-Xylose	M+FA-H	581.042657	536.044456	0.004843	8.33501627
HMDB00907	Sulfolithocholic acid	M-H20-H	437.23617	456.25456	0.00103	2.3557063
HMDB03259	Dihydrocortisol	M-H	363.217698	364.224974	0.000902	2.48335917
HMDB05081	5-HEPE	M+FA-H	363.217696	318.219495	0.000904	2.48886552
HMDB02232	8,9-Epoxyeicosatrienoic acid	M-H	319.227869	320.235145	0.001871	5.8610171
HMDB04682	11(R)-HETE	M-H	319.227869	320.235145	0.001871	5.8610171
HMDB04673	11,12-Epoxyeicosatrienoic acid	M-H	319.227869	320.235145	0.001871	5.8610171
HMDB10409	11,12-EpETrE	M-H	319.227869	320.235145	0.001871	5.8610171
HMDB02311	8,9-DiHETrE	M-H20-H	319.22732	338.24571	0.00242	7.5808048
HMDB02343	5,6-DHET	M-H20-H	319.22732	338.24571	0.00242	7.5808048
HMDB28939	Leucyl-Threonine	M-H	231.135031	232.142307	0.002231	9.65236637
HMDB29064	Threoninyl-Isoleucine	M-H	231.135031	232.142307	0.002231	9.65236637
HMDB10734	(R)-3-Hydroxy-hexadecanoic acid	M+FA-H	317.233346	272.235145	0.001746	5.50383502
HMDB10382	LysoPC(16:0)	M+FA-H	540.33069	495.332489	0.00139	2.5724987
HMDB12350	PS(14:1(9Z)/20:4(5Z,8Z,11Z,14Z))	M-3H	250.145419	753.458084	0.000459	1.83493266
HMDB12430	PS(20:4(5Z,8Z,11Z,14Z)/14:1(9Z))	M-3H	250.145419	753.458084	0.000459	1.83493266
HMDB09927	PIP(16:0/20:1(11Z))	M-H	943.531834	944.53911	0.003326	3.52505329
HMDB09972	PIP(18:1(9Z)/18:0)	M-H	943.531834	944.53911	0.003326	3.52505329
HMDB0	PIP(18:0/18:1(9Z))	M-H	943.531	944.539	0.003	3.52505

9950			834	11	326	329
HMDB09825	PI(18:1(11Z)/18:1(9Z))	M-H	861.549853	862.557129	0.006487	7.52945402
HMDB09847	PI(18:2(9Z,12Z)/18:0)	M-H	861.549853	862.557129	0.006487	7.52945402
HMDB10575	PG(16:0/18:2(9Z,12Z))	M+FA-H	791.507986	746.509785	0.003514	4.43962672
HMDB13542	PGP(18:1(9Z)/20:4(5Z,8Z,11Z,14Z))	M-2H	437.238607	876.491766	0.003467	7.92930895
HMDB13498	PGP(16:1(9Z)/22:4(7Z,10Z,13Z,16Z))	M-2H	437.238607	876.491766	0.003467	7.92930895
HMDB10583	PG(16:0/22:5(7Z,10Z,13Z,16Z,19Z))	M-H20-H	777.507045	796.525435	0.005545	7.13176818
HMDB10582	PG(16:0/22:5(4Z,7Z,10Z,13Z,16Z))	M-H20-H	777.507045	796.525435	0.005545	7.13176818
HMDB10654	PG(18:2(9Z,12Z)/20:3(8Z,11Z,14Z))	M-H20-H	777.507045	796.525435	0.005545	7.13176818
HMDB10575	PG(16:0/18:2(9Z,12Z))	M+FA-H	791.507986	746.509785	0.005086	6.42570901
HMDB10612	PG(18:0/22:5(4Z,7Z,10Z,13Z,16Z))	M-H20-H	805.538345	824.556735	0.006515	8.087759
HMDB04891	Ganglioside GA2 (d18:1/18:0)	M-3H	363.235529	1092.72842	0.002069	5.69602871
HMDB11889	Ganglioside GM1 (d18:0/26:1(17Z))	M-3H	551.328259	1657.00661	0.003609	6.54600946
HMDB07344	DG(18:4(6Z,9Z,12Z,15Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	M-2H	317.230412	636.475375	0.001188	3.74491207
HMDB07345	DG(18:4(6Z,9Z,12Z,15Z)/20:4(8Z,11Z,14Z,17Z)/0:0)	M-2H	317.230412	636.475375	0.001188	3.74491207
HMDB47904	TG(14:1(9Z)/14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))	M-3H	263.201654	792.626 79	0.000754	2.86472364
HMDB42513	TG(14:0/18:2(9Z,12Z)/16:0)	M+FA-H	861.718892	816.720691	0.006092	7.06959086
HMDB01162	Heme O	M-H20-H	819.357273	838.375663	0.000427	0.52114018

4. Conclusions

In this study, plasma from individuals with MetS subjected to four intervention diets for 12 weeks has been analyzed during a postintervention study aimed at evaluating metabolic changes during the postprandial lipemia. The complement of all statistical tools used in this study has enabled to identify that most metabolic changes occurred in the lipid fraction for all diets. This is of interest taking into account that diets were isoenergetic but with a different fat content and quality. Therefore, these standard diets could be correlated with the plasma metabolic profile of individuals subjected to them. The development of statistical models with capability to classify individuals according to the followed diet could be implemented in nutritional studies in the search for the personalized diet.

Acknowledgments

The Spanish Ministerio de Ciencia e Innovación (MICINN) and European FEDER program are thanked for financial support through project CTQ2009-07430. F.P.C. is also grateful to the MICINN for a Ramón y Cajal contract (RYC-2009-03921).

References

[1] D. I. Shaw, A. C. Tierney, S. McCarthy, J. Upritchard, S. Vermunt, H. L. Gulseth, C. A. Drevon, E. E. Blaak, W. H. M. Saris, B. Karlström, O. Helal, C. Defoort, R. Gallego, J. López-Miranda, D. Siedlecka, M. Malczewska-Malec, H. M. Roche, J. A. Lovegrove, British Journal of Nutrition 101 (2009) 750-759.

[2] A. Scalbert, L. Brennan, O. Fiehn, T. Hankemeier, B. Kristal, B. Ommen, E. Pujos-Guillot, E. Verheij, D. Wishart, S. Wopereis, Metabolomics 5 (2009) 435-458.

[3] M. J. Gibney, M. Walsh, L. Brennan, H. M. Roche, B. German, B. van Ommen, Am. J. Clin. Nutr. 82 (2005) 497-503.

[4] L. Vaclavik, O. Lacina, J. Hajslova, J. Zweigenbaum, Analytica Chimica Acta 685 (2011) 45-51.

[5] M. Betz, K. Saxena, H. Schwalbe, Current Opinion in Chemical Biology 10 (2006) 219-225.

[6] K. A. Lawton, A. Berger, M. Mitchell, K. E. Milgram, A. M. Evans, L. Guo, R. W. Hanson, S. C. Kalhan, J. A. Ryals, M. V. Milburn, Pharmacogenomics 9 (2008) 383-397.

[7] G. A. Theodoridis, H. G. Gika, E. J. Want, I. D. Wilson, Analytica Chimica Acta 711 (2012) 7-16.

[8] D. Vuckovic, Anal. Bioanal. Chem. 403 (2012) 1523-1548.

[9] J. L. Griffin, Current Opinion in Chemical Biology 10 (2006) 309-315.

[10] J. L. Griffin R. A. Kauppinen, Journal of Proteome Research 6 (2006) 498-505.

[11] G. a. Graça, I. F. Duarte, B. J. Goodfellow, I. M. Carreira, A. B. Couceiro, M. d. R. r. Domingues, M. Spraul, L.-H. Tseng, A. M. Gil, Anal. Chem. 80 (2008) 6085-6092.

[12] R. Ramautar, G. Somsen, G. de Jong, Anal. Bioanal. Chem. 387 (2007) 293-301.

[13] A. Koulman, G. Lane, S. Harrison, D. Volmer, Anal. Bioanal. Chem. 394 (2009) 663-670.

[14] W. B. Dunn, N. J. C. Bailey, H. E. Johnson, Analyst 130 (2005) 606-625.

[15] K. Sandra, A. d. S. Pereira, G. Vanhoenacker, F. David, P. Sandra, J. Chromatogr. A. 1217 (2010) 4087-4099.

[16] D. I. Shaw, A. C. Tierney, S. McCarthy, J. Upritchard, S. Vermunt, H. L. Gulseth, C. A. Drevon, E. E. Blaak, W. H. M. Saris, B. Karlström, O. Helal, C. Defoort, R. Gallego, J. López-Miranda, D. Siedlecka, M. Malczewska-Malec, H. M. Roche, J. A. Lovegrove, British Journal of Nutrition 101 (2009) 750-759.

[17] P. Bernini, I. Bertini, C. Luchinat, P. Nincheri, S. Staderini, P. Turano, Journal of Biomolecular NMR 49 (2011) 231-243.

[18] L. Vaclavik, O. Lacina, J. Hajslova, J. Zweigenbaum, Analytica Chimica Acta 685 (2011) 45-51.

[19] L. A. Berrueta, R. M. Alonso-Salces, K. Héberger, J. Chromatogr. A. 1158 (2007) 196-214.

[20] T. S. Metkus, D. Dobrosielski, K. Stewart, Journal of the American College of Cardiology 59 (2012) E1753-E1753.

DISCUSIÓN DE RESULTADOS

La normativa vigente en la Universidad de Córdoba para la elaboración de una Memoria de Tesis Doctoral en la modalidad en la que se incluyen los artículos (publicados o próximos a su publicación) como tales requiere una discusión conjunta de los resultados, lo que es función de la homogeneidad de la investigación realizada.

La investigación que constituye esta Tesis tiene como denominador común la metabolómica; no obstante, se han abarcado diferentes áreas de esta disciplina (vegetal, clínica, nutricional) y diferentes estrategias (análisis orientado o global), por lo que se hace conveniente realizar discusiones parciales atendiendo tanto a áreas como a estrategias. Finalmente se hace una discusión conjunta de la investigación.

PARTE I. DESARROLLO Y APLICACIÓN DE PLATAFORMAS ORIENTADAS: METABOLÓMICA DEL OLIVO

El conocimiento del metaboloma del olivo es de enorme importancia en la cuenca mediterránea habida cuenta de que supone su principal cultivo. Su interés innegable en Andalucía se agudiza especialmente en Córdoba, ya que en esta provincia está ubicado el mayor banco de germoplasma del olivo que existe y en el que los experimentos para la mejora de este cultivo (cruzamientos, densidad de población, maduración del fruto, etc.) con especial énfasis en la calidad del producto final, el aceite de oliva virgen, constituyen una constante.

La aportación que se ha realizado en este aspecto ha incidido en la fracción insaponificable, particularmente esteroles y alcoholes de cadena larga o alcoholes alifáticos. Estas dos familias de metabolitos secundarios son de importancia biológica, tanto en hojas como en fruto (particularmente por su presencia en el producto de este último: El aceite, en el que las dos familias son de especial interés en el establecimiento de su calidad y en la diferenciación de otros aceites vegetales para detectar fraudes).

Está ampliamente demostrado que los esteroles de plantas, también conocidos como fitoesteroles, tienen un efecto beneficioso en la salud, ya que ayudan a rebajar los niveles en sangre de lipoproteínas de baja densidad (colesterol “malo”) y a prevenir diferentes tipos de cáncer, a la vez que actúan como receptores nucleares y activan la

transcripción de genes diana. Como componentes de las membranas celulares, su papel en la regulación de su fluidez y permeabilidad es clave.

Con respecto a los alcoholes de cadena larga (desde 6 a 10 átomos de C) se emplean especialmente en cosmética, algo en alimentación y para la obtención de biocombustibles. Su uso en el área clínica o en suplementos de la alimentación es muy escaso, a pesar de sus propiedades anticancerígenas, antivíricas y antifúngicas. Por su carácter ambifílico estos alcoholes actúan como surfactantes no iónicos, de donde deriva su uso en cosmética como emulsificadores y emolientes y en la industria alimentaria.

La puesta a punto de un método que permitiera la determinación de ambas familias de compuestos en muestras sólidas tales como hojas y frutos del olivo requirió las siguientes etapas:

(i) Lixiviación, cuya aceleración mediante una sonda de ultrasonidos (US) permitió acortar unas 3 veces el tiempo necesario con respecto al del método convencional basado en agitación.

(ii) Saponificación de los analitos en el lixiviado, también acelerada por US hasta unas 10 veces respecto al método convencional basado en la utilización de un sistema de reflujo. Este efecto era previsible dado que las reacciones de saponificación en medios homogéneos ocurren a través de radicales libres y, como es sabido, la acción de los US favorece la formación de radicales libres en medios polares.

(iii) Limpieza del extracto tras la saponificación, que se llevó a cabo por SPE, utilizando un sorbente con grupos aminopropilo, dando lugar a una muestra analítica con óptimas características para la siguiente etapa.

(iv) Sililación de los analitos, también asistida por US, que se aceleró enormemente y que produjo productos adecuados para la separación subsiguiente mediante cromatografía de gases.

(v) Separación de los analitos (7 alcoholes y 10 esteroles) para su posterior detección basada en espectrometría de masas de trampa de iones en modo SIM. El tiempo total de la etapa fue de 70 min. El uso como estándares internos (IS) de eicosanol y colesterol, para alcoholes y esteroles, respectivamente, contribuyó a la precisión del análisis.

El método así desarrollado tiene como principales características las siguientes: (a) Es el primero publicado hasta la fecha que permite la determinación simultánea de esteroles y alcoholes de cadena larga; (b) el uso de US acortó la etapa de lixiviación de 24 h a 10 min; la de saponificación de 2 h a 10 min y la de sililación de 120 min a 10 min; (c) la limpieza con un sorbente aminopropilado da lugar a un eluato libre de

interferencias que pudieran dificultar las posteriores etapas de separación–detección; (d) la eficiencia de la extracción es 5 y 3 veces mayor que la que proporciona el método convencional de maceración–agitación para alcoholes y esteroles, respectivamente.

Todo ello propició el éxito del método para la determinación de los analitos en hojas y en frutos del olivo, que fue validado mediante su aplicación a la determinación de los compuestos en estudio en muestras procedentes de tres variedades de olivo (arbequina, picual y manzanilla). En concreto, el método se aplicó a frutos recogidos en cinco grados de madurez establecidos a través de su color (verde, verde amarillento, amarillo púrpura, púrpura y negro), y en hojas recolectadas en 3 periodos diferentes (otoño e invierno en el primer y último grado de maduración del fruto y en primavera). Estos estudios proporcionan la información adecuada para seleccionar la mejor época y variedad con la que obtener una materia prima (hojas, frutos, o el aceite derivado) con el contenido idóneo de ambas familias de compuestos de la fracción insaponificable.

PARTE II. DESARROLLO Y APLICACIÓN DE PLATAFORMAS ORIENTADAS: NUTRIMETABOLÓMICA

Los dos primeros trabajos de los que conforman este bloque pueden considerarse de preparación a los siguientes estudios ya que se dedican al desarrollo y aplicación de plataformas para determinar la influencia de la fritura en las fracciones insaponificable considerada tradicionalmente como tal, y fenólica (o parte más polar de la fracción insaponificable) en función del tipo de aceite y del contenido en inhibidores de la oxidación, naturales o artificiales. Estos aceites, así como el aceite de oliva virgen, fueron los utilizados en los estudios en nutrimetabolómica.

La fritura es uno de los procesos más comunes en la preparación de alimentos. Es sobradamente conocido que durante este proceso ocurren cambios físico-químicos que deterioran drásticamente la calidad de los aceites y grasas utilizados. Los cambios físicos más destacables son el aumento de la viscosidad, la formación de espuma, los cambios de color y la disminución del llamado “punto de humo”; mientras que los cambios químicos implican el incremento de la concentración de ácidos grasos libres y de componentes polares, la disminución del grado de insaturación, de sus propiedades organolépticas y del valor nutricional por degradación de componentes minoritarios. Los procesos a través de los cuales se producen estos cambios a todas luces indeseables son la autooxidación, la oxipolimerización y la degradación térmica oxidativa. La rapidez y grado de desarrollo de estos procesos dependen de la temperatura y el tiempo

de calentamiento, del alimento sometido a fritura y su contenido en humedad, del tipo de freidora utilizada y de la calidad inicial del aceite de fritura. El contenido en ácidos grasos insaturados así como el tipo y concentración de antioxidantes en el aceite son los dos aspectos de los que depende su calidad y este contenido, una vez el aceite se ha sometido a fritura, ha sido el objeto de la primera parte de este bloque: Analizar el contenido de ambos componentes antes y después de someter el aceite a fritura simulada para conocer la influencia en su comportamiento del tipo de aceite y de la presencia de inhibidores de la oxidación añadidos. Para ello se utilizaron 4 aceites: Oliva virgen con un contenido natural en fenoles de 400 μg/mL —VOO—; girasol sin contenido natural de fenoles pero enriquecido hasta 400 μg/mL con un extracto fenólico rico en inhibidores de la oxidación aislados del alperujo —ASO—; girasol enriquecido con un inhibidor de la oxidación sintético, el dimetilsiloxano —DSO— a 400 μg/mL; y de girasol puro, es decir, sin ningún tipo de enriquecimiento —SO. La fritura simulada consistió en 20 ciclos de calentamiento de 5 min cada uno y a 180 ºC, con una toma de muestra de cada aceite después de cada ciclo.

Dos fueron las fracciones de las que se estudió su evolución con el calentamiento del aceite en comparación con el blanco (el correspondiente aceite no sometido a calentamiento): La fracción insaponificable constituida por los fitosteroles, triterpenos y alcoholes de cadena larga, con un total de 14 compuestos, y la fracción fenólica, con un total de 13 compuestos. Es de destacar el diferente equipamiento analítico y preparación de la muestra en cada caso: Mientras que el análisis de fenoles sólo requirió una etapa de extracción líquido–líquido con posterior evaporación parcial del extractante previa a la introducción en un equipo LC–DAD, en el caso de la fracción insaponificable la preparación de la muestra fue más laboriosa ya que requirió una etapa de saponificación, otra de extracción líquido–líquido y una final de sililación antes de la introducción de la muestra analítica así preparada en el equipo GC–MS.

Aunque la tendencia general frente al calentamiento fue similar (degradación creciente de todos ellos con el aumento del número de ciclos), cada una de las familias tuvo un comportamiento diferente en cuanto a su resistencia a la degradación.

(i) El contenido inicial en alcoholes de cadena larga fue notablemente mayor en VOO y en el de girasol enriquecido con el extracto de alperujo. El comportamiento anómalo de muchos de estos compuestos en todos los aceites (una disminución en los primeros ciclos y un aumento en los finales) se justifica por la existencia de estructuras

conjugadas, que dejan libres los alcoholes cuando aumenta el número de ciclos de calentamiento.

(ii) Los esteroles en los aceites originales mostraron una mayor concentración (hasta 4 veces mayor para algunos de ellos, por ejemplo campesterol) en el caso de los aceites de girasol. Durante el proceso de fritura simulada, por el contrario, la menor degradación de estos compuestos se produjo en los aceites con contenido en fenoles caracterizados por su potencial como inhibidores de la oxidación (VOO y ASO).

(iii) Los triterpenos se encontraron en más altas concentraciones en el VOO, seguido de ASO. En este último el grado de enriquecimiento fue función del triterpeno en cuestión. De forma general, los más concentrados en ambos aceites fueron el ácido maslínico, eritrodiol y uvaol. El proceso de calentamiento produjo una rápida degradación de los triterpenos en ambos aceites durante los 6 primeros ciclos; llegando a ser prácticamente indetectables al cabo de 10 ciclos. Resulta paradójico el hecho de que la resistencia a la degradación de los triterpenos fuera mayor en el aceite enriquecido que en el VOO, lo que puede justificarse por la diferente composición de la fracción grasa del ASO.

El estudio de la influencia de la presencia de inhibidores de la oxidación en el proceso de fritura simulada tuvo un especial interés puesto que se incluyó la comparación entre la naturaleza del tipo de compuestos que ejercían este efecto: Los procedentes del olivo y el inhibidor sintético. Es necesario distinguir, en este caso, entre los inhibidores de la oxidación hidrofílicos, característicos de los aceites obtenidos por molturación y separación de la fracción grasa, como es el caso del VOO, y los aceites refinados, en los que la obtención del aceite se realiza mediante extracción líquido–líquido con un extractante apolar, por lo que sólo los inhibidores menos polares, como los tocoferoles, permanecen en el aceite. Teniendo en cuenta que el aceite enriquecido con inhibidores de la oxidación fenólicos, al ser un aceite refinado, contenía también los tocoferoles característicos de su condición de refinado, el método LC–DAD que se puso a punto contempló también la separación individual de estos compuestos. Para ello, el gradiente constituido por una fase A (agua acidificada con 0.2% de ácido fosfórico) y una fase B (acetonitrilo–metanol 1:1) se prolongó hasta el 100% de fase B, que se mantuvo durante 2 min. Además, a las longitudes de onda seleccionadas para la medida de los antioxidantes hidrofílicos (325 y 350 nm) se les sumaron las de 230 y 280 nm para los dos tocoferoles (α- y β-tocoferol). El tiempo total de elución fue de 57 min.

El estudio de los inhibidores de la oxidación existentes en los diferentes aceites después del enriquecimiento mostró una composición similar en el caso del VOO y del de girasol enriquecido con fenoles para algunos de estos compuestos, tales como tirosol, hidroxitirosol y su acetoderivado, luteolina, apigenina, así como los ácidos ferúlico, vainillínico y *o*- y *p*-cumáricos, que se encontraron como tales compuestos simples. Los secoiridoides derivados presentes en el VOO (formados por conjugación del tirosol o el hidroxitirosol con el ácido eleanólico), justifican la resistencia de este aceite frente a la oxidación térmica. En relación con los inhibidores de la oxidación menos polares, los aceites refinados mostraron una concentración más alta de ambos tocoferoles que el VOO, independientemente de la existencia de enriquecimiento y del tipo de inhibidor, natural o sintético.

La influencia del calentamiento puso de manifiesto la llamada "paradoja polar", que establece que los inhibidores de la oxidación polares son más efectivos en lípidos no polares, mientras que los inhibidores no polares son más activos en emulsiones polares lipídicas. En concreto, que en el caso de los aceites los inhibidores de la oxidación fenólicos protegen más efectivamente contra la oxidación que los lipofílicos debido a que estos últimos están disueltos en los aceites, mientras que los primeros permanecen en la interfase aire–aceite. El análisis en las fracciones obtenidas tras cada ciclo de calentamiento mostró, en el caso de los fenoles, dos grupos con el siguiente comportamiento: Mientras el hidroxitirosol y los derivados secoiridoideos experimentaron una degradación prácticamente total al final de la fritura simulada, el tirosol y en resto de los fenoles sólo redujeron su concentración un 30%, aproximadamente. Es de destacar que la capacidad de protección frente a la degradación en el caso de los inhibidores de la oxidación añadidos al aceite de girasol fue superior al de los existentes naturalmente en el aceite, caso del VOO. Este comportamiento puede explicarse por el hecho de que, si bien la concentración total en ambos fue la misma (400 μg/mL) la relación de componentes era distinta, ya que en el VOO predominan los menos polares (más solubles en lípidos y que quedan en mayor proporción en el aceite); mientras que, los que pasan al alperujo en la fracción acuosa del fruto son los más polares. Por tanto, el extracto usado para el enriquecimiento del aceite de girasol produjo una relación de componentes en él diferente de la que existe en el VOO.

El estudio de la evolución de los tocoferoles durante el calentamiento mostró que su degradación no se afecta por la presencia o ausencia de fenoles y que sólo depende de la

naturaleza del tocoferol. De hecho, el α-tocoferol mostró una degradación significativa después del cuarto ciclo, que llegó al 50% en el octavo ciclo y al 50% en el décimo-sexto. El comportamiento del δ-tocoferol —en menor concentración en todos los aceites— fue similar a la del α-tocoferol, pero su velocidad de degradación fue algo menor; lo que está de acuerdo con la bibliografía en la que se ha comprobado la mayor estabilidad del δ- respecto al α-tocoferol. La presencia de inhibidores de la oxidación fenólicos decrece ligeramente la velocidad de degradación de los tocoferoles; efecto que no ejerce el dimetilsiloxano.

La composición de la fracción lipídica (22 ácidos grasos), así como la de la fracción minoritaria se determinaron antes y después del calentamiento para poner de manifiesto los cambios producidos por este proceso.

Una vez conocida la composición de los aceites y sus componentes minoritarios tras el calentamiento, estos aceites se utilizaron para determinar su efecto nutricional en 26 individuos obesos mediante un estudio de intervención en el que cada individuo recibió 4 desayunos elaborados con los 4 aceites sometidos a fritura simulada, con dos semanas de intervalo entre desayunos consecutivos. Se tomaron muestras de suero de los pacientes antes de la intervención (en estado basal o blanco) y 2 y 4 h después, a través de las cuales se determinó cómo se afectaba la concentración de la fracción insaponificable y de los ácidos grasos en suero con la ingestión de los diferentes desayunos.

La determinación de los esteroles, triterpenos y alcoholes de cadena larga en se llevó a cabo adaptando, para su aplicación en suero, el método previamente puesto a punto para la determinación de parte estos compuestos en extractos de hojas de olivo y de fruto y posteriormente en los aceites sometidos a calentamiento.

La influencia que tienen estas familias de compuestos en la salud por su función preventiva, curativa y paliativa de enfermedades (y de forma concreta en los niveles de colesterol) hace conveniente conocer cómo su presencia en sangre se modifica por el tipo de grasa ingerida.

El estudio de la evolución de la fracción lipídica (ácidos grasos esterificados y no esterificados EFAs y NEFAs, respectivamente) en la sangre de los individuos sometidos a las 4 diferentes dietas es de interés dado que existen pocos métodos en la bibliografía por los que se determinen ambas familias y, especialmente escasos los datos sobre el

efecto de los aceites de fritura en individuos obesos. Otros aspectos sobre la obesidad más conocidos son la mayor probabilidad de enfermedades tales como las cardiacas, la diabetes tipo 2, la apnea del sueño, ciertos tipos de cáncer, la osteoartritis y el asma.

Dado que existe una relación directa entre obesidad y niveles de ácidos grasos en suero, es de esperar que el seguimiento de los ácidos grasos en este fluido pueda proporcionar información sobre la influencia de la dieta y sobre otros factores, tales como actividad física, susceptibilidad genética y desórdenes endocrinos. La inflamación, un proceso relacionado comúnmente con la obesidad, está dirigido por la producción de eicosanoides derivados del ácido araquidónico, cuyos niveles están totalmente controlados por la alimentación.

El método utilizado para hacer el seguimiento de los ácidos grasos en las muestras de suero provenientes de los individuos obesos sometidos a los desayunos elaborados con los aceites sometidos a fritura simulada se utilizó un método desarrollado previamente por el grupo en el que se integra la doctoranda. Consiste en la derivatización asistida por ultrasonidos y realizada en dos etapas para EFAs y NEFAs, previas a la introducción en el equipo GC–MS/MS para la separación individual y determinación mediante impacto electrónico en modo positivo. La columna cromatográfica utilizada (una SP™-2380 de sílice fundida, capilar) posibilita la separación de los isómeros *cis/trans* y permitió la separación de los 22 ácidos grasos en 63 min. La identificación estuvo basada en los espectros de masas y los tiempos de retención de cada compuesto.

Los datos así obtenidos se sometieron a una serie de estudios estadísticos para evaluar tanto el estado basal de los parámetros en los individuos en estudio, como su capacidad de distinguir el efecto que los diferentes desayunos ingeridos tuvieron sobre la concentración de los ácidos grasos en la sangre de estos individuos. El primero fue un análisis de correlación para conocer las concentraciones en los individuos de aquellos ácidos grasos estadísticamente significativos a un nivel de confianza del 95% ($p < 0.05$), que estuvieron en concordancia con los datos de la bibliografía para individuos obesos teniendo en cuenta las variables antropométricas (edad, género e índice de masa corporal o BMI).

La aplicación de un multi-ANOVA tuvo como objetivo detectar diferencias en el perfil de ácidos grasos en función del desayuno ingerido, para lo cual se estimó primero la contribución de cada una de las variables antropométricas y del desayuno ingerido en la variabilidad del perfil de ácidos grasos. Para ello se mide la contribución de cada factor

eliminando la influencia de todos los otros factores. Este estudio reveló que el factor más importante para explicar la variabilidad en los niveles de ácidos grasos (EFAs y NEFAs) encontrados en suero fue el desayuno ingerido; por el contrario, el género y el BMI no contribuyeron significativamente a la variabilidad y la edad lo hizo sólo para algunos de los ácidos grasos (C22:5n3 y C20:5n3 como EFAs, y C22:5n3 y C22:2n6 como NEFAs).

Comprobada de forma genérica la influencia del desayuno en el perfil de ácidos grasos, se diferenció cualitativamente entre cada uno de los desayunos utilizando análisis por componentes principales (PCA) para validar el estudio ANOVA utilizando los datos obtenidos de suero en el estado basal y después de la ingestión de los desayunos y restados los primeros de los segundos. Se obtuvo así un bloque drásticamente separado del resto que correspondió a los desayunos preparados con VOO. Otros sub-bloques correspondieron al aceite de girasol no enriquecido y a los dos enriquecidos, con ácidos grasos característicos de estas agrupaciones. La capacidad de discriminación del PCA se confirmó mediante un método supervisado basado en análisis discriminante por mínimos cuadrados parciales (PLS-DA) con validación cruzada. El modelo mostró una buena consistencia, ya que su capacidad de predicción fue del 98.54 y 98.41% para las series de entrenamiento y validación, respectivamente. Finalmente, la capacidad discriminatoria de los ácidos grasos significativos se evaluó mediante representaciones de caja–bigotes comparando los estados basal y post-basal para cada desayuno. Estos estudios pusieron claramente de manifiesto que los ácidos grasos con gran capacidad de discriminación, como el ácido C22:4n6 (docosatetraenoico) esterificado, tienen su concentración más alta en suero de individuos cuando ingirieron VOO y la más baja con la ingestión de aceite de girasol puro; siendo intermedia para los dos aceites enriquecidos. La situación opuesta se encontró para el ácido C20:5n3 (eicosapentaenoico). Es destacable el hecho de que el ácido C22:4n6 se sintetiza en la ruta n-6 por elongación del ácido araquidónico, mientras que el C20:5n3 es el precursor de la serie 3 de prostaglandinas. La capacidad de discriminación de estos compuestos se evaluó de forma independiente mediante representación de valores promedios y comparación de los correspondientes al estado basal con los del estado post-basal en cada caso, en los que se puso de manifiesto que los niveles más altos de omega-6 y omega-3 correspondían siempre a individuos que habían ingerido el desayuno preparado con VOO y los niveles tras la ingesta eran incluso superiores a los del estado basal. También el desayuno preparado con aceite enriquecido con extracto de alperujo la

concentración de C20:2n6 y C22:6n3 (DHA) produjo la misma tendencia que el preparado con VOO; lo que parece indicar un efecto positivo de los fenoles del olivo. Por el contrario los otros aceites promovieron en los individuos niveles de EFAs inferiores tras la ingesta de los desayunos, correspondiendo los más bajos a los individuos que ingirieron aceite que no contenía ningún tipo de inhibidor de la oxidación.

Se realizó un último estudio para comparar los niveles de los EFAs y NEFAs en suero a dos tiempos tras la ingesta de los desayunos: 2 y 4 h (en ambos casos se restaron los valores correspondientes al estado basal). Los residuales de PCAs mostraron que sólo se consigue discriminación entre ambos post-estados para la ingestión del aceite de girasol enriquecido con extracto de alperujo, con una contribución significativa del C22:5n3 o ácido docosapentaenoico (DPA). Este ácido es un inhibidor efectivo de la agregación plaquetaria y tiene una capacidad de migración en el endotelio, por lo que el aumento de su nivel en el organismo es de gran interés.

Un estudio diferente, aunque siempre en el área de la nutrimetabolómica, consistió en la puesta a punto de un método totalmente automatizado para la determinación de fenoles y sus metabolitos en plasma de individuos sanos tras la ingestión de una única pero abundante dosis de VOO (40 mL de VOO —conteniendo 400 μg/mL de compuestos fenólicos— y 60.000 IU de vitamina A/m^2 de superficie corporal). Este estudio, realizado sobre una amplia población pretendía poner de manifiesto la evolución de estos compuestos en el organismo con el ánimo de dilucidar su papel considerado crucial en el efecto protector del VOO y establecer de forma inequívoca qué parte de este efecto es atribuible a los ácidos grasos monoinsaturados y qué otra a los compuestos fenólicos.

Los numerosos estudios de la fracción fenólica han llevado a atribuir a estos compuestos una influencia clave en la mejora de la disfunción endotelial y del perfil hemostático durante el estado postprandial, la mejora de los perfiles lipídicos y la disminución del estrés oxidativo. Por tanto, la caracterización de estos compuestos y el estudio de su biodisponibilidad y metabolismo son cruciales para conocer sus implicaciones en la salud humana. La biotransformación que experimentan en el organismo, función de su polaridad, es objeto de una amplia investigación. Por una parte los fenoles polares tras su absorción en el estómago y en el tracto intestinal experimentan un metabolismo intenso en el intestino y en el hígado; mientras los menos

polares se metabolizan inicialmente para dar compuestos más polares, como el hidroxitirosol y el tirosol. Ambos tipos de metabolitos pueden, a su vez, sufrir varios tipos de conjugación tales como metilación, sulfatación y glucoronidación. Esta enorme variedad de vías metabólicas diversifica los compuestos iniciales y puede llevar a concentraciones muy pequeñas de algunos de los metabolitos formados. Por tanto, y dada la ausencia en la bibliografía de un método totalmente automatizado que hiciera posible la determinación en un número grande de muestras sin auxilio del operador y con un volumen de fluido biológico muy pequeño, se propuso el desarrollo de una plataforma analítica que supliera esta carencia. Para conseguirlo se utilizó un sistema que implicó una estación comercial de SPE que trabaja a alta presión y que está conectada en línea con la columna cromatográfica de tal forma que una vez optimizada esta etapa de preparación de la muestra, la propia fase móvil cromatográfica eluye los compuestos de interés que se han retenido en la columna de forma que todo el eluato se aprovecha en la etapa siguiente. El uso de un detector muy sensible como es el espectrómetro de masas de triple cuadrupolo tras la separación cromatográfica permitió el desarrollo de un método que sólo requirió 100 μL de plasma para conseguir límites de detección de los 18 analitos problema entre 0.02 y 0.28 ng/mL, con coeficientes de correlación superiores a 0.996 y con factores de recuperación entre 84.2 y 99.4%. Estas características analíticas fueron fruto de una exhaustiva optimización, tanto de la etapa de SPE (selección del sorbente entre 8 tipos cubriendo el rango de materiales polares a los de polaridad media; del medio para la introducción de la muestra en el cartucho, de la etapa de lavado de la columna para eliminar los compuestos no retenidos, y del modo y tiempo de elución de los analitos. El inconveniente debido a la no idoneidad de la fase móvil cromatográfica para eluir los analitos que abarcaban un amplio rango de polaridad y que produjo un ensanchamiento notable de los picos, se solventó con el uso del modo de elución mediante enfoque. Esta modalidad consiste en la mezcla de la fase móvil cromatográfica con la disolución apropiada para conseguir la elución en el menor volumen de mezcla posible, disminuyendo así la anchura de los picos de forma drástica. La composición de esta disolución auxiliar se optimizó ensayando diferentes mezclas etanol–agua en el rango de pH 2–5.

El método se validó mediante su aplicación a muestras de plasma obtenidas de los voluntarios que habían ingerido el alimento rico en VOO tomadas antes de la ingestión y 1 h después, previa evaluación de los efectos matriz aprovechando la configuración de doble cartucho que presenta la estación automática de SPE, que

demostró una retención mínima en el primer cartucho del 84.2%. El uso de ácido siríngico como patrón interno permitió mejorar la precisión del método. La cuantificación de los metabolitos conjugados para los que no existen patrones comerciales se realizó de forma relativa a la de los metabolitos libres. El método, que sólo requiere situar las muestras en el automuestreador de la estación de trabajo SPE, está listo para su aplicación de forma automática a un número grande de muestras.

PARTE III. DESARROLLO DE PLATAFORMAS ORIENTADAS EN METABOLÓMICA CLÍNICA: VARIEDAD DE MUESTRAS

Esta Parte III de la Memoria recoge la investigación dirigida al área clínica de forma concreta para poner de manifiesto varios aspectos: (i) La variedad de equipos de coste de adquisición y mantenimiento asequible, que tras una adecuada optimización dan lugar a métodos de interés en clínica por sus excelentes características, especialmente sensibilidad y pequeño volumen de muestra, que constituyen dos de los aspectos más valorados en esta área. (ii) La variedad de tipos de muestras que pueden utilizarse para la determinación en ellas de compuestos cuya concentración en cada fluido biológico puede dar información sobre una determinada situación del individuo en estudio (con pequeñas o nulas modificaciones del método para su adaptación a la muestra en cuestión). (iii) La capacidad de los grandes equipos para adecuar los métodos a un formato totalmente automatizado con el que analizar un gran número de muestras requiriendo mínimo o nulo concurso del operador y, por tanto, con una capacidad de trabajo de 24 h/día.

Los compuestos objeto de este estudio fueron los ácidos siálicos (SIAs), de los que se seleccionaron entre los 43 que existen comúnmente en la naturaleza los que sólo se encuentran en humanos: Los ácidos N-acetilneuramínico (Neu5Ac) y N-glicolilneuramínico (Neu5Gc). La función en el organismo humano de estos compuestos no es bien conocida. Se ha considerado que los niños tienen una capacidad limitada para sintetizar SIAs en la edad temprana, que la leche materna humana contiene altas concentraciones de estos compuestos sin que se conozca su destino final ni su papel biológico, y que su concentración alta en suero está relacionada con desórdenes inflamatorios, mientras que el individuos sanos la concentración es baja.

Su potencial papel como biomarcadores de ciertas patologías ha disparado el interés clínico de los SIAs y el desarrollo de métodos para su determinación en muestras

biológicas, que implican dos etapas básicas: Liberación de los monosacáridos a los que se encuentran unidos mediante hidrólisis enzimática o ácida, con posible derivatización posterior, y análisis propiamente dicho, que implica fraccionamiento, caracterización y cuantificación.

Teniendo en cuenta el amplio rango de concentraciones en que pueden encontrarse estos compuestos en el organismo humano en función del fluido en cuestión y del estado patológico o saludable del individuo, se proyectó un método implicando las siguientes etapas, que configuran la plataforma experimental adecuada: Una etapa de hidrólisis (que dada la experiencia del grupo y de la propia doctoranda se consideró podría acelerarse mediante US) y que sería previa a otra de limpieza y preconcentración realizada con un sistema dinámico de "laboratorio en válvula" o LOV. Se decidió realizar la separación mediante un equipo μ-LC y la detección mediante fluorescencia asistida por láser (LIF), con lo que se aseguraba un volumen de muestra pequeño y una muy buena sensibilidad, respectivamente, requiriendo esta última la derivatización previa de los analitos para convertirlos en productos fotoemisores (derivados de la quinoxalina en este caso).

La selección de una hidrólisis ácida (medio HCl) en lugar de la enzimática (más cara) y la asistencia de US redujeron el tiempo de esta etapa de 60 a 20 min y aumentaron al doble el rendimiento de la reacción. La etapa SPE de limpieza y preconcentración (realizada en el sistema LOV) se optimizó exhaustivamente considerando diferentes sorbentes, su acondicionamiento, la disolución de lavado una vez retenidos los analitos para eliminar restos de la matriz, el eluyente, así como la fracción de eluato en la que aparecen los analitos. Esta última se optimizó manualmente recogiendo fracciones de eluato de 50 μL que se derivatizaron e inyectaron en el μ-LC–LIF, resultando la fracción tercera la que contenía totalmente lo analitos y una fracción suficiente y reproducible del SI (el éster metílico del Neu5Ac). La eficiencia de esta etapa se evaluó con muestras de los fluidos biológicos en estudio (suero, leche materna, orina y saliva) fortificadas y no fortificadas con los analitos, en los que el factor de recuperación fue siempre superior al 92%. La etapa de derivatización se optimizó también con asistencia de US, lo que redujo el tiempo para su desarrollo de 180 a 20 min, realizada en condiciones suaves de irradiación ultrasónica, ya que condiciones drásticas producen degradación de los analitos. La separación cromatográfica de los dos productos y su detección a 448 nm, con excitación a 325 nm (longitud de onda nominal del láser de

nitrógeno utilizado), que no difiere significativamente del máximo de excitación de los productos (373 nm), requirió 20 min.

El desarrollo en régimen dinámico del proceso se realizó mediante un montaje tal como el que se recoge en la figura 1 del Capítulo 9, en el que la misma sonda de US se utiliza para las dos etapas asistidas por este tipo de energía, una válvula de selección permite que sólo la fracción tercera del eluato prosiga en el camino hacia el cromatógrafo, que el agente derivatizante disuelto en el eluyente favorezca la elución e inicie la derivatización que se complete por parada del flujo en el serpentín 2, y que a partir de éste se realice la inyección en el μ-LC.

Las características del método son excelentes: Rangos lineales de calibración de 0.1–100 ng/mL y 0.5–500 ng/mL para Neu5Ac y Neu5Gc, respectivamente, con límites de detección y cuantificación que dependen de la matriz de la muestra, pero que en ningún momento sobrepasan los 0.8 pg en columna, más bajos que prácticamente todos los reportados hasta la fecha y siempre con menor volumen de muestra. Estas características hacen al método aplicable a cualquier fluido biológico, incluso a suero de individuos sanos, en los que los niveles son bajos (en el intervalo 1–3.4 mM). Así se ha demostrado en su aplicación a muestras de suero, pero también de leche materna, orina y saliva.

Este método constituye un claro ejemplo de que plataformas simples, con coste relativamente bajo, pueden dar lugar a métodos con excelentes características (sensibilidad, selectividad y consumo de muestra) y posibilidades para su aplicación clínica.

Un equipamiento más caro, pero con unas mejores prestaciones, requirió el desarrollo de la plataforma SPE–LC–MS/MS con un espectrómetro de masas de triple cuadrupolo para el análisis de los mismos SIAs.

En este caso se persiguió, además de utilizar como primera etapa la derivatización asistida por US de la que ya se había comprobado su eficiencia para acortar el tiempo necesario para su desarrollo, otros objetivos, tales como: (i) Realizar de manera totalmente automática la etapa de SPE para una limpieza y preconcentración de los analitos más eficaz al llevarse a cabo sin contacto con la atmósfera y evitando por tanto la potencial degradación de los metabolitos por esta causa. (ii) Conectar en línea la etapa de SPE con la cromatográfica, realizando la primera a alta presión para conseguir que la propia fase móvil de la segunda eluyera los analitos retenidos en el cartucho de

limpieza–preconcentración, aprovechando así de forma completa los metabolitos retenidos, con lo que el volumen de muestra pudo disminuirse sin menoscabo de la sensibilidad. (iii) Utilizar un detector de masas de triple cuadrupolo en el modo SRM para conseguir la máxima sensibilidad también en esta etapa.

La optimización exhaustiva del tipo de sorbente para la etapa de SPE, así como de la de detección, permitió los siguientes logros respecto al método anterior: (i) Reducir el volumen de muestra de 100 a 50 μL; (ii) aumentar la sensibilidad en un orden de magnitud al reducir de forma concomitante los límites de detección y de cuantificación; (iii) disminuir el tiempo de análisis al acortar el tiempo requerido para cada etapa y conseguir el solapamiento entre ellas para muestras sucesivas; (iv) adecuar el método a los requerimientos del análisis sistemático con jornadas de trabajo ininterrumpido de 24 h/día.

PARTE IV. DESARROLLO Y APLICACIÓN DE PLATAFORMAS GLOBALES EN NUTRIMETABOLÓMICA

La Parte IV de esta Memoria se dedica a una plataforma desarrollada para el análisis metabolómico global y también viene a engrosar las aplicaciones en nutrimetabolómica. En este caso ambas se han dedicado a muestras de plasma de 76 voluntarios con síndrome metabólico sometidos a cuatro dietas características durante 12 semanas, dos diseñadas para que un 38% de la energía total proviniera de grasa y en las otras sólo un 28%. La composición y nombre dado a cada dieta fueron los siguientes: (1) HSFA: Rica en grasa con el porcentaje más alto (16%) correspondiendo a ácidos grasos saturados (SFA), pero también con un 12% de monoinsaturados (MUFA) y un 6% de poliinsaturados (PUFA). (2) HMUFA: Rica en grasa con el porcentaje más alto de MUFA (20%), 8% de SFA y 6% de PUFA. (3) LFHCC: Bajo contenido en grasa y alto contenido en carbohidratos (8% SFA, 11% MUFA, 6% PUFA), con un suplemento de 1 g/d de aceite de girasol alto en ácido oleico. (4) LFHCC (n-3): Bajo contenido en grasa y alto contenido en carbohidratos (8% SFA, 11% MUFA, 6% PUFA), con un suplemento de 1.24 g/d de PUFA de cadena larga n-3. Las condiciones estrictas exigidas a los participantes se recogen en los Capítulos 11 y 12. Las muestras de plasma se tomaron antes (PRE) y después (POS) de la dieta de intervención con el objetivo de poder eliminar la variabilidad entre individuos y poder hacer así los resultados dependientes de la dieta en cada caso. Ambos tipos de muestras (PRE y POS) se tomaron en ayunas. En el caso de las muestras tomadas después del estudio de

intervención con cada dieta los voluntarios fueron citados a las 12 semanas y tomaron una comida de las mismas características que las dietas planteadas. Las muestras se tomaron durante el período postprandial a las 0, 4 y 8 h después de la ingesta de la comida planificada. Los dos estudios realizados se orientaron a la comparación de los perfiles metabólicos del plasma de los voluntarios en función de la dieta (Capítulo 11 que implicaba la comparación de perfiles metabólicos asociados a las muestras PRE y POS0) y a la evaluación del efecto de cada una de las dietas durante el período postprandial en los individuos que las ingirieron (Capítulo 12 que implicaba la comparación de los perfiles metabólicos asociados a las muestras tomadas en el período de postintervención POS0, POS4 y POS8). En ambos casos los cromatogramas de las fracciones orgánica y acuosa obtenidas por fraccionamiento del plasma con una disolución metanol–cloroformo se obtuvieron mediante LC–TOF/MS en los modos de ionización positivo y negativo para las fracciones metanol y cloroformo (para una diferenciación preferencial entre compuestos polares y no polares), respectivamente.

El tratamiento de los datos brutos en ambos estudios implicó la extracción de las entidades moleculares (MFs) basada en un algoritmo que localiza los posibles aductos de todos los iones relacionados con la misma molécula neutra. Cada característica viene dada por un mínimo de dos iones, por lo que iones con perfiles de elución idénticos y valores de *m/z* relacionados (que representan diferentes aductos o isótopos del mismo compuesto) se extraen como entidades caracterizadas por el tiempo de retención (RT), intensidad máxima del pico cromatográfico y valor de masa/carga con alta resolución. La contribución del ruido de fondo se eliminó por sustracción de las MFs relacionadas con impurezas de los disolventes, trazas de plástico, etc., con lo que se crearon archivos de datos brutos (.cef files) para cada muestra que se exportaron al paquete de software Mass Profiler Professional (MPP). Con este software los datos se sometieron a un preproceso de alineamiento de MFs (pares de valores de *m/z* y tiempos de retención) en una matriz de datos utilizando una ventana de tolerancia de 1.0 min y 30 ppm de exactitud de masa, que puede refinarse mediante realineamiento con una ventana de tolerancia de 0.2 min con la misma exactitud de masa. La reducción subsiguiente de las MFs se basó en la aplicación de un filtro por frecuencia con el fin de eliminar todas aquellas entidades moleculares no presentes en un número representativo de muestras para cada una de las clases consideradas (75%). Este protocolo de preprocesado permitió obtener matrices de datos representativas de cada clase estudiada (dietas y estado de preintervención). A partir de cada matriz de datos se llevó a cabo el análisis

estadístico asociado a cada estudio mediante herramientas multivariantes de análisis no supervisado (PCA) y supervisado (PSL-DA).

En el primero de los estudios (Capítulo 11) se discriminaron los diferentes estados de postintervención que demostraron la presencia de variabilidad asociada a la dieta ingerida. Además, se llevaron a cabo estudios mediante PCA y PLS-DA para cada dieta con el fin de discriminar el estado de postintervención frente al estado de preintervención. La diferenciación entre ambos estados fue patente para todas las dietas en ambos modos de ionización, a excepción de la dieta LFHCC (n-3) en modo de ionización positivo. Con ambos estudios quedaron demostradas las diferencias en el perfil metabólico del plasma como consecuencia de una dieta controlada. Este estudio abre la posibilidad de utilizar el análisis nutrimetabolómico para discriminar el tipo de dieta que sigue un determinado individuo y que en el momento actual se lleva a cabo mediante análisis genéricos y otras fuentes de información como la consulta personal. Mediante la utilización de diagramas de Venn se puso de manifiesto el impacto metabólico de cada una de las dietas mediante comparación cualitativa de los perfiles de metabolitos frente al estado previo al estudio de intervención. Con esta herramienta se mostró que la influencia de la dieta HSFA fue la que causó una mayor alteración metabólica considerando el número de potenciales metabolitos detectados de forma exclusiva en aquellos individuos sometidos a ella . En una etapa final se llevó a cabo la identificación de aquellos potenciales metabolitos que contribuyen de forma significativa a la discriminación de los individuos sometidos a cada dieta frente al estado de preintervención. Para ello se aplicó un análisis ANOVA con valor de significancia fijado en el 99% ($p<0.01$) y un valor de cambio de concentración relativa de 2.0. Las entidades moleculares resultantes para cada dieta se buscaron en bases de datos (METLIN y base de datos del metaboloma humano o HDMB) para su identificación de acuerdo con los valores de *m/z* de los iones precursores. La identificación de metabolitos permitió revelar el efecto clave que las dietas objetivo tenían sobre familias concretas de metabolitos tales como los triglicéridos o diglicéridos, fosfolípidos o eicosanoides en la fracción clorofórmica. En concreto se observó un aumento notable de ácidos grasos saturados en triglicéridos, diglicéridos y fosfolípidos detectados en plasma de individuos sometidos a la dieta HSFA. De la misma forma, la proporción de ácidos grasos monoinsaturados y poliinsaturados en los mismos lípidos fue mayor en plasma de individuos sometidos a las dietas HMUFA, LFHCC y LFHCC (n-3). También se detectó una mayor concentración de derivados

eicosanoides, considerados marcadores de la inflamación y del estrés oxidativo, en individuos sometidos a la dieta LFHCC (n-3). El efecto de cada dieta en la fracción metanólica (formada por compuestos de mayor polaridad) fue menos evidente que en el caso de la fracción preferentemente lipídica. Así se puso de manifiesto el efecto en determinadas familias de metabolitos tales como azúcares, aminoácidos y vitaminas. Sin embargo, el análisis de esta fracción estuvo más marcado por la presencia de compuestos exógenos procedentes de los alimentos ingeridos.

En el segundo estudio (Capítulo 12) destinado a evaluar el efecto postprandial de cada dieta permitió detectar diferencias entre el estado POS0, correspondiente al estado antes de la ingesta de la comida suministrada, frente a los estados POS4 y POS8, correspondientes a tiempos postprandiales. El mismo esquema de trabajo que el aplicado en el Capítulo 11 permitió establecer diferencias entre ambos estados POS4 y POS8 frente al estado inicial al estudio POS0. Por tanto, las diferencias metabólicas durante el período postprandial fueron patentes. Además, para cada dieta se comprobó si existían diferencias entre ambos tiempos de muestreo postprandiales, lo cual no siempre resultó positivo. Este estudio permitió comparar los periodos postprandiales para cada dieta diferenciando entre ambas fracciones del plasma. De esta forma se pusode manifiesto ver que los cambios metabólicos a nivel postprandial eran diferentes para cada dieta con posibilidad, en algunos casos, de discriminar entre las muestras tomadas a 4 y 8 horas. De la misma forma que en el capítulo anterior, los cambios metabólicos fueron más representativos en la fracción lipídica con notables diferencias en el perfil cualitativo de los compuestos que formaban esta fracción.

Ambos estudios pueden ampliarse mediante la aplicación de un método basado en LC–MS/MS para la identificación y el análisis confirmatorio de los metabolitos significativos para cada uno de los estudios. Con esta información se puede realizar la búsqueda de marcadores asociados a cada dieta en una etapa más avanzada de los estudios recogidos en esta Memoria.

La investigación desarrollada en este Capítulo complementa a la realizada en el Capítulo 11 demostrando el potencial de la metabólomica en estudios nutricionales destinados a: (i) Obtener un perfil metabólico asociado a una dieta patrón mediante el análisis de una muestra obtenida de forma poco invasiva como es el plasma, y (ii) evaluar la influencia de cada dieta en el perfil metabólico del plasma durante el período postprandial después de la ingesta de una comida modelo. Ambos estudios suponen un

paso adelante en la búsqueda de la dieta personalizada para cada individuo en función de sus necesidades.

Aspectos comunes que merecen una discusión conjunta al constituir prácticamente una constante en la investigación realizada son los siguientes:

(i) El uso de una sonda de ultrasonidos para acelerar diferentes etapas del proceso analítico, que ha supuesto siempre una reducción significativa, si no drástica, de la duración de la etapa y en la mayoría de los casos un aumento en el rendimiento de la etapa en cuestión: Lixiviación, hidrólisis, sililación, metilación y formación de fluoróforos han sido las etapas que más se han beneficiado con la aplicación de este tipo de energía.

(ii) Aplicación de diseños multivariantes para la optimización de variables interrelacionadas, consiguiendo un valor óptimo verdadero y una visión de conjunto de la influencia de las diferentes variables implicadas en el proceso en cuestión. La presencia de variables discontinuas ha obligado en algunos casos a utilizar diseños de optimización univariantes.

(iii) El diseño de plataformas basadas en equipos analíticos actuales que han proporcionado unas características analíticas a los métodos orientados que permiten su aplicación a la determinación de compuestos que se encuentran en muy baja concentración en muestras tanto vegetales como fluidos biológicos de muy variada naturaleza. Esta doble vertiente de los métodos ha hecho posible, en el caso de la nutrimetabolómica, la comparación entre la concentración y forma química en el alimento ingerido y ambos parámetros en los fluidos tras una determinada etapa o incluso a lo largo de la ruta metabólica.

(iv) La puesta a punto y aplicación de plataformas analíticas globales, que han permitido mediante un tratamiento exhaustivo de los datos brutos obtenidos de ellas y utilizando los softwares y bases de datos propias y las existentes a disposición del usuario (e.g. la base de datos del Metaboloma Humano), una interpretación de los resultados que ha arrojado luz sobre aspectos en estudio que no se conocían o no estaban suficientemente dilucidados antes de la investigación presentada en esta Memoria.

CONCLUSIONES

La aportación que se ha realizado con la investigación que constituye esta Tesis ha incidido en tres áreas de la metabolómica (metabolómica vegetal, clínica y nutricional), para las que se han utilizado las dos estrategias más comunes en esta disciplina: el análisis orientado y el global o perfil metabolómico, en las que se han desarrollado y aplicado plataformas analíticas basadas en herramientas de alta sensibilidad y selectividad. Las conclusiones que pueden extraerse de este conjunto son las siguientes:

(i) Se ha contribuido al conocimiento del metaboloma del olivo aportando información sobre la concentración de metabolitos tales como los esteroles y los alcoholes de cadena larga tanto en hojas como en frutos y su evolución en función de la variedad del olivo, el tiempo de maduración del fruto y el tiempo de recolección de las hojas.

(ii) Se ha puesto de manifiesto cómo el enriquecimiento con inhibidores de la oxidación naturales (fenoles del olivo) ayudan a mejorar las cualidades en la fritura de diferentes tipos de aceites mediante el análisis del efecto del calentamiento en las fracciones insaponificable y polar (fenoles y tocoferoles) en comparación con los aceites no enriquecidos o los que se han enriquecido con inhibidores de oxidación sintéticos.

(iii) Se ha aportado información clínica sobre el efecto que los aceites de fritura enriquecidos con inhibidores de la oxidación naturales o sintéticos ejercen sobre individuos obesos analizando esteroles, alcoholes alifáticos y triterpenos en muestras de suero de estos individuos tras la ingesta de alimentos preparados con estos aceites. Se ha comprobado el efecto beneficioso de los inhibidores de la oxidación naturales en la síntesis de ácidos grasos esenciales.

(iv) Se ha comprobado que la evolución de los ácidos grasos en el organismo (procesos anabólicos y metabólicos) dependen en parte de las características individuales, pero en mayor proporción de la dieta y especialmente de la naturaleza de los inhibidores de la oxidación existentes en los aceites ingeridos.

(v) Se ha puesto a punto un método totalmente automatizado y basado en SPE–LC–MS/MS para la determinación de compuestos fenólicos presentes en aceite de oliva virgen y metabolitos derivados en plasma, que se ha validado por aplicación en muestras de un estudio de intervención con aceite de oliva virgen, que ha resultado ser idóneo para su aplicación en nutrimetabolómica.

(vi) Se han desarrollado dos plataformas para la determinación de ácidos siálicos en muestras clínicas caracterizadas por el diferente grado de automatización y con el denominador común del pequeño volumen de muestra necesario, la sensibilidad y la selectividad. El diferente coste de los equipos utilizados y el distinto grado de implicación del usuario en cada caso pueden ser decisivos a la hora de su implantación en laboratorios clínicos.

(vii) Se ha visto el potencial de la metabolómica en estudios nutricionales destinados a discriminar perfiles metabólicos sanguíneos asociados a una dieta particular, así como para evaluar el efecto de una dieta durante el período postprandial. La investigación desarrollada en esta Tesis se puede convertir en una herramienta deseada por los expertos clínicos dedicados a la búsqueda de la dieta personalizada.

(viii) Aspecto común y destacable de todas las plataformas desarrolladas es la mejora realizada en la preparación de la muestra, en la que se han acelerado de forma drástica, especialmente mediante el uso de ultrasonidos, etapas tales como la lixiviación y la derivatización, acortando así significativamente el tiempo global del análisis.

ANEXOS

Comunicaciones a congresos

NoSSS2009

5th Conference on Separation and Related Techniques by Nordic Separation Science Society

26-29 August, 2009

Tallinn University of Technology

Estonia

Abstract Book and Program

A METABOLOMICS APPROACH TO TRITERPENIC STEROLS AND ALCOHOLS IN OLIVE FRUITS

Mara Orozco Solano*[1], *José Ruiz-Jiménez*, *María Dolores Luque de Castro

[1] *Department of Analytical Chemistry, Marie Curie Building, Annex C-3. Campus of Rabanales University of Córdoba,14071 Córdoba, Spain*

*M*etabolites in plants are generated as part of a tremendously complex biochemical network, supported by the expression closely regulated of specific genes and the precise biosyntetic enzyme modulation. Between the different metabolites present in the olive tree (*i.e.* fatty acids, phenolic compounds, alcohol, waxes, esteroles and terpenos, gliceroles, carotenoid, antocianos vitamins, chlorophylls and sugars) the determination of triterpenic sterols and alcohols is of great interest. The content of these compounds influences the quality of vegetal oils, including olive oil. Clinical studies have demonstrated that plant sterols, also known as fitosterols, ingested in the normal diet or as dietetic supplement, decrease cholesterol levels in blood, by inhibiting its absorption in the thin gut and can act as anti cancinogenic. Also, sterol and alcohol profiles are used to characterize virgin olive oil, and, specially, to detect adulteration of olive oil by hazelnut oil. The techniques used for the development of the strategies characteristic of metabolomics in vegetals are the same as other areas of this discipline. The individual separation, if required, is carried out by gas chromatography, for volatile (or easy to convert into volatile) compounds , liquid chromatography or capillary electrophoresis for thermolabile compounds, polar compounds and high-molecular-weight compounds. The detection-quantification by means mass detectors of different complexity is the commonest.

A method based on separation by gas chromatography and detection by spectrometry of masses in tandem for the study of the metabolic profile of the unsaponifiable fraction of olive fruit (namely, alcohols and sterols) has been developed. The study has been extended to 3 varieties of olive tree (arbequina, picual, manzanilla) cultivated in the same zone, so that differences in the unsaponifiable fraction can be attributed to characteristics of the target varieties, the climate, type of soil and other environmental aspects.

In this study, an alternative method to that accepted by the EU is reported. has been developed. The developed method is faster than the EU method but keeps its analytical characteristics. The sample preparation procedure is based on the following steps: (i) leaching of the raw material accelerated by ultrasound; (ii) saponificación of the leachate also accelerated by ultrasound, and separation of the unnsaponifiable; (iii) cleaning of the extract by solid-phase extraction; (iv) sililation of the target analytes prior to injection into the gas chromatograph for identification/simultaneous quantification of the two compound families.

REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA
SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

XII GRASEQA 2010

Córdoba, 10 y 11 de junio

LIBRO DE RESÚMENES

UNIVERSIDAD D CORDOBA

Fully Automated Method for Determination of Olive Phenols in Human Serum by HPLC–MS/MS

M. Orozco-Solano, C. Ferreiro-Vera, J.M. Mata-Granados[a,b,c], F. Priego-Capote*[a,b], M.D. Luque de Castro[a,b]

[a]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain [b]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071, Córdoba, Spain [c]Sanyres I+D+i Department (Grupo Prasa), Córdoba, Spain

Phenolic compounds are bioactive substances widely distributed in the vegetable kingdom and present in typical components of the Mediterranean diet. Phenols such as hydroxytyrosol, oleuropein, luteolin, apigenin, vanillin, caffeic acid, vanillic acid, *p*- and *o*-coumaric acids and ferulic acid are a part of the minoritary fraction of virgin olive oil, also present at higher concentrations in the waste from oil production and leaves of olive trees. The antioxidant properties of these phenols have been associated to a reduced risk of cardiovascular diseases and several types of cancer. They also exert a proved beneficial effect on the treatment of these diseases and other such as Alzheimer, liver diseases and atherosclerosis, among others. A fast automated method is here proposed for verification and quantitative analysis of phenolic antioxidants in human serum. The developed approach is based on targeting mass spectrometry by using selected reaction monitoring. The approach was carried out by an on-line system composed by a Prospekt-2 unit coupled to a triple quadrupole mass analyser. Samples were prepared in the Prospekt module by solid-phase extraction on a cationic cartridge for cleanup and preconcentration. The analytical process was completed in approximately 30 minutes. The limits of detection (LLOD) and quantitation (LLOQ) ranged from 9.7 to 42 ng/L and from 32.6 to 144 ng/L, respectively. The between-day precision, expressed as relative standard deviation (RSD), ranges between 2.4–3.1% and the within laboratory variability, also expressed as RSD, between 2.2 and 2.6%. A dual cartridge configuration was tested to estimate recovery values of phenols in serum samples which ranged from 86.5 to 98.6%.. The proposed method is reliable, robust, and has an excellent potential for high-throughput use in both clinical and research laboratories. The approach proposed here has been applied to investigate the profile of phenolic antioxidants in human blood of volunteers subjected to four different diets based on four edible oils (*i.e.* olive oil with overall content in phenols either naturally existing in the oil —oil a— or by enrichment of high oleic sunflower oil without these compounds with an alperujo extract —oil b—, the latter, high oleic sunflower oil, enriched with an artificial antioxidant —oil c—and sunflower oil without any type of enrichment —oil d—).

Targeted Mass Spectrometry Analysis of Olive Oil Phenols and Fatty Acids in Human Serum for Application in Nutritional Studies

M. Orozco Solano[1,2], C. Ferreiro Vera[1,2], F. Priego-Capote [1,2], M.D. Luque de Castro[1,2]

[1]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071, Córdoba, Spain

[2]Maimónides Institute of Biomedical Research (IMIBIC), Reina Sofía University Hospital, University of Córdoba, E-14071, Córdoba, Spain

Among olive oil constituents, phenols and fatty acids are crucial in the balanced composition of olive oil, which is responsible for its nutraceutical value. Methods for determination of olive oil phenols and fatty acids in human serum by LC–MS/MS and CG–MS, respectively, have been optimized and validated for application to nutritional studies. The first method allows determining phenols and metabolites in an automated manner by on-line coupling of solid-phase extraction and LC–MS/MS detection in selected reaction monitoring (SRM) mode. The second method allows separation of *cis*/*trans* isomers of esterified and non-esterified fatty acids (EFAs and NEFAs, respectively) based on GC–MS in single ion monitoring (SIM) mode through a two-step extraction/derivatization procedure assisted by ultrasound, which is selective for sequential analysis of EFAs and NEFAs.

1. Phenols in human serum

An automated and fast method based on the on-line coupling between SPE as sample preparation approach and LC–MS/MS with a triple quadrupole mass spectrometer was developed for detection and quantitation of phenols and metabolites in human serum (Figure 1). Table 1 shows the analytical features of the method, which has been characterized in terms of sensitivity, accuracy and precision, and their capability has

been evaluated by application in intervention studies destined to elucidate the metabolism of phenols in individuals after intake of virgin olive oil.

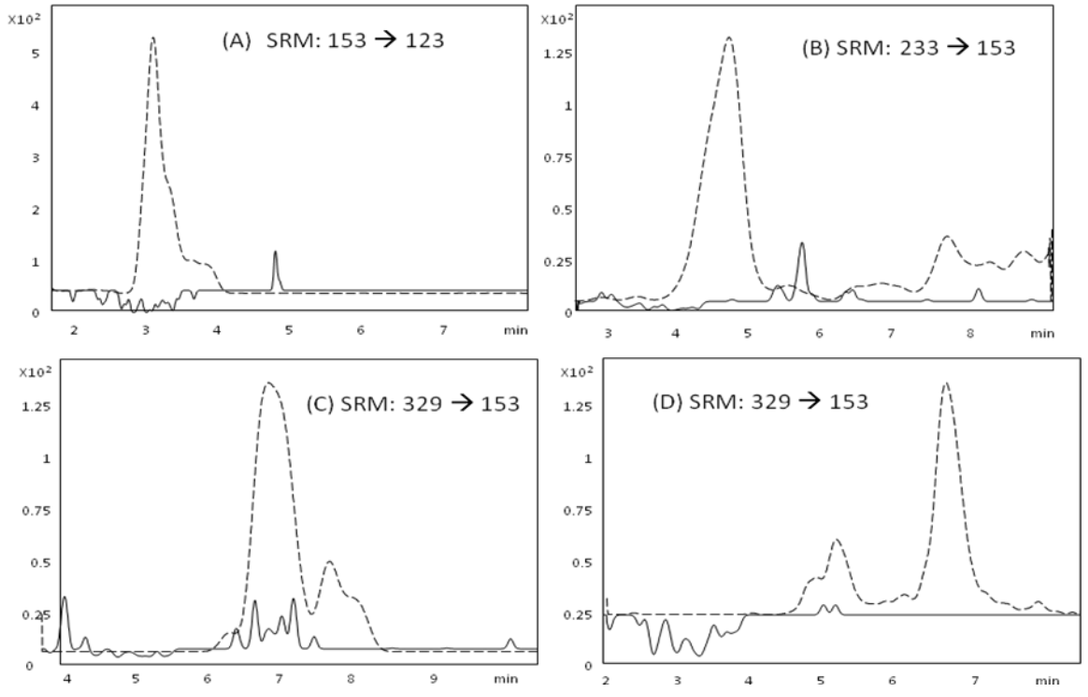


Figure 1. SRM chromatograms for hydroxytyrosol and metabolites in plasma from control individuals (continuous line) and individuals after intake of VOO-based breakfast (dashed line). (A) Hydroxytyrosol; (B) hydroxytyrosol sulfate; (C) hydroxytyrosol monoglucuronide I; (D) hydroxytyrosol monoglucuronide II.

2. Fatty acids in human serum

Table 1 also shows the analytical features of the proposed gas chromatographic method for determination of fatty acids, which is sensitive, selective and robust and thus suitable for the analysis of EFAs and NEFAs in human serum for application in clinical and nutritional studies. In this method, sample preparation plays a main role due to the fact that ultrasound energy, decreases the derivatization time to 5 and 15 min for EFAs and NEFAs, respectively. In this way, the proposed method makes possible to study separately the status of esterified and non-esterified fatty acids in humans. The method also allows the separation and quantitation of *cis/trans* isomers, making it useful to evaluate the effect of these isomers on health. This method has been applied in nutritional studies destined to evaluate the incidence of breakfasts prepared with different fried oils on the profile of fatty acids of a cohort formed by obese individuals. A strong influence of the type of fried oil was observed in the serum concentration of specific fatty acids.

Table 1. Analytical features of the methods. [a] *expressed as µg/mL;* [b] *expressed as percent of relative standard deviation.*

<	**Range LOD (ng/mL)**	**Range LOQ (ng/mL)**	**Range Regression coefficient**	**Range Recovery (%)**	**Range[b] Repeatability (%)**	**Range[b] Reproducibility (%)**
Phenols	0.020 - 0.274	0.054 - 0.832	0.996 - 0.999	84.2 – 99.4	1.7 – 2.9	2.7 – 3.8
Fatty acids	0.002 - 0.028[a]	0.005 - 0.082[a]	0.986 - 0.999	81.2 – 92.7	1.9 – 7.1	2.9 – 7.5

As conclusion, two methods based on mass spectrometry are here presented for application in nutritional studies. The first method takes benefits from SRM mode for quantitation of phenols and metabolites in human serum, which allows to evaluate the metabolism of individuals after intake of virgin olive oil. The second method, based on GC–MS, involves a dual derivatization protocol for sequential analysis of EFAs and NEFAs, including *cis*/*trans* isomers, in human serum. The proposed methods can be applied for high-throughput use in both clinical and research laboratories.

References

1. Bonanome A., Pagnan A., Caruso D., Toia A., Xamin A., Fedeli E., Berra B., Zamburlini A., Ursini F., Galli G., *Nutrition Metabolism & Cardiovascular Diseases*, **10,** 111 – 117 (2000).

2. Del Boccio P., Di Deo A., De Curtis A., Celli N., Iacoviello L., Rotilio D., *Journal Chromatographia. B*, **785,** 47 – 54 (2003).

3. De la Torre Carbot K., Chávez Servín J. L., Jaúregui O., Castellote A. I., Lamuela R. M., Fitó M., Covas M. I., Muñoz Aguayo D., López Sabater M. C., *Analytycal Chimica Acta*, **583,** 402 – 408 (2007)..

4. Smedman A. E., Gustafsson I.B., Berglund L. G., Vessby B. O., *American Journal Of Clinical Nutrition*, **69,** 22-29 (1999).

5. Mensink R. P., Zock P. L., Kester A. D., Katan M. B., *American Journal Of Clinical Nutrition*, **77,** 1146-1155 (2003).

LISTA DE ABREVIATURAS

Lista de abreviaturas

AA ácido araquidónico

ACE intercambiador de cartuchos automático

ANOVA análisis de la varianza

API ionización a presión atmosférica

ASO aceite de girasol alto oleico enriquecido con fenoles extraídos del alperujo

DMB 1,2-diamino-4,5-methylenedioxybenzene

BPC cromatograma obtenido con el pico base de cada espectro

CDA análisis discriminante cuadrático

CE–MS electroforesis capilar-espectrometría de masas

CID disociación inducida por colisión

DAD detector de diodos en fila

DHA ácido docosahexaenoico

DSO aceite de girasol enriquecido con dimetilsiloxano

EIC cromatograma obtenido mediante extracción de un determinado ión de cada espectro

EPA ácido eicosapentaenoico

ESI ionización por electrospray

ESI–MS/MS ionización por electrospray-espectrometría de masas en tándem

FA ácido graso

SIAs ácidos siálicos

FDA Food and Drug Administration (Organismo de los EEUU)

FID detector de ionización de llama

FWHM ancho de pico en la semialtura

GC-MS cromatografía de gases-espectrometría de masas

HPD jeringa dispensadora de alta presión

HPETE ácido hidroperoxieicosatetraenoico

HPLC-DAD cromatografía de líquidos de alta resolución-espectrometría de masas en tándem

HPODE ácido hidroperoxioctadecadienoico

JSESI ionización por electrospray focalizado

LA ácido linoleico

LC–MS cromatografía de líquidos-espectrometría de masas

LC–MS/MS cromatografía de líquidos-espectrometría de masas en tándem

LC–TOF MS cromatografía de líquidos-espectrometría de masas con analizador de tiempo de vuelo

LC–QqTOF MS/MS cromatografía de líquidos-espectrometría de masas en tándem con analizador de cuadrupolo– tiempo de vuelo

LDA análisis discriminante lineal

LLE extracción líquido–líquido

LOD límite de detección

LOQ límite de cuantificación

MANOVA análisis multivariante de la varianza

METLIN base de datos de metabolitos del Scripps Center for Metabolomics and Mass Spectrometry

MF entidad molecular

MPP Mass Profiler Professional

MRM multiple reaction monitoring

MS espectrometría de masas

MSCs células madres mesenquimales

MUFA ácido graso monoinsaturado

NMR resonancia magnética nuclear

PAF factor activador de plaquetas

PBS tampón fosfato salino

PCA análisis de componentes principales

PC fosfatidilcolina

PC componente principal en PCA

PEEK polyether ether ketone

PLS-DA ánalisis discriminante por mínimos cuadrados parciales

PPAR receptor activado por proliferadores de peroxisomas

PS fofatidilserina

PUFA ácido graso poliinsaturado

QqTOF analizador de masas híbrido cuadrupolo–tiempo de vuelo

QqQ detector de triple cuadrupolo

RIA radioinmunoanálisis

RSD desviación estándar relativa

SDS dodecilsulfato sódico

SFA ácidos grasos saturado

SAI ácidos siálicos

SMRS Standard Metabolomic Reporting Structures

SO aceite de girasol refinado

SOP procedimiento de operación estandarizado

SPE extracción en fase sólida

SRM Selected Reaction Monitoring

TIC cromatograma de iones totales

UV ultravioleta

VOO aceite de oliva virgen extra

HMUF high monounsaturated fatty acids

HFA high saturated fatty acids

LFHCC low-fat, high-complex carbohydrate diet

LFHCC (n-3) low-fat, high-complex carbohydrate diet with PUFA supplement