“Assessment of the influence of soybean oil minor components, and of certain added tocopherols and dietary amino compounds on the oil oxidation process under thermoxidative conditions and/or on its evolution during *in vitro* digestion”

Ana San Martín Rubio

DISSERTATION SUBMITTED TO OBTAIN THE DOCTORAL DEGREE
BY THE UNIVERSITY OF THE BASQUE COUNTRY (UPV/EHU)

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Universidad del País Vasco
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NAZIOARTEKO BIKAINTASUN CAMPUSA
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AREA OF FOOD TECHNOLOGY

ASSESSMENT OF THE INFLUENCE OF SOYBEAN OIL MINOR COMPONENTS, AND OF CERTAIN ADDED TOCOPHEROLS AND DIETARY AMINO COMPOUNDS ON THE OIL OXIDATION PROCESS UNDER THERMOXIDATIVE CONDITIONS AND/OR ON ITS EVOLUTION DURING *IN VITRO* DIGESTION.

DISSERTATION SUBMITTED TO OBTAIN THE DOCTORAL DEGREE WITH INTERNATIONAL MENTION BY THE UNIVERSITY OF THE BASQUE COUNTRY (UPV/EHU)

Ana María San Martín Rubio

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RESUMEN

Estudio bajo condiciones de almacenaje acelerado

La oxidación de lípidos alimentarios es un proceso que conlleva tanto la degradación de componentes lipídicos mayoritarios y minoritarios, como la generación de una amplia variedad de compuestos, los cuales no solo pueden afectar negativamente al color, olor o sabor de los alimentos, sino que también pueden tener efectos tóxicos. Por estas razones, la búsqueda de estrategias para evitar, o al menos reducir, la oxidación lipídica ha despertado el interés de la industria alimentaria y de los investigadores durante años. En este contexto**, el uso de aceites con mayor estabilidad oxidativa** podría considerarse como una estrategia para limitar estas reacciones de oxidación. En este sentido, aunque la influencia de la composición en grupos acilo (componentes mayoritarios) de aceites comestibles sobre su estabilidad oxidativa está bien documentada, se conoce poco sobre cómo el conjunto de compuestos minoritarios del aceite podría influir en el proceso de oxidación de éste. En esta línea se planteó el **primer objetivo** de esta tesis doctoral, el cual consiste en analizar hasta qué punto el **conjunto de compuestos minoritarios presentes de forma natural en aceite de soja comercial puede influir en su estabilidad oxidativa y en su proceso de oxidación** bajo condiciones de almacenamiento acelerado. Para alcanzar este objetivo, dos aceites de soja, uno virgen y otro refinado, con proporciones muy similares de los diferentes tipos de grupos acilo, pero diferentes en cuanto al perfil de componentes minoritarios, fueron sometidos a un proceso de almacenamiento acelerado a 70 ºC. El estudio de los componentes minoritarios del aceite se llevó a cabo mediante inmersión directa de una fibra de microextracción en fase sólida, seguida del análisis de los componentes extraídos mediante cromatografía de gases con detector de espectrometría de masas (ID MEFS-CG/EM). Dado que un factor crucial a la hora de estudiar los procesos de oxidación lipídica es la metodología, la técnica empleada fue la Resonancia Magnética Nuclear de Protón (RMN de ^{1}H); ésta permite seguir, por un lado, la degradación de los grupos acilo del aceite, y por otro, la generación y posterior evolución de una amplia variedad de compuestos de oxidación, tanto primarios (hidroperóxidos) como secundarios tales como epóxidos, aldehídos, keto-dienos o compuestos con grupos alcohol. Una característica muy importante de esta técnica es que permite estudiar

simultáneamente todos estos aspectos sin que sea necesaria ninguna modificación de la muestra y prácticamente sin el uso de reactivos.

Los resultados de este estudio demuestran que, aunque los aceites de soja virgen y refinado estudiados tienen composiciones similares en grupos acilo, los niveles más bajos de tocoferoles y esteroles presentes en el aceite virgen, junto con su mayor contenido de ácidos grasos libres, dan como resultado una menor estabilidad oxidativa y una degradación más rápida de éste en comparación con el aceite refinado bajo las condiciones analizadas. Por lo tanto, el conocimiento de la composición de aceites comestibles en componentes minoritarios podría resultar útil para establecer niveles de calidad, en relación con la estabilidad oxidativa y el contenido en compuestos bioactivos beneficiosos para la salud humana, dentro de aceites de un mismo origen botánico y con grados de insaturación similares. En este contexto, la metodología basada en ID MEFS-CG/EM podría constituir una herramienta útil.

Otra estrategia comúnmente empleada para evitar o reducir la oxidación de lípidos, es la **adición de compuestos con potencial capacidad antioxidante**. En este sentido cabe señalar a los **tocoferoles**, debido a las propiedades antioxidantes que se les han atribuido clásicamente, y de hecho, son ampliamente empleados por la industria con este fin. La cantidad de estos compuestos que puede ser añadida a los aceites vegetales está regulada por la legislación europea, que permite la adición de **α-tocoferol (α-T) y γ-tocoferol (γ-T)** bajo el principio *quantum satis*, es decir sin límite establecido, a los aceites refinados, excepto los de oliva. Sin embargo, esta normativa contrasta con la información proporcionada por la literatura científica, según la cual, este tipo de compuestos pueden comportarse como prooxidantes en determinadas circunstancias. A esto hay que añadir que en muchos casos las metodologías utilizadas para evaluar el efecto de estos compuestos ofrecen una visión muy limitada del proceso de oxidación, y que además, la mayoría de los estudios sobre este tema se han realizado con aceites desprovistos de sus tocoferoles originales, y por tanto diferentes de los aceites comerciales. Teniendo en cuenta todo esto, se establecieron los **objetivos 2 y 3**. Éstos tienen como finalidad profundizar en el conocimiento del efecto de la adición de proporciones variables de **α-T (0,002, 0,02, 0,2, 2 y 5% en peso) y de γ-T (0,02, 0,2 y 2% en peso) sobre la estabilidad oxidativa y el proceso de oxidación de aceite de soja comercial** bajo condiciones de almacenamiento acelerado. Para ello se ha empleado la RMN de ^{1}H, prestando atención tanto a la degradación de los grupos acilo

como a la formación y posterior evolución de una amplia variedad de productos de oxidación.

Los resultados obtenidos con esta metodología muestran que proporciones de α-T entre el 0,02 y el 5%, y de 0,2 y 2% de γ-T reducen la estabilidad oxidativa del aceite de soja, más cuanto mayor es la concentración de tocoferol. Sin embargo, dicho efecto es más pronunciado para el α-T. Esto se deduce de una mayor velocidad de degradación de los grupos acilo del aceite y de un aumento más rápido de la concentración de hidroperóxidos al aumentar la cantidad de tocoferol. A pesar de ello, un mayor grado de enriquecimiento con tocoferol provoca un aumento en el tiempo necesario para alcanzar la polimerización total del aceite, y este efecto es más marcado en el caso del γ-T. El enriquecimiento de aceite de soja con α- o γ-T modifica el proceso de oxidación del mismo según aumenta la concentración de tocoferol, forzando la formación de (*Z*,*E*)-hidroperóxidos, reduciendo y retrasando la de sus isómeros (*E*,*E*), que no aparecen hasta etapas más avanzadas del proceso de oxidación, de manera más marcada en el caso del α-T. Esto tiene consecuencias directas en la naturaleza y proporciones relativas de los productos de oxidación secundarios originados a partir de los hidroperóxidos, de tal forma que, según aumenta el nivel de tocoferol añadido, se observa una mayor generación de compuestos con configuración (*Z*,*E*), como hidroxi-dienos conjugados y keto-dienos, e incluso aparecen compuestos no detectados generalmente bajo las condiciones de almacenaje acelerado estudiadas, tales como (*Z*, *E*)-2,4-alcadienales.

A pesar de estas similitudes entre los efectos de α- y γ-T sobre la evolución del aceite de soja, las diferentes intensidades con que cada uno de estos tocoferoles afecta a la velocidad de degradación de grupos acilo y a la generación y descomposición de hidroperóxidos, dan lugar a diferencias importantes en la tasa de generación de la mayoría de los productos de oxidación secundarios en los aceites enriquecidos con α- y γ-T. Así, contrariamente al α-T, el γ-T retrasa la aparición de la mayoría de los productos de oxidación secundarios en relación con el aceite sin enriquecer, más cuanto mayor es la proporción añadida, con la excepción de algunos epóxidos, que se detectan antes en el aceite con un 2% de γ-T que en el original. Estos resultados ponen de manifiesto la dificultad de definir el efecto de estos tocoferoles como antioxidante o prooxidante, ya que, aunque aceleran la degradación del aceite, más cuanto mayor es la concentración de tocoferol, la polimerización total se observa más tarde.

En vista de los efectos de α- y γ-T en la oxidación de aceite de soja, y teniendo en cuenta que, tal y como se ha descrito en la literatura científica, ciertos **aminoácidos** podrían constituir una alternativa para reducir la oxidación de aceites, se planteó el **objetivo 4**. El propósito fue investigar el potencial **efecto antioxidante de la L-lisina**, un aminoácido esencial, en el **proceso de oxidación de un aceite de soja refinado**. Para lograr dicho objetivo, tanto el aceite de soja en su estado original como este mismo aceite enriquecido con lisina en un 1 ó 2% en peso se sometieron a calentamiento a 70 ° C en condiciones de agitación, y sus respectivas evoluciones fueron estudiadas mediante RMN de ^{1}H. Se prestó atención tanto a la degradación de grupos acilo del aceite como a la generación y evolución de compuestos de oxidación. Además, también se analizó mediante RMN de ^{1}H la evolución del principal tocoferol presente en el aceite de soja, γ-T, a lo largo del proceso de oxidación.

Los resultados muestran que la adición de lisina retrasa considerablemente la degradación de los grupos acilo del aceite, así como la generación de compuestos de oxidación primarios y secundarios, a la vez que conserva durante más tiempo el contenido de γ-T del aceite. Todo esto alarga la vida útil del aceite de soja, de manera similar para los dos niveles de lisina probados. En las muestras enriquecidas con este aminoácido también se observó una disminución en la concentración máxima alcanzada por algunos tipos de productos de oxidación secundarios, especialmente en el caso de los bien conocidos tóxicos aldehídos oxigenados α,β-insaturados, probablemente debido a su reacción con la lisina.

Cuando los procesos de oxidación de lípidos tienen lugar en presencia de aminoácidos, estos últimos pueden sufrir cambios en su estructura debido a reacciones de oxidación o a reacciones con compuestos procedentes de la oxidación lipídica, tales como hidroperóxidos, aldehídos o epóxidos, entre otros. No obstante, en la mayoría de los estudios llevados a cabo para evaluar la influencia de la presencia de aminoácidos en la oxidación de lípidos no se tiene en cuenta el efecto de este proceso en el aminoácido en sí. Teniendo en cuenta que el estudio de las posibles modificaciones de la lisina durante el proceso de oxidación del aceite de soja podría proporcionar información sobre el mecanismo de acción de este aminoácido, se abordó el **objetivo 5**. Para ello, se estudió el **proceso de cooxidación de la lisina y del aceite de soja** a lo largo de un tratamiento térmico a 70 ºC con agitación. El seguimiento de la evolución del aceite se llevó a cabo mediante RMN de ^{1}H, mientras que para investigar los posibles cambios en

la lisina se emplearon la RMN de ^{1}H y también cromatografía líquida acoplada a espectrometría de masas (CL/EM). Esta última técnica, debido a su mayor especificidad y sensibilidad comparada con la RMN de ^{1}H, podría proporcionar más información acerca de las modificaciones sufridas por el aminoácido.

Los resultados de este estudio confirman los del anterior con respecto al efecto de la lisina en el proceso de oxidación del aceite de soja. Con respecto a la lisina, el estudio de una serie de extractos acuosos obtenidos a lo largo del proceso de oxidación del aceite enriquecido con este aminoácido, muestra que las principales modificaciones de la lisina detectadas bajo estas condiciones se deben a la generación de aductos con diversos aldehídos. Los más abundantes y los primeros en detectarse fueron aquéllos formados por el extremo Nε de la lisina con n-alcanales de bajo peso molecular y malondialdehído, seguidos por los formados con aldehídos oxigenados α,β-insaturados y con (*E*)-2-alquenales. Cabe destacar que es la primera vez que se pone de manifiesto la presencia de varios aductos de lisina con aldehídos de naturaleza variable en un sistema modelo lipídico complejo. De entre todos estos aductos, solamente el más abundante, Nε-formil-lisina, se detectó también por RMN de ^{1}H.

Estudio bajo condiciones de digestión *in vitro*

La oxidación de lípidos, además de ocurrir durante el almacenamiento y/o procesado de alimentos, también puede darse durante la digestión. De hecho, tanto el desarrollo de oxidación, como el grado de lipólisis alcanzado durante este proceso, son factores que determinan la bioaccesibilidad de los nutrientes de origen lipídico. En este contexto, aunque se ha demostrado que la composición en grupos acilo de aceites vegetales influye en el grado de oxidación observado bajo condiciones de digestión *in vitro*, aún se sabe muy poco sobre el impacto de los componentes minoritarios que se encuentran presentes de forma natural en los aceites comerciales en las reacciones que ocurren durante la digestión. Teniendo esto en cuenta, se planteó el **objetivo 6**, en el que se analiza **la influencia del conjunto de componentes minoritarios presentes naturalmente en aceite de soja comercial sobre su proceso de digestión *in vitro***, prestando atención a las reacciones de lipólisis y de oxidación. Para lograr el objetivo propuesto, dos aceites de soja, uno virgen y otro refinado, con grados de insaturación similares y diferentes perfiles de componentes minoritarios, se sometieron a un proceso de digestión *in vitro*. El estudio simultáneo de los procesos de lipólisis y de oxidación lipídica, y de la evolución del γ-T naturalmente presente en los aceites de soja se llevó a

cabo mediante RMN de ^{1}H. Además, se empleó también la técnica de MEFS-CG/EM, aplicada al espacio de cabeza de las muestras, para obtener información adicional relativa a los procesos de oxidación, debido a su mayor sensibilidad y especificidad en relación con la RMN de ^{1}H. Los resultados de este estudio muestran que, aunque en el grado de lipólisis no se observan diferencias entre los dos aceites estudiados, el menor contenido de tocoferoles y escualeno y el mayor nivel de ácidos grasos libres en el aceite virgen parecen favorecer un grado de oxidación ligeramente más alto durante la digestión del aceite de soja virgen que durante la del refinado. Esto se concluye a partir de la aparición de hidroperóxidos e hidroxidienos conjugados en el digestato del aceite virgen, pero no en el del refinado, y por una mayor generación de aldehídos volátiles en el primero. Además, también se observó una reducción algo mayor de la bioaccesibilidad de γ-T durante la digestión del aceite virgen.

El grado de oxidación y la extensión del proceso de lipólisis durante los procesos de digestión *in vitro* también pueden verse afectados por el grado inicial de oxidación del aceite, así como por la presencia de otros nutrientes en el sistema, entre éstos las proteínas. Además, el estudio de la evolución de los diferentes tipos de productos de oxidación lipídica durante la digestión, así como de los parámetros que influyen en esta evolución, se consideran temas de especial interés debido a la potencial toxicidad de algunos de estos compuestos, los cuales podrían llegar a reaccionar con diferentes componentes de los tejidos del tracto gastrointestinal o incluso ser absorbidos. Teniendo en cuenta todo esto se abordaron los **objetivos 7 y 8**. El **objetivo 7** está encaminado al estudio del **comportamiento durante la digestión *in vitro* de aceites de soja virgen y refinado levemente oxidados**, obtenidos mediante un tratamiento térmico a 70 ºC, mediante RMN de ^{1}H y MEFS-CG/EM. El interés se centró en las reacciones de lipólisis, en la evolución del proceso de oxidación durante la digestión, y en evaluar el impacto de dos proporciones diferentes de ovoalbúmina, una proteína ampliamente utilizada en la industria alimentaria, en ambos procesos. Asimismo, se siguió por RMN de ^{1}H la evolución del γ-T en los casos en que fue posible. Con respecto al **objetivo 8,** su propósito fue **investigar la biodisponibilidad de los principales nutrientes lipídicos y compuestos de oxidación presentes en muestras de aceite de soja altamente oxidadas después de la digestión *in vitro***. Dichas muestras fueron obtenidas después de un proceso de almacenamiento acelerado prolongado a 70 ºC. El estudio se llevó a cabo mediante RMN de ^{1}H, analizando el grado de lipólisis obtenido,

el desarrollo de oxidación durante la digestión, la evolución de diferentes clases de productos de oxidación presentes en las muestras estudiadas y la generación de compuestos de oxidación adicionales, si los hubiera. Además, se abordó el estudio de la influencia en todos estos procesos de la presencia de dos proporciones diferentes de ovoalbúmina.

Los resultados obtenidos revelan que el grado de oxidación inicial del aceite de soja influye negativamente en la extensión de la lipólisis durante la digestión *in vitro*, reduciendo la biodisponibilidad de los principales nutrientes lipídicos, entre los que se incluyen algunos ácidos grasos esenciales como los ω-3. Esto podría deberse a la reacción de ciertos productos de oxidación con enzimas lipolíticas. Sin embargo, no se observa una gran diferencia entre las muestras con grado de oxidación inicial más alto o más bajo. Además, en el caso de los aceites altamente oxidados, se produce un mayor grado de oxidación a lo largo del proceso de digestión *in vitro*, evidenciable por la degradación de grupos acilo poliinsaturados, lo cual también contribuye a reducir su bioaccesibilidad.

Con respecto a la evolución de los productos de oxidación, no todos muestran el mismo comportamiento durante la digestión. Así, mientras que la concentración de hidroperóxidos disminuye considerablemente, los epóxidos, keto-dienos, dihidroxi-derivados y n-alcanales persisten en gran medida, mostrando algunos de ellos un incremento en su concentración después de la digestión. Entre éstos, los epóxidos son los compuestos de oxidación más abundantes presentes en los digestatos. Por el contrario, cuando los aceites sometidos a digestión *in vitro* presentan cantidades notables de aldehídos oxigenados α,β-insaturados tóxicos, su concentración disminuye considerablemente, posiblemente debido a su reacción con proteínas que se encuentran en los jugos digestivos. A pesar de ello, parte de ellos sigue estando bioaccesible para su absorción.

Si bien la menor proporción de ovoalbúmina probada no afecta significativamente a la evolución de los compuestos de oxidación o al grado de lipólisis, cuando la proporción de ovoalbúmina es alta, la lipólisis aumenta de forma importante en los aceites ligeramente oxidados, mejorando la bioaccesibilidad de los principales nutrientes lipídicos y también de γ-T. Sin embargo, este efecto es mucho menos pronunciado en las muestras con un alto grado de oxidación. No obstante, la presencia de una alta proporción de ovoalbúmina durante la digestión de aceites muy oxidados

mejora ligeramente la bioaccesibilidad de ciertos nutrientes lipídicos, ya que se reduce la extensión de las reacciones de oxidación, y provoca una reducción en los niveles de aldehídos tóxicos presentes en los digestatos. Estos resultados evidencian la importancia de consumir alimentos que posean una composición variada de nutrientes tales como proteínas, las cuales, debido a su acción durante la digestión, pueden reducir el nivel de compuestos tóxicos y facilitar el proceso de lipólisis.

ABSTRACT

Study under accelerated storage conditions

The oxidation of food lipids entails both the degradation of major and minor lipid nutrients, and the generation of a very broad range of oxidation compounds that not only can negatively affect the color, odour or flavour of food products, but also can have toxic effects. Therefore, the search for strategies to limit the occurrence of lipid oxidation has attracted the interest of food industry and researchers for years. In this respect, **the use of oils with greater oxidative stability** could be a means to limit oxidative reactions. In this context, although the influence of the composition in acyl groups of vegetable oils on their oxidation rate has been well documented, much less is known about the effect that the pool of minor components present in edible oils may have on their oxidative stability. In this context, the **first objective** of this doctoral thesis was raised. It was aimed at analyzing the **influence of the minor components present in commercial soybean oil**, considered as a whole, **on its oxidative stability and its oxidation process** under accelerated storage conditions. In order to fulfill this objective, two soybean oils, one virgin and the other refined, having very similar proportions of the different kinds of acyl groups but different profiles of minor components, were subjected to an accelerated storage process at 70ºC and their evolution analyzed throughout time. The analysis of the oil in minor components was performed by means of Direct Immersion Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (DI SPME-GC/MS). Given that a crucial issue when assessing lipid oxidation is the methodology employed, ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) was used to monitor on the one hand the degradation rate of oil acyl groups, and on the other the generation and further evolution of a wide variety of oxidation compounds, both primary (hydroperoxides) and secondary such as epoxides, keto-dienes, hydroxy-derivatives and aldehydes. One very important feature is that this technique allows one to study simultaneously all these subjects without any modification of the sample and without the use of reagents.

The outcomes of this study reveal that, although both the virgin and the refined soybean oils have similar compositions in acyl groups, the lower levels of tocopherols and sterols found in the virgin oil, together with its higher free fatty acid content, result in a lower oxidative stability and in a faster oil degradation under the assayed conditions

when compared to the refined one. Therefore, the knowledge of the edible oil composition in minor components could be useful to establish quality levels, relative to oxidative stability and content in bioactive compounds beneficial for human health, within oils of the same botanical origin and similar unsaturation degrees. In this context, DI SPME-GC/MS could constitute a useful tool.

A commonly employed strategy to avoid or at least reduce the occurrence of edible oil oxidation is **the addition of compounds with potential antioxidant ability**. In this regard **tocopherols** stand out due to their classically attributed antioxidant properties, and indeed they are widely employed by industry with this aim. The amount of these compounds that can be added as antioxidants to vegetable oils is regulated by the European legislation, which allows the addition of both **α-tocopherol (α-T) and γ-tocopherol (γ-T)** to refined oils on the basis of the *quantum satis* principle, this is without an established limit, except for refined olive oils. This contrasts with the information provided by the scientific literature, according to which prooxidant effects of these compounds have been reported under certain conditions. To this, it must be added that, in many cases, the methodologies used to assess the effect of these compounds only provide a very limited view of the oxidation process, and that most of the studies regarding this issue have been conducted with oils devoid of their original tocopherols, which are different from commercial oils. Bearing all this in mind, **objectives 2 and 3** were established. These were aimed at obtaining further knowledge about the **effect of varying proportions of α-T (0.002, 0.02, 0.2, 2 and 5% in weight) and of γ-T (0.02, 0.2 and 2% in weight) on the oxidative stability and the oxidation process of commercial soybean oil** under accelerated storage conditions by means of ^{1}H NMR, paying attention to both acyl group degradation and oxidation product formation and subsequent evolution.

The results obtained show that α-T proportions between 0.02 and 5% and γ-T levels of 0.2 and 2% reduce the oxidative stability of soybean oil, more as higher is the tocopherol concentration. However, the effect is more pronounced for α-T. This has been proved through a higher rate of degradation of acyl groups and a more elevated pace of hydroperoxide concentration increase in line with tocopherol enrichment level. In spite of this, as the tocopherol concentration gets higher, an enlargement in the time needed to reach oil total polymerization is observed, more marked in the case of γ-T.

Both the α- and the γ-T enrichments modify the oxidation pathway of soybean oil in line with tocopherol concentration, forcing the generation of increasing levels of (*Z*,*E*)-hydroperoxides while reducing and postponing that of their (*E*,*E*)-counterparts to the most advanced stages of the oxidation process, more markedly in the case of α-T. This has direct consequences in the nature and relative proportions of secondary oxidation products, in such a way that oxidation compounds with (*Z*,*E*)-isomerism such as conjugated hydroxy-dienes and (*Z*,*E*)-keto-dienes are also generated earlier and in higher concentration as the tocopherol level rises, and even (*Z*,*E*)-2,4-alkadienals, not usually detected under the accelerated storage conditions used, appear.

Despite these similarities between the actions of α- and γ-T on soybean oil evolution, the above mentioned variations in the intensity of the effects provoked by each of these tocopherols on the rates of acyl group degradation and of hydroperoxide generation and decomposition, lead to important differences in the generation rate of most secondary oxidation products in the oils enriched with α- and γ-T. Thus, contrary to α-T, γ-T delays the appearance of most secondary oxidation products compared to the non enriched oil, the higher the enrichment degree, with the exception of some epoxides, which are detected earlier than in the non-enriched oil. All these findings evidence the difficulty to define the effect of these tocopherols either as antioxidant or prooxidant since, although they accelerate oil degradation in line with tocopherol concentration, the oil total polymerization is observed later the higher the enrichment level.

In view of the outcomes regarding the effect of α- and γ-T on soybean oil oxidation, and taking into account that, on the basis of their described antioxidant ability on edible oils, **amino acids** could constitute an alternative to reduce edible oil oxidation, **objective 4** was raised. The purpose was to investigate the potential antioxidant **effect of L-lysine**, an essential amino acid, **on the oxidation process of a refined soybean oil**. To achieve this objective, systems composed of soybean oil and 1% or 2% proportions of lysine were subjected to heating at 70º C under stirring conditions, and their evolution monitored by ^{1}H NMR and compared with that of soybean oil. Attention was focused on oil acyl group degradation and oxidation compound generation and evolution. In addition, the progress throughout the oxidation process of the concentration of the main tocopherol present in soybean oil, γ-T, was also monitored by ^{1}H NMR.

The results of this study show that the addition of lysine considerably delays the degradation of the oil acyl groups and the generation of both primary and secondary oxidation compounds, while preserving the oil content of γ-tocopherol, especially at the highest enrichment level. All this extends the shelf life of soybean oil to a similar degree for the two lysine levels tested. A diminution in the maximum concentration reached by some types of secondary oxidation products is also observed in the lysine-enriched samples. This is particularly noticeable for aldehydes, whose concentration is markedly reduced, above all that of the toxic oxygenated α,β-unsaturated ones, probably due to their reaction with lysine.

On another issue, when lipid oxidation processes take place in presence of amino acids, these latter can also undergo oxidation, or react with lipid oxidation products like hydroperoxides, aldehydes or epoxides among others, giving rise to a wide variety of compounds. Nonetheless, most of the studies focused on the effect of amino acids on lipid oxidation disregard the effect of this process on the amino acid itself. Considering that the knowledge of the modifications undergone by lysine in parallel with the soybean oil oxidation process could provide information about the mechanism of action of this amino acid, **objective 5** was tackled. This involves the study of the **cooxidation process of lysine and soybean oil** throughout a thermal treatment at 70 ºC under stirring conditions. The evolution of the oil was monitored by ^{1}H NMR, while that of lysine was investigated by ^{1}H NMR and also by Liquid Chromatography followed by Mass Spectrometry (LC/MS), which due to its greater specificity and sensitivity in relation to ^{1}H NMR, could provide more information about amino acid modifications.

The results of this study confirm those of the previous one regarding lysine effect on soybean oil oxidation process. Concerning lysine evolution, the analysis by LC/MS of a series of aqueous extracts obtained from the oil containing lysine throughout the oxidation process reveals that, under the studied conditions, the main modifications of lysine detected are due to the generation of lysine-aldehyde adducts. The most abundant ones and the first to be detected were those formed at the reactive Nε position with low molecular weight n-alkanals and malondialdehyde, followed by the ones with oxygenated α,β-unsaturated aldehydes and (*E*)-2-alkenals, this being the first time that several lysine adducts with aldehydes of varying nature have simultaneously been detected in a complex food model system. Of all these lysine-aldehyde adducts, only Nε-formyl-lysine, the most abundant one, was detected by ^{1}H NMR.

Study under *in vitro* digestion conditions

In addition to storage and processing, digestion could also lead to lipid oxidation. Thus, the occurrence of oxidation, together with the extent of lipolysis achieved during this process, are determining factors for the bioaccessibility of lipid nutrients. In this context, the composition in acyl groups of vegetable oils has been shown to have a great influence on the oxidation extent, but very little is known about the **impact of naturally present oil minor components on the reactions occurring during digestion** of edible oils having similar compositions in acyl groups. Bearing this in mind, in **objective 6** the **influence of the pool of minor components present in soybean oil on its *in vitro* digestion process** was addressed, paying attention to lipolysis and oxidation reactions. To achieve the proposed goal, two commercial virgin and refined soybean oils, having similar unsaturation degrees and different minor component profiles, were submitted to a gastrointestinal *in vitro* digestion process. ^{1}H NMR was used to study simultaneously the lipolysis extent, the occurrence of lipid oxidation and the evolution of the γ-T naturally present in the oil. In addition, headspace SPME-GC/MS was employed to obtain further information relative to the occurrence of oxidation, due to its higher sensitivity and specificity in relation to ^{1}H NMR. The results arising from this study shows that, although no differences in the lipolysis extent were observed between the two oils subject of study, the lower content of tocopherols and squalene and the higher level of free fatty acids in the virgin oil seems to lead to a slightly greater oxidation extent during digestion than in the refined type. This is evidenced by the appearance of conjugated hydroperoxy- and hydroxy-dienes in the digestate of the virgin oil but not in that of the refined one, and by a greater generation of volatile aldehydes in the former. In addition, a somewhat greater reduction of γ-T bioaccessibility during digestion was also observed in the virgin oil.

The extent of oxidation during the *in vitro* digestion of oils, as well as the lipolysis degree, can also be affected by the initial degree of oxidation of the oil and by the presence of other nutrients in the system, among them proteins. Moreover, the study of the evolution of different types of oxidation products during digestion, and of the parameters influencing this evolution, are considered relevant issues due to the potential toxicity of some of these compounds, which could react with different biological tissues of the gastrointestinal tract and/or be absorbed reaching other targets. Taking all this into account **objectives 7 and 8** were outlined. **Objective 7** tackles the study of the

behaviour of slightly oxidized virgin and refined soybean oils, coming from a thermal treatment at 70 ºC, during *in vitro* digestion by means of ^{1}H NMR and SPME-GC/MS. The main objectives were to analyze lipolysis extent and oxidation during digestion, and to assess the impact of two different proportions of ovalbumin, a protein widely employed by food industry, on both processes. At the same time γ-tocopherol fate was monitored, when possible, by ^{1}H NMR. Regarding **objective 8**, its purpose was to **investigate the bioaccessibility after *in vitro* digestion of major lipid nutrients and oxidation compounds present in highly oxidized soybean oil samples**, obtained after a prolonged accelerated storage process at 70 ºC. This was assessed by means of ^{1}H NMR, through the extent of lipolysis, the occurrence of oxidation during digestion, the fate of different classes of lipid oxidation products already present in the samples subject of study and the generation of additional oxidation compounds, if any. Moreover, the influence on all these processes of the presence of two different proportions of ovalbumin was tackled.

The results obtained reveal that an initial oxidation degree in soybean oil negatively influences the lipolysis extent during *in vitro* digestion, reducing the bioaccessibility of the major lipid nutrients, which include some essential fatty acids like the ω-3 ones. This could be due to the reaction of certain oxidation products with lipolytic enzymes. However, great differences are not observed between samples with lower and higher initial oxidation degrees. Moreover, in the case of the highly oxidized oils, a greater extent of oxidation occurs throughout *in vitro* digestion, perceivable from the degradation of polyunsaturated acyl groups, which also contributes to reduce the bioaccessibility of of these latter.

With regard to the evolution of oxidation products, not all of them exhibit the same behavior during digestion. Thus, while hydroperoxide concentration decreases considerably, epoxides, keto-dienes, dihydroxyderivatives and n-alkanals persist to a great extent, some of them showing a concentration increase after digestion. Among these, epoxides are the most abundant oxidation compounds found in the digestates. Conversely, when the very reactive and toxic oxygenated α,β-unsaturated aldehydes are present in noticeable amounts in the oils subjected to digestion, their concentration markedly diminish, possibly to their reaction with proteins of the digestive juices. In spite of this, part of them remain bioaccessible for absorption.

While the lower ovalbumin proportion tested does not significantly affect lipolysis or oxidation or oxidation compound evolution, lipolysis is greatly enhanced when ovalbumin is added at a high level to slightly oxidized oils, improving the bioaccessibility of major lipid nutrients and also of γ-T. This effect is much less pronounced in the samples exhibiting a high oxidation degree. However, the presence of a high proportion of ovalbumin during the digestion of highly oxidized oils slightly improves lipid nutrient bioaccessibility by reducing the extent of oxidative reactions and causes a diminution in the levels of toxic aldehydes present in the digestates. These results evidence the importance of the intake of foods that contain a varied composition of nutrients like some proteins, due to their action during digestion, reducing the level of toxic compounds and increasing the lipolysis extent.

ABBREVIATIONS

A	Area
AG	Acyl groups
AS	Accelerated storage
Bp	Base peak
CD-OH	Conjugated dienes associated to hydroxides
CD-OOH	Conjugated dienes associated to hydroperoxides
DG	Diglycerides
DI	Direct immersion
DJ	Juices submitted to digestion conditions
D_2O	Deuterium oxide
DVB/CAR/PDMS	Divinylbenzene/ Carboxen/Polydimethylsiloxane
FA	Fatty acids
FDP-lysine	Nε-(3-formyl-3,4-dehydropiperidino)lysine
GC/MS	Gas Chromatography/ Mass Spectrometry
GOL	Glycerol
HNA-lysine	Nε-4-hydroxynonanoic acid-lysine
^{1}H NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
4-HPNE	4-Hydroperoxy-(*E*)-2-nonenal
L	Linoleic
Ln	Linolenic
L_{BA}	Lipid bioaccessibility
LC/MS	Liquid Chromatography/Mass Spectrometry
LYS	Lysine
MDA	Malondialdehyde
MG	Monoglycerides
MU	Monounsaturated
MW	Molecular weight
O	Oleic
OP	Oxidation product
RSO/RO	Refined soybean oil
S+M	Saturated plus modified acyl groups
SO	Soybean oil
SPME	Solid Phase Microextraction
TBARS	Thiobarbituric acid reactive substance assay
TG	Triglycerides
TMS	Tetramethylsilane
U	Unsaturated acyl groups
VSO/VO	Virgin soybean oil
α-T	Alpha-tocopherol
γ-T	Gamma-tocopherol

LIST OF MANUSCRIPTS

The present doctoral thesis relies on the 8 manuscripts listed below:

1- Assessment of soybean oil oxidative stability from rapid analysis of its minor component profile.
A.S. Martin-Rubio; P. Sopelana; María D. Guillén (**Submitted for publication**)

2- Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L., & Guillén, M. D. (2018). Prooxidant effect of α-tocopherol on soybean oil. Global monitoring of its oxidation process under accelerated storage conditions by ^{1}H nuclear magnetic resonance. ***Food Chemistry***, *245*, 312-323.

3- Martin-Rubio, A. S., Sopelana, P., & Guillén, M. D. (2018). A thorough insight into the complex effect of gamma-tocopherol on the oxidation process of soybean oil by means of 1h nuclear magnetic resonance. Comparison with alpha-tocopherol. ***Food Research International***, *114*, 230-239.

4- Martin-Rubio, A. S., Sopelana, P., & Guillén, M. D. (2019). The potential of lysine to extend the shelf life of soybean oil evidenced by ^{1}H Nuclear Magnetic Resonance. ***LWT- Food Science and Technology***, *105*, 169-176.

5- Study of the effect of the cooxidation of soybean oil and lysine on their respective evolutions: a combined assessment by ^{1}H NMR and LC/MS.
A.S. Martin-Rubio; P. Sopelana; F. Nakashima; T. Shibata; K. Uchida; M. D. Guillén (**Submitted for publication**)

6- Martin-Rubio, A. S., Sopelana, P., & Guillén, M. D. (2019). Influence of minor components on lipid bioaccessibility and oxidation during *in vitro* digestion of soybean oil. ***Journal of the Science of Food and Agriculture***. https://doi.org/10.1002/jsfa.9734

7- The key role of ovalbumin in lipid bioaccessibility and oxidation product profile during the *in vitro* digestion of slightly oxidized soybean oil.
A. S. Martin-Rubio; P. Sopelana; María D. Guillén (**Under revision in *Food and Function***).

8- ^{1}H NMR study of the *in vitro* digestion of highly oxidized soybean oil, focusing on the bioaccessibility of major lipid nutrients and of some oxidation compounds. Effect of the presence of ovalbumin.
A. S. Martin-Rubio; P. Sopelana; María D. Guillén (**Prepared for submission**)

INTRODUCTION

1. Lipid oxidation: the need to seek solutions to avoid this deleterious process for the nutritional value and safety of food

Foods are complex matrices that are composed of macronutrients like carbohydrates, proteins and/or lipids of diverse nature and of micronutrients like different kinds of vitamins, pigments or minerals. Among all these components, lipids have a special impact on food quality and safety, since their oxidation entails the degradation of their main and minor components and the generation of a very broad range of oxidation compounds that not only can negatively affect the colour, odour or flavour of food products, but also can have toxic effects (Esterbauer, 1993).

The oxidation course and, in consequence, the nature, amount and relative proportions of the compounds generated can vary depending on different factors such as lipid composition, temperature, time, aeration, etc (Martínez-Yusta et al., 2014). Thus, when oxidation occurs at low or medium temperatures, the degradation of lipids gives rise, in a first step, to the so-called primary oxidation compounds, which include hydroperoxides, and in some cases hydroxides, supporting conjugated diene systems with either (*Z*,*E*)- or (*E*,*E*)-isomerism. However, at high temperatures like the frying ones, studies performed by using ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) reveal that hydroperoxides are not generated, or if they are so, their degradation rate is too fast for them to be detected with this technique (Guillén & Ruiz, 2008). From the breakdown of these primary oxidation products a wide variety of secondary ones can be generated, such as different kinds of aldehydes, epoxides, alcohols and ketones (Frankel et al., 1982, 1988, 1990).

Among all these secondary products, aldehydes have been the most studied, and practically the only ones considered in most of the studies dealing with the monitoring of lipid oxidation (Carocho & Ferreira, 2013). Aldehyde determination constitutes a relevant issue, since this type of compounds not only can adversely affect the sensory properties and the acceptance of food, but also some kinds of aldehydes like the oxygenated α,β-unsaturated ones are well-known toxic compounds, due to their great ability to react with biological molecules like proteins and DNA (Guillén & Goicoechea, 2008).

Another group of oxidation products that include potentially toxic compounds is that constituted by epoxides, even though they are hardly considered in oxidation studies.

Only over the last few years this type of compounds are receiving increasing attention from some researchers like Brühl and coworkers (2016), according to whom epoxidized fatty acids are suspicious to be linked with different diseases, such as cardiac failure or respiratory distress syndrome, among others. Moreover, very recently, the citotoxicity on HepG2 cells of epoxystearic acid, an oxidation product derived from oleic acid, has been reported (Liu et al., 2018). The toxicity of epoxy fatty acids has been considered to rely on the high reactivity that the oxirane ring confers to these molecules (Greeene et al., 2000), and some studies have suggested that they may even cause cancer (Wilson et al., 2002). Also in recent years, several authors are stressing the need to determine epoxides to accurately assess the extent of lipid oxidation (Grüneis & Pignitter, 2018; Schaich et al., 2017). In fact, monoepoxides have been found to be one of the major groups of oxidized compounds formed at frying temperatures (Velasco et al., 2004), and epoxides have also been detected in considerable amounts during the oxidation of sunflower oil under accelerated storage (AS) conditions (Goicoechea & Guillén, 2010).

In addition to degradation of essential lipidic macronutrients and generation of deleterious products, oxidation can also provoke losses of minor lipidic nutrients considered to have bioactive properties like, for example, certain compounds with antioxidant ability such as tocopherols (Gottstein & Grosch, 1990; Wagner & Elmadfa, 2000; Wagner et al., 2004). Therefore, the occurrence of lipid oxidation reactions can be determinant for the nutritional value, shelf life and safety of foods.

For all the reasons commented above, the reduction of lipid oxidation has raised the interest not only of researchers but also of the food industry for decades. This has led to the search for strategies aimed at limiting the occurrence of oxidation reactions and alleviating their impact on the quality of lipidic food, especially edible oils, which make a great contribution to dietary lipids. In this respect, the use of oils with greater oxidative stability can constitute a medium to limit oxidative reactions. Concerning this issue, many works have been conducted where the influence of the composition in acyl groups of vegetable oils on their oxidation rate has been evidenced (Martínez-Yusta et al., 2014). However, less is known about the impact that the pool of minor components present in certain types of oils, which are the result of their botanical origin (Alberdi-Cedeño et al., 2017; Johnson et al., 2015; Phillips et al., 2002) and also of their processing (Jung et al., 1989), can exert on their behaviour under oxidative conditions. Regarding this topic, studies are usually intended to address the effect of specific

individual compounds or groups of compounds that, in general, are exogenously added to oils devoid of their original minor components (Dolde & Wang, 2011; Jung & Min, 1990; Koskas et al., 1984; Yoshida & Niki, 2003), so the extrapolation of the results obtained to commercial oils may not be entirely satisfactory. It must also be noticed that, aside from compounds with antioxidant ability, vegetable oils can also contain other minor components able to favour oxidation like, for example, free fatty acids (Waraho et al., 2011). Therefore, the separate analysis of the influence of different types of minor components on the oxidative stability of oils could not be enough to assess the impact that the group of minor components present in commercial oils could have on their behaviour under oxidative conditions. Having this in mind, it would be interesting to go deeper into the effect that the pool of minor components naturally present in edible oils, considered as a whole, could have on the oxidative stability and the behaviour under oxidative conditions of oils with similar compositions in acyl groups, as would be expected to be the case of oils of the same botanical origin.

2. The use of compounds with antioxidant ability to reduce the occurrence of lipid oxidation

A strategy to avoid, or at least reduce, the occurrence of lipid oxidation in edible oils is the addition of compounds with potential antioxidant ability. This latter practice has intensified over time due, firstly, to the existing trend of enriching food in polyunsaturated lipids, which are especially prone to oxidation, and secondly, to the general belief that there are compounds that act as antioxidants under any conditions and that the intensity of this effect can be increased in line with the enrichment level. In this regard, some of the compounds that have been subject of myriads of studies over years are tocopherols, the main compounds with antioxidant ability present in vegetable oils.

2.1. The effect of tocopherols on lipid oxidation

Among the four different tocopherols that can be found in vegetable oils (α-, β-, γ- and δ-), either α-tocopherol (α-T) or γ-tocopherol (γ-T) are the major ones depending on the type of oil (Alberdi-Cedeño et al., 2017; Gliszczynska-Swiglo et al., 2007), although α-T has been considered to have greater activity at the biological level than γ-T. This latter fact can be explained by the existence of an α-T transfer protein in the liver highly selective for this compound that causes the excretion of other tocopherols as

bile, or by the urine (Birringer et al., 2002), resulting in an α-T concentration in tissues 10 fold higher than that of γ-T (Wolf, 2006). These two compounds, whose chemical structures can be observed in Figure 1, differ in the number of methyl groups attached to the chromanol ring, which also determines differences in their hydrogen donor ability (Gottstein & Grosch, 1990) and therefore in their effect on lipid oxidation (Huang et al.,1994).

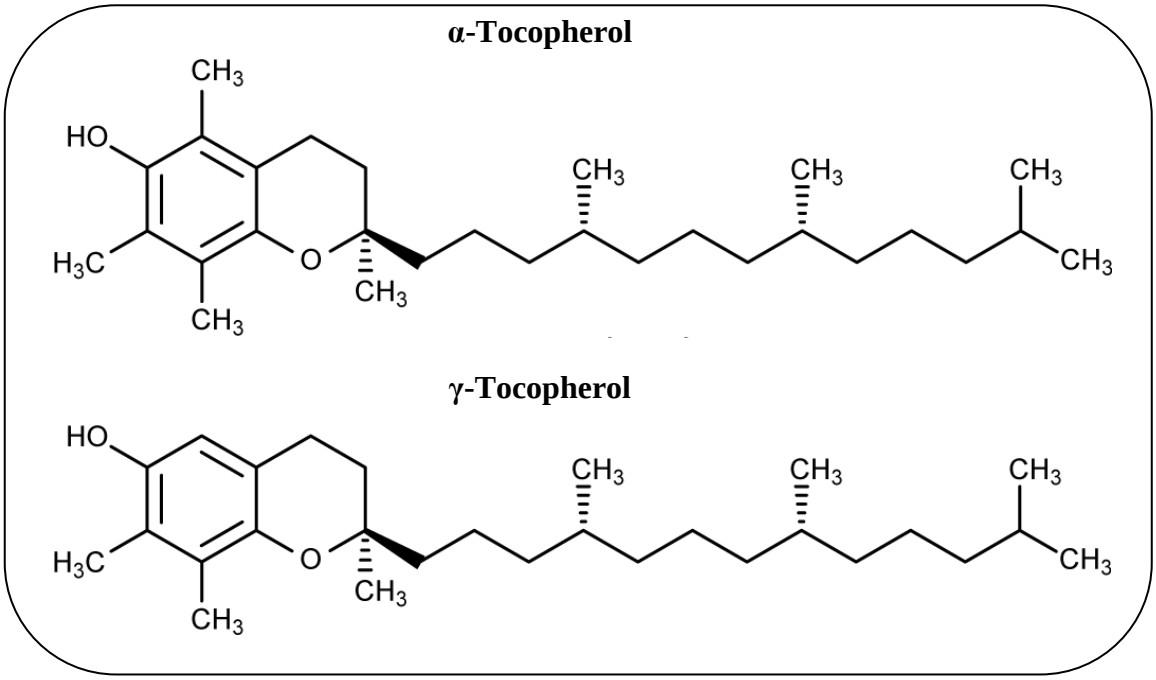

Figure 1. Chemical structures of α- and γ-tocopherols.

Due to their classically attributed antioxidant properties (Seppanen et al., 2010), tocopherols are commonly employed by industry with this aim. The amount of these compounds that can be added as antioxidants to vegetable oils is regulated by the European legislation, which allows the addition of both α-T and γ-T to refined oils on the basis of the *quantum satis* principle (Commission Regulation 1129/2011), this is without an established limit, except for refined olive oils. However, this lack of restriction in the use of tocopherols, and concretely of α-T, contrasts with that found when analyzing the extensive scientific literature regarding the effect of α-T on the oxidative stability of lipids, since controversial results have been obtained concerning this issue. Indeed, the outcomes of multiple **scientific works dealing with the antioxidant ability of α-T**, performed with various matrices like linoleic acid and its methyl ester (Cillard, et al., 1980; Koskas et al.,1984; Mäkinen et al., 2000; Morales et al., 2012; Terao & Matsushita, 1986), purified oil triglycerides (Blekas et al., 1995;

Dolde & Wang, 2011; Evans et al., 2002; Fuster et al.,1998; Huang et al., 1994; Isnardy et al., 2003, 2016; Jung & Min, 1990, 2015; Kulas & Ackman, 2001; Lampi et al., 1999; Ohm et al., 2005), vegetable oils (Tabee et al., 2008; Wagner & Elmadfa, 2000), fish oils (Zuta et al., 2007), lard (King et al., 2011), oil-in-water emulsions (Dwyer et al., 2012; Heinonen et al., 1997; Huang et al., 1994; Jayasinghe et al., 2013; Kamal-Eldin & Budilarto, 2014; Osborn-Barnes & Akoh, 2003; Wagner et al., 2004; Winkler-Moser et al., 2014), fried food systems (Neff et al., 2003; Nogala-Kalucka et al., 2005) and other types of food products like corn chips or avocado puree (Elez-Martínez et al., 2007; Rogalski & Szterk, 2015), evidence the prooxidant effect of α-T under certain circumstances, and especially depending on its concentration (Blekas et al., 1995; Cillard et al., 1980; Dolde & Wang, 2011; Huang et al., 1994; Isnardy et al., 2003; Jung & Min, 1990; King et al., 2011; Koskas et al., 1984; Terao & Matsushita, 1986; Wagner et al., 2004). Therefore, it is evident that an antioxidant effect of this compound should not be generally assumed.

In this context, it is worthwhile noticing that some researchers have warned about the risk of altering the balance among the several antioxidants present in oils by increasing the concentration of only one of them (Kamal-Eldin & Pickova, 2008). In the case of tocopherols, this could be due to the fact that the donation of an hydrogen atom to neutralize radicals derived from unsaturated lipid molecules could result in the generation of tocopheroxyl radicals, which are also able to give rise to the generation of lipid radicals under certain circumstances (Isnardy et al., 2003). However, when there are other compounds present in the medium able to regenerate tocopherols from tocopheroxyl radicals, the potential participation of these radicals in prooxidant reactions can be limited, what it would not be possible if they are in excess, leading to the promotion of further reactions of lipid oxidation (Kamal-Eldin & Appelqvist, 1996).

Although as mentioned above, α-T has been the most widely studied tocopherol, **γ-T has also received quite attention**. With respect to this tocopherol, it has been shown to exhibit a greater antioxidant efficiency in comparison with α-T (Gottstein & Grosch, 1990; Wagner et al., 2004), together with a lower susceptibility to exert a prooxidant action (Gottstein & Grosch, 1990; Huang et al., 1994; King et al., 2011). In fact, among the studies performed to investigate the effect of γ-T on the oxidative stability of a wide range of lipidic systems like linoleic acid and/or methyl linoleate (Gottstein & Grosch, 1990; Koskas et al., 1984), various types of purified vegetable oils (Fuster et al., 1998;

Huang et al., 1995; Isnardy et al., 2003; Jung & Min, 1990; Lampi et al., 1999), oil-in-water emulsions (Heinonen et al., 1997; Huang et al., 1994), olive and linseed oils (Wagner & Elmadfa, 2000), margarine (Azizkhani et al., 2011), fish oil enriched salad dressing (Let et al., 2007) and lard (King et al., 2011), only a few of them have shown a prooxidant effect of γ-T in different types of purified oils (Huang et al., 1995; Isnardy et al., 2003; Jung & Min, 1990), as well as in linseed oil (Wagner & Elmadfa, 2000).

As evidenced in the multiple studies mentioned, it is noteworthy and, to a certain extent, striking, that diverging results can be sometimes attributed to the methodology employed to monitor lipid oxidation, since the effect of any compound with antioxidant ability should be independent of the analytical tool employed to examine such effect. This evidences the unsuitability of some methodologies to assess accurately the real action of potential antioxidants like tocopherols. Indeed, some of the studies are based on the performance of non-specific classical methods, such as the peroxide value, the spectrophotometrical measurement of conjugated dienes (Cillard et al., 1980; Dolde & Wang, 2011; Isnardy et al., 2003; Jung & Min, 1990, Lampi et al., 1999), the *p*-anisidine value to measure the concentration of certain aldehydes altogether (Isnardy et al., 2003; Lampi et al., 1999) or the thiobarbituric acid reactive substance (TBARS) assay (Zuta et al., 2007). In addition, although other techniques able to provide specific information about the nature of the oxidation products generated such as High Performance Liquid Chromatography (HPLC) have also been used to assess the effect of tocopherols on lipid oxidation, the studies are in general limited to certain individual hydroperoxides, hydroxy-dienes or keto-dienes (Banni et al., 1996; Koskas et al., 1984; Mäkinen et al., 2000). In the case of aldehydes, despite the great variety of these compounds that can be generated during lipid oxidation processes, their evolution is in some cases addressed by only examining the behaviour of hexanal (Heinonen et al., 1997; Huang et al., 1994; Isnardy et al., 2003; Lampi et al., 1999; Ohm et al., 2005). Moreover, most studies exclude some of the most toxic oxidation products, as is the case of the oxygenated α,β-unsaturated aldehydes (Guillén & Goicoechea, 2008). In addition to all the commented limitations, another factor that can affect the conclusions obtained from these studies is the use of only one type of oxidation marker, generally hydroperoxides or a reduced number of aldehydes. It must be taken into account that **paying attention just to a very limited number of oxidation compounds could lead to misleading conclusions**, since the whole oxidation process is not being monitored

with a broad perspective (Martínez-Yusta & Guillén, 2019). In fact, the limitations of the classical methodologies traditionally used to assess lipid oxidation have been highlighted by some researchers with extensive experience in this field (Frankel, 1993; Frankel & Meyer, 2000; Schaich et al., 2017).

All the aforementioned demonstrates **the need to employ approaches that provide a global view of the oxidation process by monitoring a broad range of compounds**, in order to make an assessment of the effect of α-T and γ-T on the oxidative stability of food lipids as accurate and complete as possible. In this regard, ^{1}H NMR has been proved to be a powerful technique in the study of oils and fats, and it has significantly contributed to knowledge of the oxidation processes of oils and fats under very different conditions (Guillén & Ruiz, 2005; Guillén & Uriarte, 2009; Martínez-Yusta et al., 2014), providing insight not only into the degradation rate of the main oil and/or food lipids components (acyl groups), but also into the nature and concentration of a wide range of oxidation compounds, both primary and secondary, some of which are not usually determined by the methodologies generally employed to assess lipid oxidation. This evidences the great ability of ^{1}H NMR to offer a comprehensive view of the oxidation course, without the need for chemical changes in the sample or the use of reagents.

Considering all the above mentioned about the prooxidant potential, especially of α-T but also of γ-T, under certain conditions, and that their addition without an established limit is allowed in most refined edible oils, the concern arises about whether this practice could negatively affect oil stability and even human health. Moreover, given that some of the conclusions that can be extracted from the literature could result somehow contradictory, it would be of interest obtaining further knowledge about the effect of these two tocopherols on the oxidation process of edible oils.

In this context, another worth mentioning issue, which could also affect the results obtained when studying the effect of tocopherols on the oxidative stability of oils, is the type of oil used. In this sense, it must be noticed that in the available scientific literature most of the studies deal with the effect of tocopherols on stripped oils, which are devoid of their original tocopherol content (Dolde & Wang, 2011; Fuster et al., 1998; Huang et al., 1995; Isnardy et al., 2003; Jung & Min, 1990; Lampi et al., 1999; Ohm et al., 2005). It is obvious that the varying levels and proportions of endogenous tocopherols and of

other minor compounds could condition the effect observed in oils when exogenous tocopherols are added, but it is also true that oils available for consumption are not subjected to any stage aimed at removing tocopherols. Hence, the study of the tocopherol enrichment using oils containing all their components would reflect better the reality of the food industry and of the consumers. Otherwise, it would be difficult to extrapolate the results obtained to real practice and erroneous conclusions could be obtained about the most appropriate level of tocopherols to improve the oxidative stability of edible oils. To exemplify the importance of considering the original tocopherol content of the oil in this type of studies, it is worthwhile mentioning the work conducted by Frankel and coworkers in 1959, where the oxidative stability of stripped soybean oil was found to be greater than that of the original oil. Having this in mind, it would be possible that the effect of the same tocopherol dose was different depending on whether it is added to a stripped or to a non-stripped oil. In this regard, little is known about the effect of tocopherols on the oxidative stability and the oxidation process of real, commercial edible oils.

2.2. Amino acids as an alternative to other types of classical plant-derived compounds able to act as antioxidants

The interest in developing strategies to minimize lipid oxidation has led to the search for multiple sources of compounds of varying nature able to act as antioxidants. The classical compounds with antioxidant ability most widely employed consist of micronutrients like phenolic compounds or carotenoids among others, which once added to food, are supposed to delay the advancement of oxidation reactions (Carocho & Ferreira, 2013). However, as discussed in section 2.1, some compounds with antioxidant ability like α-T, but also others like carotenoids and even polyphenols, are surrounded by certain controversy due to their prooxidant effects under certain conditions (Eghbaliferiz & Iranshahi, 2016; Haila et al., 1996; Sakihama et al., 2002; Warner & Frankel, 1987). In this context, a great interest has been focused in the last decades on amino compounds like milk and soy proteins and a wide variety of protein hydrolysates of diverse origin, whose antioxidant ability has been proved in various food systems (Al-Shamsi et al., 2018; Bakota & Winkler-Moser, 2017; Cheetangdee & Benjakul, 2015; Elias et al., 2006; Hu et al., 2003; Singh et al., 2018).

With regard to the usefulness of free amino acids to reduce lipid oxidation, the antioxidant activity of several amino acids has been demonstrated for example in vegetable oils and oil-in-water emulsions (Ahmad et al., 1983; Hidalgo et al., 2006; Hwang & Winkler-Moser, 2017), even though prooxidative effects for some amino acids under certain conditions have also been reported (Ahmad et al., 1983; Marcuse, 1962; Riisom et al., 1980). Although it is true that the structure of amino acids and the presence of additional functional groups can determine their ability to react not only with oxygen and/or lipid-derived radicals, but also with already formed oxidation compounds (Stadtman, 1993; Viljanen et al., 2005), the medium and the conditions under which oxidation takes place can also have a great influence on the amino acid behaviour towards oxidation. In fact, dissenting results regarding the effect of amino acids can be observed in different systems and even in the same system but under varying conditions (Marcuse, 1962; Yee & Shipe, 1982). This reveals the importance of carrying out studies using models as similar as possible to those under which it is intended to reduce lipid oxidation.

As just stated, the functional groups present in certain amino acids affect their reactivity and the way these compounds act on the lipid oxidation course. In this sense, one of the amino acids that have been shown to exert antioxidant actions on food lipids is L-lysine (Ahmad, et al., 1983; Hwang & Winkler-Moser, 2017). Regarding this amino acid, whose structure is shown in Figure 2, it contains a free amino group at the Nε-position, considered to be very reactive, and it can take part in the chelation of transition metals (Ahmad al., 1983; Gutteridge et al., 1981), scavenge free radicals or even regenerate antioxidants like tocopherols (Kamal-Eldin & Appelqvist 1996). What is more, this amino acid could also play a role as antioxidant when it reacts with carbonyl compounds derived from lipid oxidation, since some products of this type

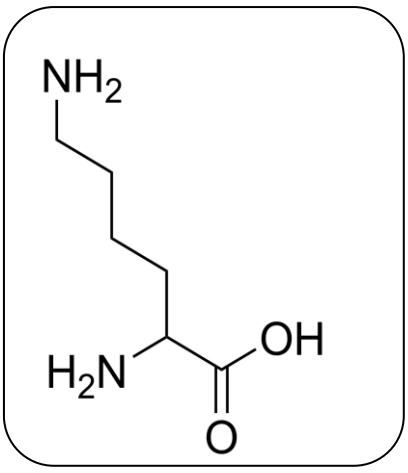

Figure 2. Chemical structure of L-Lysine

of reaction are said to slow down lipid oxidation (Alaiz et al., 1995a,b).

Although several **studies** have been conducted **on the effect of proteins, peptides or amino acids on lipid oxidation**, (Filippenko & Gribova, 2011; Gopala Krishna & Prabhakar, 1994; Hidalgo et al., 2006; Karel et al., 1966; Marcuse, 1962; Riisom et al., 1980,), similarly to that pointed out in section 2.1, the assessment of this action is based, in general, on classical methodologies that offer only a limited view of the oxidation process and fail to enable the advancement in the knowledge about the mechanism through which different amino acids might exert antioxidant effects. Among these, oxygen consumption, peroxide value measurement, spectrophotometrical determination of conjugated dienes or TBARS assay can be mentioned (Ahmad et al., 1983; Chen & Nawar, 1991; Farag et al., 1978; Filippenko & Gribova, 2011; Gopala Krishna & Prabhakar, 1994; Hidalgo et al., 2006a,b; Karel et al.,1966; Marcuse, 1962; Park et al., 2005 Riisom et al.,1980). This issue becomes particularly important in the study of the antioxidant potential of amino acids, because some of them, like for example lysine, are able to react with certain lipid oxidation products (Gardner, 1979; Schaich, 2008; Shimozu et al., 2011; Uchida, 2015; Zamora & Hidalgo, 1994); this fact can somewhat alter the perception of lipid oxidation and then underestimate the extent of this process, especially if only one type of oxidation marker is used. Therefore, an accurate assessment of the effect of amino acids on lipid oxidation requires the monitoring of a wide range of oxidation products throughout a prolonged period under oxidative conditions.

On another issue, **when lipid oxidation processes take place in presence of amino acids, these latter can also undergo oxidation**, and in fact, free radical transfer from lipids to proteins has been reported to occur early in lipid-protein cooxidation processes (Schaich & Karel, 1975, 1966, 1980; Schaich, 2008). In addition, as pointed out above, amino acids can react with lipid oxidation products like hydroperoxides (Gardner, 1979), aldehydes (Hidalgo & Zamora, 2016; Shimozu et al., 2011; Schaich, 2008; Uchida, 2015) and epoxides (Gardner et al., 1985; Schaich, 2008), this provoking changes in their structure, crosslinking reactions and the generation of a wide variety of compounds (Hidalgo & Zamora, 2016; Schaich, 2008), among other effects. Moreover, the compounds derived from these reactions can in turn take part in other processes like for example polymerization ones (Davies, 2005; Zamora & Hidalgo, 2005). At this point, it is worthwhile noticing that the cooxidation of lipids and proteins constitutes a

relevant issue in the area of food science, since it can cause losses in the biological value of foods, as well as changes in some organoleptic features (Schaich, 2014).

Lipid oxidation is a dynamic process, constantly changing and contributing new oxidants. In consequence, the mechanisms of action, and in case of taking place, the rate and type of reactions of amino acids with lipid oxidation products that are being generated, can change with the extent of oxidation. Thus, the analysis of how amino acids can be modified when they are involved in a cooxidation process with lipids constitutes a challenging task. In line with this, and with the aim of obtaining a global picture of the cooxidation process of lipids and amino acids, the oxidative modifications of amino acids, which represent the "other side of the coin" of these dynamic processes, constitute a very interesting subject that, due to its complexity, is often ignored despite the relevance of its consequences (Schaich, 2008). In this context, the study of the cooxidation process of amino acids and lipids with techniques that could provide a global perspective of the reactions taking place both in the oil and in the amino acid itself would be of great interest in order to advance within this field of knowledge.

3. The oxidation process and lipolysis during *in vitro* digestion: relevant issues related to the health effects of dietary lipids and influenced by several factors

The changes undergone by food lipids throughout the digestion process is an issue of great interest, since on the one hand they are sources of essential nutrients, and on the other because some lipids are very prone to oxidative degradation. Indeed, lipid digestion constitutes a critical step conditioning the bioaccessibility and health effects of dietary lipids. Thus, this physiological process not only determines the proportion of absorbable molecules, including essential fatty acids, but also it can promote lipid oxidation depending on the composition of the ingested products; this refers, among other factors, to lipid concentration (Li et al., 2011), the structure on which fatty acids are supported (triglycerides or partial glycerides), the length of the acyl group chains (Li et al., 2011; Zhu et al., 2013), the presence of free fatty acids (Golding & Wooster, 2010) or the initial oxidation degree of the lipids (Nieva-Echevarría et al., 2017a,b). Actually, it has been evidenced that **oxidation takes place during digestion** (Larsson et al., 2012; Nieva-Echevarría, et al., 2017a,b; Steppeler et al., 2016), and that this can provoke losses not only of major lipidic nutrients like fatty acids (Nieva-Echevarría et al., 2017a,b), but also of certain micronutrients considered to be able to exert an

antioxidant action such as tocopherols (Kenmogne-Domguia et al., 2014), reducing the bioaccessibility of all of them. This evidences the **relevance of the knowledge of the factors that can affect this process**. In this sense, similarly to that observed under AS conditions, the **lipid composition in acyl groups** has a great influence on the oxidation extent during digestion. Thus, a higher degree of oxidation has been observed during the digestion process of linseed oil than in that of sunflower oil (Nieva-Echevarría et al., 2017a,b), this latter being less unsaturated than the previous one.

In the case of **vegetable oils**, in addition to their unsaturation degree, attention should also be given to the influence of their **minor components** on the reactions occurring during digestion, particularly on oxidation. Indeed, as commented in section 1, these have a noticeable impact on the oxidative stability of oils and on their behaviour under oxidative conditions (Choe & Min, 2006). Among them, tocopherols, phytosterols, free fatty acids or metal ions can be mentioned, which despite their low concentrations, can either increase the oxidative stability of oils, or on the contrary, reduce it. Therefore, similarly to that argued under AS conditions, the composition in minor components could also affect the processes taking place during digestion. Bearing all this in mind, the analysis of the influence of the pool of minor components present in vegetable oils, on their *in vitro* digestion process paying attention to lipolysis and oxidation reactions is considered to be an interesting research issue.

Also in the context of lipid digestion, it is worth noticing that the **presence of oxidation products of different nature in the food bolus**, either generated *in situ* during digestion or arising from the diet, not only could impair to a certain extent the lipid digestive process by reducing lipolysis and, in consequence, the bioaccessibility of essential lipophilic nutrients (Márquez-Ruiz et al., 2008; Nieva-Echevarría et al., 2017a,b; Nik et al., 2010; Sánchez-Muniz et al., 2000), but also could have negative consequences for human health. In this respect, it is widely known that some oxidation products, like is the case of oxygenated α,β-unsaturated aldehydes, are very toxic compounds, (Guillén & Goicoechea, 2008). Moreover, although studied to a much lower extent than aldehydes, certain types of epoxides coming from both linoleic and oleic groups are also suspicious to have deleterious effects on human health (Brühl et al., 2016; Greene et al., 2000; Liu et al., 2018). Due to their reactivity, all these compounds could different modify biological components of the gastrointestinal mucosa (Kanazawa et al., 1988), and also be absorbed, thus reaching different targets

(Awada et al., 2012; Penumetcha et al., 2000; Wilson et al., 2002). Notwithstanding, in spite of the relevance of this topic, it could be said that very little is known about the evolution of oxidation products throughout digestion since, as far as we know, only a few studies have been carried out on this subject (Awada et al., 2012; Chalvardjian et al., 1962; Goicoechea et al., 2008; Kanazawa & Ashida, 1998a,b; Wilson et al., 2002), most of them focused on determining the absorbed fraction of only a few compounds (Awada et al., 2012; Wilson et al., 2002), so the information provided is very limited.

Finally, **another crucial factor** that can influence the evolution of digested lipids in the gastrointestinal tract, particularly difficult to assess due to the countless options that can be formulated, **is the presence of other nutrients, among them proteins**. As reported by Nieva-Echevarría and coworkers (2017c), the presence of proteins during the *in vitro* digestion of slightly oxidized sunflower and linseed oils can affect the extent both of lipolysis and of oxidation occurring during this process, favouring and limiting, respectively, both types of reactions. Moreover, the antioxidant potential of proteins could be enhanced during digestion due to the hydrolytic processes taking place, which allow the release of peptides, whose antioxidant potential can be even higher than that of proteins (Elias et al., 2006). Notwithstanding, it must also be noticed that the impact of proteins on oxidation reactions also depends on their composition, since for example iron-containing proteins (heme proteins) could promote oxidation during digestion (Van Hecke et al., 2014).

Regarding the **methodologies to study lipid digestion**, both i*n vivo* and *in vitro* systems have been used (Kostewicz et al., 2014). However, due to practical and ethical reasons, *in vitro* models, with their intrinsic advantages and drawbacks, are widely employed, either in its dynamic (Larsson et al., 2016; Maestre et al., 2013) or static versions (Steppeler et al., 2016; Van Hecke et. al., 2014a,b, 2016); these *in vitro* digestion models have been shown to correlate well with the data obtained *in vivo* (Kostewicz et. al., 2014). In the context of ***in vitro*** **methodologies**, it is worth noticing **the importance of simulating the complete digestion process** including especially gastric and duodenal stages. In this respect, several studies dealing with lipid oxidation during digestion focus only on the gastric phase (Gobert et al., 2014; Lorrain et al., 2012; Tirosh et al., 2015), disregarding the reactions occurring in the gut.

Among the existing techniques to monitor lipid hydrolysis during digestion, ^{1}H NMR has several advantages over titration and chromatographic methods (Nieva-Echevarría et al., 2018), and it has been proved to be extremely useful to estimate at the same time the different types of glycerides generated and the proportion of absorbable molecules. Thus, based on ^{1}H NMR analysis, several works have been recently conducted that have succeded in optimizing the methodology to simulate lipid digestion (Nieva-Echevarría et al., 2014, 2017a,b, 2018), based on the method of Versantvoort and coworkers (2005), achieving lipolysis degree close to those found *in vivo* (Golding & Wooster, 2010). Moreover, ^{1}H NMR allows one to monitor, simultaneously with lipolysis, the occurrence of lipid oxidation and the evolution of oxidation products already present in the samples (Nieva-Echevarría et al., 2017a,b).

It is worthwhile noticing that the deeper knowledge about the reactions taking place throughout digestion, the better assessment of the transformations undergone by lipids before their absorption and of their potential effect on human health. However, the multitude of factors that can affect this process still makes the study of lipid digestion a very complex and challenging task.

In summary, issues related to the importance of minor oil components in the oxidative stability of edible oils, as well as the in-depth study of the role played in the oxidation process of edible oils by some compounds to which antioxidant ability has been classically attributed and by others barely considered such as amino acids, will be the object of attention. Furthermore, the influence of several factors on the *in vitro* digestion of oils, considering both lipolysis and oxidation, will also be addressed.

AIMS AND OBJECTIVES

The structure of this doctoral thesis relies on **three main aims**, some of them encompassing several objectives.

AIM 1: STUDY OF THE INFLUENCE OF THE MINOR COMPOUNDS NATURALLY PRESENT IN COMMERCIAL SOYBEAN OIL ON ITS EVOLUTION UNDER ACCELERATED STORAGE CONDITIONS.

This aim is based on the achievement of one only objective: **Objective 1**

1. Analysis of the effect of the pool of minor components present in commercial virgin and refined soybean oils, determined by Direct Immersion SPME-GC/MS, on their evolution under accelerated storage conditions (70 ºC), monitored by ^{1}H NMR (Manuscript 1)

The consecution of this objective will give information about the influence that the minor components naturally present in oils of the same botanical origin with very similar composition in acyl groups can have on their evolution under oxidative conditions. This might contribute to establish criteria for the selection of vegetable oils with better oxidative stability among those from the same botanical source.

AIM 2: ASSESSMENT OF THE EFFECT OF ADDING ALPHA-TOCOPHEROL, GAMMA-TOCOPHEROL OR L-LYSINE ON THE OXIDATIVE STABILITY AND THE OXIDATION PROCESS OF COMMERCIAL SOYBEAN OIL SUBMITTED TO ACCELERATED STORAGE CONDITIONS.

For this purpose, the following objectives were established:

2.1. Study of the effect of enriching a commercial soybean oil with different proportions of α-tocopherol (0.002, 0.02, 0.2, 2 and 5% in weight) on its evolution under accelerated storage conditions at 70 ºC by means of ^{1}H NMR, paying attention to both acyl group degradation and oxidation compound generation and evolution. (Manuscript 2)

2.2. Assessment by means of ^{1}H NMR of the influence of different levels of γ-tocopherol (0.02, 0.2 and 2% in weight) added to a commercial refined soybean oil on acyl group degradation and oxidation compound generation and further evolution throughout an accelerated storage process at 70 ºC, and comparison with that of α-tocopherol. (Manuscript 3)

Up to now, the assessment of the effect of tocopherols on the oxidative stability of edible oils and on lipid oxidation processes has often been carried out on the basis of one type, or at most two, of oxidation markers, determined by means of classical methodologies that generally do not provide much information about how the oxidation process takes place, and that in some cases could lead to erroneous conclusions regarding the antioxidant ability of the tocopherols tested. Moreover, most of the studies on this issue have been performed with stripped oils, devoid of their original tocopherols, which are different from those commercially available.

With the consecution of the two goals here proposed, conducted with commercial oils already containing tocopherols and other types of minor components, the effect of α- and γ-tocopherols on soybean oil oxidation process will be analyzed in depth by ^{1}H NMR. This will allow to establish relationships among the evolutions of different types of oxidation products, thus obtaining a global view of the oxidation course. Moreover, the differences in the mechanism of action of α- and γ-tocopherols will become evident, as well as their effect on soybean oil at different concentration levels. This could provide useful practical information relative to the suitability of these types of tocopherols to be used as antioxidants in commercial edible oils. It should also be mentioned that the European Union allows the addition of both α- and γ-tocopherols to most refined oils on the basis of the *quantum satis* principle, this is without an established limit. In this sense, these studies will show if this rule could be considered adequate or if, on the contrary, it would be necessary to revise it.

2.3. Investigation of the effect of different proportions of L-lysine (1 and 2% in weight) on the evolution of a commercial refined soybean oil throughout a thermal treatment at 70 ºC by means of ^{1}H NMR, paying attention to the evolution of oil acyl groups and γ-tocopherol, and to the generation of a wide range of oxidation products. (Manuscript 4)

With the accomplishment of this goal, the action of L-lysine on the oxidation pathway of a commercial refined soybean oil will be assessed. Thanks to the global perspective of the oxidation processes that ^{1}H NMR allows one to obtain, a wide vision of the influence of this amino acid on oil oxidation, not achieved before, will be obtained. This will be useful to advance in the knowledge of the antioxidant action of amino acids in lipid matrixes and will provide information about the potential of this type of dietary amino compounds as antioxidants in edible oils and maybe in other lipid systems. Moreover, this study will enable to compare the antioxidant ability of lysine with that of the tocopherols before mentioned.

2.4. Study of the effect of the cooxidation of soybean oil and L-lysine, added in a proportion of a 2% in weight, on their respective evolutions throughout a thermal treatment at 70 ºC by combining ^{1}H NMR and LC/MS analyses. (Manuscript 5)

The simultaneous study of the cooxidation of L-lysine and soybean oil throughout the thermal treatment will provide some information about the potential modifications that this amino acid can suffer when it is involved in a dynamic oil oxidation process, and will make it possible to establish relationships between oil and amino acid evolutions. Therefore, this work might contribute to gaining further insight into the cooxidation process of lipids and amino acids, which is considered a relevant issue. In addition, the achievement of this objective will provide information about the potential of ^{1}H NMR to study lysine modifications.

AIM 3: STUDY OF THE *IN VITRO* DIGESTION PROCESS OF COMMERCIAL SOYBEAN OIL AND OF THE INFLUENCE OF THE OIL COMPOSITION IN MINOR COMPONENTS, OF ITS INITIAL OXIDATIVE STATUS AND OF THE PRESENCE OF DIFFERENT PROPORTIONS OF OVALBUMIN ON LIPOLYSIS AND OXIDATION REACTIONS.

The achievement of this aim relies on 3 objectives:

3.1. Study of the influence of the minor components present in commercial virgin and refined soybean oils, determined by Direct Immersion SPME-GC/MS, on lipid bioaccessibility and oxidation during gastrointestinal *in vitro* digestion, studied by ^{1}H NMR and SPME-GC/MS. (Manuscript 6)

The achievement of this objective will provide knowledge about to what extent the pool of minor components present in soybean oil, and by extension in other vegetable oils, could affect some important reactions undergone by lipids during *in vitro* digestion: lipolysis and oxidation, and also about the bioaccessibility of compounds with antioxidant ability like γ-tocopherol, the most abundant tocopherol in soybean oil.

Thus, the results of this study will show how the quality of edible oils, referred to their abundance in compounds able to exhibit antioxidant ability and to their concentration of free fatty acids, influences oil evolution during *in vitro* digestion and the bioaccessibility of compounds able to influence human health.

The information extracted from this study could also be useful to identify which types of edible oils might be more suitable in order to both limit the occurrence of oxidative reactions during digestion and favour the preservation of their bioactive components.

3.2. Analysis of the lipolysis extent, of γ-tocopherol fate and of oxidation compound evolution during the *in vitro* digestion of slightly oxidized virgin and refined soybean oils, and of the influence of two proportions of ovalbumin on these processes by combining ^{1}H NMR and SPME-GC/MS analyses. (Manuscript 7)

The consecution of this objective will provide information contributing to create a body of knowledge on the *in vitro* digestion process of edible oils with regard to the influence of lipid oxidation degree on some important reactions taking place during

digestion and to the evolution of oxidation products. It will also shed light on the influence of different amounts of ovalbumin, a non-heme protein, on lipolysis, lipid oxidation and γ-tocopherol bioaccessibility, what could be useful in order to optimize lipidic macronutrients and micronutrients bioaccessibility.

It should also be remembered the relevant role of the methodology used to carry out this study, since it provides results very difficult to obtain by other methods.

3.3. Study by ^{1}H NMR of the *in vitro* digestion of highly oxidized soybean oil, focusing on the bioaccessibility of major lipid nutrients and of some oxidation compounds, as well as of the influence of the presence of two different proportions of ovalbumin on this process. (Manuscript 8)

The study of the evolution of different types of oxidation products during digestion, and of the parameters influencing this evolution, as well as of the effect that a high initial oil oxidation level can have on lipid hydrolysis and oxidation during digestion, can be considered issues of primary interest in furthering the intricate task of assessing how dietary lipids can affect human health. Furthermore, the analysis of the fate of the several kinds of lipid oxidation compounds throughout the *in vitro* digestion process will provide a very valuable knowledge about which classes of dietary lipid oxidation products could decompose in the gut *versus* which ones could remain, thus being available for absorption in the intestinal lumen and/or for reaction with biological components.

Furthermore, the potential effect on health of many of the compounds present in the samples subject of study, and also of those able to be generated during digestion, increases the interest of studying the proposed issues.

EXPERIMENTAL DESIGN

Aim 1- STUDY OF THE INFLUENCE OF THE MINOR COMPOUNDS NATURALLY PRESENT IN COMMERCIAL SOYBEAN OIL ON ITS EVOLUTION UNDER ACCELERATED STORAGE CONDITIONS.

Objective 1 - Manuscript 1: Assessment of soybean oil oxidative stability from rapid analysis of its minor component profile.

1. Samples

The samples subject of study were two commercial soybean oils, one of them virgin (VSO), and the other one refined (RSO).

2. Characterization of the oils subject of study

2.1. Analysis of the the main oil components (acyl groups)

The molar percentages of the different types of oil acyl groups were determined by ^{1}H NMR, as in previous works (Guillén and Ruiz, 2003; Guillén and Uriarte, 2009, 2012).

2.2. Analysis of the minor oil components

Extraction of the minor oil components was performed by means of Direct Immersion Solid-Phase Microextraction (DI-SPME), following the methodology described by Alberdi-Cedeñoand coworkers (2017a). To this aim, a fiber of 65 μm StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB), acquired from Supelco (Bellefonte, PA, U.S.), was immersed directly into 6 ml of edible oil at room temperature for 45 minutes. The thermal desorption process of the extracted oil components and their subsequent separation was carried out in a gas chromatograph equipped with a mass spectrometry detector (GC/MS) in the same way described in the above mentioned work.

Identification of most of the extracted components was made by comparison of their retention times and mass spectra with those of commercial standards acquired from Sigma-Aldrich (St. Louis, MO, USA) and Larodan Fine Chemicals AB (Malmo, Sweden). Others were identified by matching of their mass spectra with spectra from a commercial library by more than 85% (W9N08, Wiley ver. 9.0 and NIST ver. 8.0 library) and also with those obtained from the literature.

Semi-quantification was based on arbitrary units of the base peak ion area counts divided by 10^5. The base peaks of the several compounds identified, together with their respective molecular weights, are displayed in Table S1 (see supplementary material of manuscript 1). All the determinations were carried out in duplicate in order to obtain a mean value with the corresponding standard deviation for each of the components studied.

3. Accelerated storage (AS) process

10 g portions of each oil sample were poured into plastic Petri dishes of 80 mm diameter for each of the days monitored throughout the AS process. These were heated at 70º C in a convection oven with circulating air but without forced convection, simulating AS conditions. The evolution of the samples was followed by ^{1}H NMR until their total polymerization

4. Monitoring by ^{1}H NMR of the evolution of VSO and RSO throughout the AS process

4.1. Operating conditions

The ^{1}H NMR spectra of the starting oils and of the corresponding aliquots taken throughout the AS process were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz. The weight of each aliquot was approximately 0.16 g. These were mixed in a 5 mm diameter tube with 400 µl of deuterated chloroform that contained 0.2% of non deuterated chloroform and a small amount (0.03%) of tetramethylsilane (TMS) as internal references. The acquisition parameters used were: spectral width 5000 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.744 s and pulse width 90º, with a total acquisition time of 8 min 55 s. The relaxation delay and acquisition time selected allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making possible their use for quantitative purposes. The experiments were carried out at 25 ºC, as in previous works (Guillén & Ruiz, 2003, 2005). Each sample was analyzed in duplicate, in order to obtain a mean value for the concentration of each of the studied components.

4.2. Identification of some components

The identification of the oil acyl groups and of the products formed throughout the AS process was carried out on the basis of the signal assignment shown in Table S2 (see

supplementary material of manuscript 1), made from bibliographic data and with the aid of several standard compounds. These were: (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, 4,5-epoxy-(*E*)-2-decenal and 12,13-epoxy-9(*Z*)-octadecenoic acid methyl ester (isoleukotoxin methyl ester), acquired from Sigma-Aldrich, 4-hydroxy-(*E*)-2-nonenal, 4-hydroperoxy-(*E*)-2-nonenal, *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, purchased from Cayman Chemical (Ann Arbor, MI, USA) and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica (Barcelona, Spain).

4.3. Quantitative data derived from ^{1}H NMR spectra

The molar percentages of the several kinds of oil acyl groups were estimated throughout the AS process as in previous studies (Guillén and Uriarte, 2012), by means of the following equations:

$Ln\% = 100(A_H/3A_I)$;

$L\% = 100(2A_G/3A_I)$;

$O\%\ (\text{or } MU\%) = 100(A_E/3A_I)\text{-}Ln\%\text{-}L\%$,

where A_H and A_G are the areas of the signals of *bis*-allylic protons of linolenic and linoleic groups, respectively (signals “H” and “G” in Table S2 of manuscript 1); given that their respective signals overlap to a certain extent, the total area corresponding to each of them was calculated using pure trilinolein and trilinolenin (Sigma-Aldrich) as references. A_I, in turn, is the area of the signal of the protons at *sn*-1 and *sn*-3 positions in the glycerol backbone of triglycerides, while A_E corresponds to that of mono-allylic protons (signals “I” and “E”, respectively, in Table S2 of manuscript 1).

The molar percentage of saturated acyl groups can be obtained by difference.

The concentrations of the different types of oxidation products were estimated as millimoles per mole of triglyceride (mmol/mol TG). The general equation to carry out this determination was the following:

$[OP] = [(A_{OP}/n)/(A_I/4)]*1000$,

where A_{OP} is the area of the signal selected for the quantification of each oxidation product (OP) and n the number of protons that generate the signal.

AIM 2: ASSESSMENT OF THE EFFECT OF ADDING ALPHA-TOCOPHEROL, GAMMA-TOCOPHEROL OR L-LYSINE ON THE OXIDATIVE STABILITY AND THE OXIDATION PROCESS OF COMMERCIAL SOYBEAN OIL SUBMITTED TO ACCELERATED STORAGE CONDITIONS.

Objective 2.1 - Manuscript 2: Prooxidant effect of α-tocopherol on soybean oil. Global monitoring of its oxidation process under accelerated storage conditions by ^{1}H nuclear magnetic resonance.

1. Samples

The starting oil was a soybean oil (SO) purchased from a local supermarket. Subsequently several samples of this oil enriched with α-T were prepared by adding 0.002, 0.02, 0.2, 2 and 5% in weight of α-T to the oil, and given the following designations: SO + 0.002, SO + 0.02, SO + 0.2, SO + 2 and SO + 5, respectively. α-Tocopherol with a purity of 98.2% was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The molar percentages of the different types of oil acyl groups were determined by ^{1}H NMR, as in previous works (Guillén and Ruiz, 2003, Guillén and Uriarte, 2009). These were 6.6 ± 0.4 for linolenic, 42.8 ± 0.4 for linoleic, 32.4 ± 1.0 for oleic and 18.2 ± 1.9 for saturated groups.

The average concentrations of the four tocopherols, in mg/kg, estimated by using a DI-SPME-CG/MS methodology previously developed in our laboratory (Alberdi-Cedeño et al., 2017) were the following: 130.0 for α-T, 781.7 for γ-T, 316.1 for δ-T and 21.9 for β-T.

2. AS process

Regarding the AS process, it is the same as that described in section 3. of the experimental design of objective 1.

3. Monitoring by ^{1}H NMR of the evolution of SO and of the SO enriched with different proportions of α-T

3.1. Operating conditions

Operating conditions were the same as those described in the section 4.1. of manuscript 1.

3.2. Identification of oil components

The identification of the various oil components was made on the basis of the signals assignments shown in Table S1 of manuscript 2. For this purpose, several standard compounds were used; these were (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E,E*)-2,4-hexadienal, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, (*E*)-2-penten-1-ol and 1-hexanol, acquired from Sigma-Aldrich, and 4-hydroperoxy-(*E*)-2-nonenal, 4-hydroxy-(*E*)-2-nonenal and 4,5-epoxy-(*E*)-2-decenal, purchased from Cayman Chemical (Ann Arbor, MI, USA).

3.3. Quantitative data estimated from ^{1}H NMR spectra

The molar percentages of the several kinds of acyl groups throughout the oils thermodegradative process were estimated as in previous studies (Guillén & Uriarte, 2012) employing the equations described in section 4.3. of the experimental design of objective 1. Some of the α-T signals (Baker & Myers, 1991) partially overlap with some signals of triglycerides, so their contribution was subtracted when determining the molar percentages of oil acyl groups.

The concentrations of the different types of oxidation products generated were estimated as mmol/mol TG, as described in the section 4.3. of manuscript 1.

4. Statistic and kinetic studies

The Microsoft Office Excel 2007 software was used to find equations that fit heating time and the concentrations of saturated+modified acyl groups, of hydroperoxides and of the conjugated dienes associated to them, in all the studied samples.

Objective 2.2 - Manuscript 3: A thorough insight into the complex effect of gamma-tocopherol on the oxidation process of soybean oil by means of ^{1}H Nuclear Magnetic Resonance. Comparison with alpha-tocopherol.

1. Samples

The samples subject of study were two refined soybean oils (RSO), of the same brand but from two batches, and those prepared by adding different proportions by weight of γ-T to one oil and of α-T to the other. The designations of the samples were the following: RSO1 (0% of γ-T added), RSO1+0.02γT (0.02%), RSO1+0.2γT (0.2%), RSO1+2γT (2%), RSO2 (0% of α-T added), RSO2+0.2αT (0.2%) and RSO2+2αT

(2%). γ-T with a purity ≥ 90% and α-T with a purity of 98.2% were acquired from Eisai Food & Chemical Co. Lltd (Tokyo, Japan) and from Sigma-Aldrich, respectively. Furthermore, γ-T with a purity of 98% was also purchased from Sigma-Aldrich to conduct a complementary assay aimed at checking if γ-T purity could have any influence on the results obtained.

2. AS process

This was carried out in the same way as that described in section 3 of the experimental design of objective 1.

3. Monitoring by ^{1}H NMR of the evolution of RSO and of the RSO oil samples enriched with different proportions of either γ-T or α-T

3.1. Operating conditions

Operating conditions were the same as those described in the section 4.1. of objective 1.

3.2. Identification of some components

The identification of the oil acyl groups and of the products formed throughout the AS process was carried out on the basis of the signal assignment shown in Table S1 of manuscript 3, made from bibliographic data and with the aid of several standard compounds.

3.3. Quantitative data derived from ^{1}H NMR spectra

The molar percentages of the several kinds of acyl groups throughout the oils thermodegradative process were estimated as indicated in section 3.3. of the experimental design of objective 2.

The concentrations of the different types of oxidation products generated were estimated as as millimoles per mole of triglyceride mmol/mol TG, as described in the section 4.3. of objective 1.

4. Statistical analysis

The significance of the differences in the concentrations of the different kinds of oxidation products was determined between samples RSO1 and in RSO1+0.02γT by t-student test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

Objective 2.3 - Manuscript 4: The potential of lysine to extend the shelf life of soybean oil evidenced by ^{1}H Nuclear Magnetic Resonance.

1. Samples

The oil employed was a refined soybean oil (RSO). Each one of the samples enriched with L-lysine (RSO+LYS) was prepared by adding either 1% or 2% (RSO+LYS1 or RSO+LYS2) by weight of lysine.

L-lysine with a purity ≥ 98% was purchased from Cymit Quimica (Barcelona, Spain).

2. Oxidation process

10 g samples of RSO and of the RSO+LYS oils were prepared in several beakers of 6.5 cm diameter (250 ml), one per day of sampling. These were placed in a multiple magnetic stirrer with calefaction and heated at 70º C. Aliquots were taken periodically from each respective beaker throughout the oil oxidation process for their study by ^{1}H NMR. The evolution of the samples was monitored until the stirring magnet stopped rotating due to the polymerization of the oil. The oxidation process was carried out in duplicate in order to obtain average values for all the studied compounds.

3. Monitoring by ^{1}H NMR of the evolution of RSO and of the lysine-enriched oils throughout the oxidation process

3.1. Operating conditions

Operating conditions were the same as those described in the section 4.1. of manuscript 1.

3.2. Identification of some compounds

The identification of the oil acyl groups, of γ-tocopherol and of the oxidation products formed throughout the oxidation process was carried out on the basis of the signal assignment shown in Table S1 (see supplementary material of manuscript 4).

3.3. Quantitative data estimated from the ^{1}H NMR spectra

The molar percentages of the several kinds of oil acyl groups throughout the oxidation process were estimated as in previous studies (Guillén & Uriarte, 2012), employing the equations described in section 4.3. of the experimental design of manuscript 1.

The concentrations of γ-T and of the different types of oxidation products generated were estimated as mmol/mol TG, in the way described in the supplementary material of manuscript 4.

Objective 2.4 - Manuscript 5: Study of the effect of the cooxidation of soybean oil and lysine on their respective evolutions: a combined assessment by ^{1}H NMR and LC/MS.

1. Samples

The samples subject of study were a refined soybean oil (RSO) purchased from a multinational company and the same oil enriched with 2% by weight of L-lysine (RSO+2LYS).

The L-lysine used had a purity ≥ 98% and was purchased from Sigma-Aldrich. The molar percentages of the different types of oil acyl groups were determined by ^{1}H NMR, as in previous works (Guillén & Uriarte, 2012). These were 6.3±0.1 for linolenic, 49.5±0.1 for linoleic, 25.5±0.3 for oleic and 18.7±0.3 for saturated groups. Samples of RSO and of RSO+2LYS (8 g of oil in all cases) were put in beakers of 5 cm diameter, one per day of sampling. Due to the difficulty of uniformly distributing lysine into the oil, RSO+2LYS samples were prepared by directly weighing 8 g of RSO in each beaker and then adding 2% by weight of lysine.

2. Oxidation process

Samples of RSO and of RSO+2LYS (8 g of oil in all cases) were placed on a multiple magnetic stirrer heated at 70 ºC and stirred at 180 rpm with magnets of 4.5 cm long. Samples submitted to oxidative conditions for different periods were taken throughout the oxidation process for their study. Their evolution was monitored until the stirring magnet stopped rotating due to the polymerization of the oil.

3. Monitoring by ^{1}H NMR of the evolution of RSO and of RSO+2LYS samples throughout the oxidation process

The ^{1}H NMR spectra both of the original RSO and RSO+2LYS samples and of these samples after being submitted to oxidative conditions over different periods of time were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz, as in

previous works (Guillén & Uriarte, 2012). Operating conditions were the same as those described in the section 4.1. of objective 1.

The determination of the oil acyl groups and of some of their derived products formed throughout the oxidation process was carried out from the ^{1}H NMR spectra signal assignments shown in Table S1 (see supplementary material of manuscript 5).

The molar percentages of the several kinds of oil acyl groups throughout the oxidation process were estimated as in previous studies (Guillén & Uriarte, 2012) following the procedure described in section 4.3. of the experimental design of objective 1. The concentrations of the different types of oxidation products generated were estimated as mmol/mol TG, as described in the section 4.3. of objective 1.

4. Extraction of lysine and some of its derivatives from the RSO+2LYS sample after being submitted for different periods of time to oxidative conditions

Lysine and some of its derivatives formed during the oxidation process were extracted from the corresponding RSO+2LYS sample as follows: 0.16 g of each sample were poured into a 1.5 ml Eppendorf microtube and mixed with 800 µl of the extraction solvent. Several deuterated solvents were tested to extract the amino acid and its derivatives from the system and to study their recovery by means of ^{1}H NMR: water, water with different methanol percentages, methanol and acid water (0.5 M of HCl, pH close to 1.5). The best extraction efficiency was achieved using acid water, so this was the solvent chosen. After adding the solvent, each Eppendorf microtube was shaken for 10 min with an automatic tube stirrer and then centrifuged for another 10 min. The aqueous phase was taken out with a pipette, filtered through a 0.45 µm filter (GL Science Inc., Tokyo, Japan) using a 1 ml syringe (Terumo corporation, Tokyo, Japan) and poured into another Eppendorf microtube. The extracts were analyzed both by LC/MS and by ^{1}H NMR. It must be pointed out that milliQ water was used to obtain the extracts intended for the LC/MS analysis, while deuterated water was used for ^{1}H NMR.

4.1. Study of the extracts by LC/MS

The aqueous extracts obtained from the RSO+2LYS sample throughout the oxidation process were studied by LC/MS. However, due to analytical requirements, in this case it was necessary to add milliQ water to these extracts before their analysis in order to make the pH less acid (near 2.0).

The LC/MS chromatograms of the aqueous extracts of RSO+2LYS sample were obtained using a Waters Xevo TQD LC/MS equipment. Sample volumes of 10 μl each were injected into an Imtakt, WAA24 Intrada Amino Acid column (100 mm x 2 mm x 3 μm). A discontinuous gradient of solvent A (H_2O containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) was used as follows: 20% B at 0 min, 75% B at 7 min and 99% B at 7.1 min. Mass spectrometric analysis was performed in TIC mode using positive ion chemical ionization (cone potentials 20 V and 35 V).

The identification of lysine derivative**s** was achieved, on the one hand, by comparing the mass spectra of the compounds detected in the TIC chromatograms obtained with cone potentials 20 V and 35 V with those of lysine adducts obtained in the laboratory by making react lysine with n-alkanals (formaldehyde, propanal, butanal, hexanal, heptanal, octanal and nonanal), as in the study conducted by Kawai and coworkers (2006). For this purpose, lysine (50 mM) was incubated with 50 mM of each alkanal in the presence of H_2O_2 (50 mM) in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C. Furthermore, bibliographic data were also used to tentatively identify some of the compounds present in the aqueous extracts.

All the identified lysine derivatives were quantified by measuring the area of their corresponding mass spectra base peaks in the chromatograms obtained with cone potential 20 V. The quantifications were made for them to be useful for comparisons between the samples, not to achieve absolute concentrations of each of the compounds formed.

The evolutions of lysine and their derivatives were monitored only up to the 22nd day of the oxidation process; afterwards, the oil polymerization degree impaired the extraction of lysine and lysine derivatives in the aqueous phase, so it was not possible to obtain extracts directly comparable to the previous ones.

4.2. Study of the extracts by ^{1}H NMR

The aqueous extracts obtained from the RSO+2LYS sample throughout the oxidation process were also studied by ^{1}H NMR. The procedure followed was the same as for the lipid samples (see section 3) but, in this case, 600 μl of the aqueous extract were taken directly from the Eppendorf microtube (see section 4) and placed in a NMR tube for analysis.

Identification of the compounds in these extracts was made from the assignment of the ^{1}H NMR spectral signals obtained taking as reference standard compounds. To this aim L-lysine, Nε-formyl-lysine, Nα-acetyl-lysine and Nε-acetyl-lysine were purchased from Cymit Quimica. The chemical shifts, multiplicities and assignments of their signals are given in Table S2 (see supplementary material of manuscript 5).

The quantification of lysine and its derivatives by ^{1}H NMR constitutes one of the goals of this work, so the procedure developed for this purpose is described in the "Results and discussion" section of manuscript 5.

AIM 3: STUDY OF THE *IN VITRO* DIGESTION PROCESS OF COMMERCIAL SOYBEAN OIL AND OF THE INFLUENCE OF THE OIL COMPOSITION IN MINOR COMPONENTS, OF ITS INITIAL OXIDATIVE STATUS AND OF THE PRESENCE OF DIFFERENT PROPORTIONS OF OVALBUMIN ON LIPOLYSIS AND OXIDATION REACTIONS.

Objective 3.1 - Manuscript 6: Influence of minor components on lipid bioaccessibility and oxidation during *in vitro* digestion of soybean oil.

1. Samples subject of study

The samples subject of study were two commercial soybean oils: one of them virgin (VO) and the other refined (RO).

2. *In vitro* digestion

Samples (0.5 g) of the two oils were digested following the same procedure as in previous works (Nieva-Echevarría et al., 2017a) based on the static *in vitro* gastrointestinal model developed by Versantvoort and coworkers (2005) and slightly modified in our laboratory in order to reach a higher level of lipolysis (Nieva-Echevarría et al., 2016). This involves a three-step procedure to simulate digestive processes in the mouth, stomach and small intestine, by sequentially adding the corresponding digestive juices (saliva, gastric juice, duodenal juice and bile), whose composition is given in Table S1 (see supporting information of manuscript 6). The digestion experiment started by adding 6 mL of saliva to each of the oil samples. After 5 min of incubation, 12 mL of gastric juice were added and the mixture was rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. 1 hour after starting the gastric digestion, pH was set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring *in vivo*. After 2 h of gastric digestion, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice were added. Subsequently, pH was set between 6 and 7, and the mixture was rotated again at 40 rpm and incubated at 37±2ºC for 4 h.

All the reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich: α-amylase from *Aspergillus oryzae* (10065, ~30 U/mg); pepsin from porcine gastric mucosa (P7125, ≥400 U/mg protein); amano lipase A from *Aspergillus niger* (534781, ≥120,000 U/g); pancreatin from porcine pancreas (P1750);

lipase type II crude from porcine pancreas (L3126, 100-500 U/mg protein (using olive oil, 30 min incubation)) and bovine bile extract (B3883). Two digestion experiments, each including duplicate samples of the two oils, were performed. Blank samples corresponding to the mixture of juices submitted to digestive conditions were also taken for further analysis.

3. Lipid extraction of the digestates

Lipids of the digestates were extracted using dichloromethane (CH_2Cl_2) as solvent (HPLC grade, Sigma-Aldrich) and following the methodology employed by Nieva-Echevarría and coworkers (2015), slightly modified in a later study (Nieva-Echevarría et al., 2017a). This involves a three-stage liquid-liquid extraction process with 20 ml of CH_2Cl_2 each. Afterwards, to ensure a complete protonation of fatty acids and/or the dissociation of the potential salts formed, the remaining water phase was acidified to pH 2 with HCl (37%) and a second extraction was carried out, also in three steps.

4. Analysis by ^{1}H NMR

4.1. Operating conditions

The ^{1}H NMR spectra of the starting oils (VO and RO) and of the lipid extracts of their digestates (DVO and DRO) were acquired in quadruplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned lipid samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform, which contained TMS as internal reference (Cortec, Paris, France). The acquisition conditions were the same as those indicated in section 4.1 of the experimental design of objective 1.

4.2. Identification of some oil components

The identification of γ-T and of the oxidation products formed throughout digestion was carried out on the basis of the signal assignments shown in Table S2 (see supporting information of manuscript 6), made from bibliographic data and with the aid of standard compounds. These were γ-T, acquired from Sigma-Aldrich, and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica (Barcelona, Spain).

4.3. Quantification from ^{1}H NMR spectral data

4.3.1. Concerning the various types of glycerides

The molar percentages of triglycerides (TG%), diglycerides (1,2-DG% and 1,3-DG%), monoglycerides (2-MG% and 1-MG%) and glycerol (Gol%) in relation to the total of glyceryl structures present in the lipid samples were determined using the equations developed and validated in previous studies (Nieva-Echevarría et al., 2014, 2015). All these equations are given as supporting information of manuscript 6 (see equations [S1-S10]). Lipid bioaccessibility (L_{BA}), another parameter concerning lipolysis extent, was calculated by using equations [S11] and [S12].

4.3.2. Concerning lipid composition

The molar percentages of linolenic (Ln%), linoleic (L%), oleic (O%), and saturated plus modified (S+M%) acyl groups (AG) or fatty acids (FA), in relation to the total moles of AG+FA (NT_{AG+FA}) present in the various lipid samples were estimated as in a previous study (Nieva-Echevarría et al., 2017a) by using the following equations:

$$NT_{AG+FA}=Pc*A_F/2$$
$$U\%=100*(Pc*A_E/4)/NT_{AG+FA}$$
$$Ln\%=100*(Pc*A_H/4)/NT_{AG+FA}$$
$$L\%=100*(Pc*A_G/2)/NT_{AG+FA}$$
$$O\%=U\%–L\%–Ln\%$$
$$(S+M)\%=100–U\%$$

where A_F, A_E, A_H and A_G are the areas of signals F, E, H and G indicated in Table S1. It must be noted that due to partial overlapping of signals H and G, a previous correction of both areas must be carried out to properly assess the area corresponding to each one of them. For this purpose, trilinolenin and trilinolein were used as references.

4.3.3. Concerning oxidation compounds and γ-T

The concentrations of (*Z*,*E*)- and (*E*,*E*)-conjugated dienic systems supported on chains having either hydroperoxy or hydroxy groups, and of epoxides, expressed as millimoles per mole of AG+FA present (mmol/mol AG+FA), were also estimated as in a previous study (Nieva-Echevarría et al., 2017a) by using the following equation:

$$[OP] = [(A_{OP}/n)/(A_F/2)]*1000$$

where A_{OP} is the area of the signal selected for the quantification of each oxidation product (OP), shown in Table S1, and n the number of protons that generate each signal. This same approach was used to estimate the concentration of γ-T:

$$[\gamma\text{-T}] = [A_{\gamma T}/(A_F/2)]*1000$$

5. Study of oxidation during *in vitro* digestion by Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (SPME-GC/MS)

5.1. SPME-GC/MS analysis

The extraction of the volatile components of the several samples (0.5 g in a 10 ml screw-cap vial) was accomplished automatically by using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA).), in the same way as in previous works (Nieva-Echevarria et al 2017a).Given that the nature of the samples subjected to the digestion process (oil samples) is very different from that of the digested ones (basically aqueous samples), it is necessary to prepare mixtures of the non-digested oil samples with the digestive juices submitted to the digestion process, in the same proportions as in the digestates; this enables one to accurately assess the changes occurring throughout the *in vitro* digestion process. Therefore, the samples subject of study, which were analyzed in duplicate, were the following:

i) the digestates of the two types of soybean oil samples (DVO and DRO)
ii) the juices submitted to digestion conditions (DJ); and
iii) the mixtures made up of starting oil samples and juices submitted to digestion conditions (VO+DJ and RO+DJ).

The fiber used, coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm long), was acquired from Supelco (Sigma-Aldrich); this was inserted into the headspace of the sample and was maintained for 55 min at 50 ºC, after a pre-equilibration time of 5 min. The fiber containing the extracted components was desorbed for 10 min in the injection port (splitless mode with 5 min purge time) of a 7890A gas chromatograph equipped with a 5975C inert MSD with Triple Axis Detector (Agilent Technologies) and a computer operating with the ChemStation program.

The column used was a fused-silica capillary column (60 m long x 0.25 mm inner diameter x 0.25 μm film thickness, from Agilent J&W Advanced Capillary GC Columns), coated with a non-polar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The operating conditions were as follows: the oven temperature was set initially at 50º C (5 min hold) and increased to 290 ºC at 4 ºC/ min (2 min hold); the temperatures of the ion source and of the quadrupole mass analyser were kept at 230 ºC and 150 ºC respectively; helium was used as carrier gas at a pressure of 18.611 psi; injector temperature was held at 250 ºC; mass spectra were recorded at an ionization energy of 70 eV, and the data acquisition mode employed was scan. In order to avoid carry-over problems between samples, after each run the fiber was submitted to heating at 250 ºC for 20 min in the Fiber Cleaning and Conditioning Station of the CombiPAL autosampler.

A reference sample of known composition was periodically analyzed in order to verify not only the extraction efficiency and repeatability of the SPME fiber but also the performance of the equipment.

5.2. Identification of the compounds present in the headspace of the samples

Most of the components were identified by using commercial standards, acquired from Sigma-Aldrich. These were: pentanal (base peak: 86), hexanal (100), heptanal (114), octanal (128), nonanal (142), (*E*)-2-pentenal (84), (*E*)-2-hexenal (98), (*E*)-2-heptenal (112), (*E*)-2-octenal (126), (*E*)-2-nonenal (140), (*Z*,*E*)-2,4-heptadienal (110), (*E*,*E*)-2,4-heptadienal (110), (*Z*,*E*)-2,4-nonadienal (138), (*E*,*E*)-2,4-nonadienal (138), (*Z*,*E*)-2,4-decadienal (152), (*E*,*E*)-2,4-decadienal (152) and 2-pentyl-furan (138).

When standards were not available, matching of the mass spectra with those obtained from scientific literature or from a commercial library at higher than 85% (Wiley W9N08, Mass Spectral Database of the National Institute of Standards and Technology (NIST)), was taken as identification criterion.

5.3. Semi-quantification of the compounds present in the headspace of the samples

This was based on the area counts of the base peak (Bp) of the mass spectrum of each compound divided by 10^6. When the Bp of a compound overlapped with the same ion peak of the mass spectrum of another compound, an alternative ion peak was selected for the semi-quantification of the former. The area counts thus determined are useful for the comparison of the abundance of each compound in the different samples.

6. Statistical analysis

The significance of the differences on the several determinations made among the samples was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM).

Objective 3.2 - Manuscript 7: The key role of ovalbumin in lipid bioaccessibility and oxidation product profile during the *in vitro* digestion of slightly oxidized soybean oil.

1. Samples subject of study

The samples subject of study were two slightly oxidized commercial soybean oils: one virgin (VSx) and the other refined (RSx). In order to obtain these slightly oxidized oils, 10 g of each of them were weighed in glass Petri dishes of 80 mm diameter and placed in a convection at 70 ºC with circulating air for 4 and 5 days in the case of the virgin and the refined oils, respectively.

In addition, samples were prepared by mixing each of these two slightly oxidized oils with two different proportions of ovalbumin; 0.26 g of ovalbumin per g of oil in the samples with the low level of ovalbumin (LO) and 2.6 g of ovalbumin per g of oil in the ones with the high ovalbumin proportion (HO). Food grade ovalbumin was acquired from a protein manufacturer (Apasa SA, Astigarraga, Spain).

2. *In vitro* digestion

Samples (0.5 g) of the two oils were digested following the same procedure as in previous works (Nieva-Echevarría et al., 2017a,b), as described in section 2. of the experimental design of objective 3.1.

3. Lipid extraction of the digestates

Lipids of the digestates were extracted as indicated in section 3. of the experimental design of objective 3.1. Given that in the case of the samples with a high proportion of ovalbumin a strong emulsion is formed, the extraction was performed with the aid of a centrifuge in order to break up this emulsion. For this purpose, a Sigma 3K30 centrifugal machine working at 10,000 rpm was used (Sigma Laboratory Centrifuges, Germany), each extraction step lasting 10 min. This same extraction procedure was used for all the samples, without any differences in extraction efficiencies achieved when using either centrifugation or extraction with separating funnels in the case of the

samples without ovalbumin and those with a low proportion of this protein. All the dichloromethane extracts of each sample were mixed and the solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. Afterwards, these extracts were stored at -80 ºC until their analysis.

4. Analysis by ^{1}H NMR

4.1. Operating conditions

The ^{1}H NMR spectra of all the samples were acquired in quadruplicate using a Bruker Avance 400 spectrometer as indicated in section 4.1 of the experimental design of objective 3.1.

4.2. Identification of some oil components

The identification of the oil acyl groups, of partial glycerides, of γ-T and of the oxidation products present in the various samples was carried out on the basis of the signal assignments shown in Table S1 (see supplementary material of manuscript 7), made from bibliographic data and with the aid of several standard compounds. These were: γ-T, acquired from Sigma-Aldrich, and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica (Barcelona, Spain).

4.3. Quantification from ^{1}H NMR spectral data

4.3.1. Concerning the various types of glycerides

It was made as indicated in section 4.3.1. of the experimental design of objective 3.1.

4.3.2. Concerning lipid composition

The concentrations of linolenic (Ln) and linoleic (L) acyl groups and fatty acids were estimated in mmol/mol AG+FA present both in the starting oils and in the lipid extracts of the digested samples, by using the following equations:

$$[Ln] = [(A_H/4)/(A_F/2)]*1000$$

$$[L] = [(A_G/2)/(A_F/2)]*1000$$

where A_H and A_G are the areas of signals H and G indicated in Table S1. It must be noted that due to partial overlapping of signals H and G, a previous correction of both areas must be carried out to properly assess the area corresponding to each one of them. For this purpose, trilinolenin and trilinolein were used as references.

4.3.3. Concerning oxidation compounds

The concentrations of the different types of oxidation products, either present in the starting samples or generated throughout digestion, expressed as mmol/mol AG+FA, were also estimated as in a previous study (Nieva-Echevarría et al., 2017c) in the same way described in section 4.3.3. of the experimental design of objective 3.1. In the case of the samples digested with a high proportion of ovalbumin, signals due to components coming from this protein are perceived in the ^{1}H NMR spectra of their corresponding digestates, which overlap with those of some oil components. Therefore, some corrections must be made, especially in the case of epoxides. For this purpose, ovalbumin was added to the digestive juices after undergoing the digestion process and this mixture was extracted in the same way as the rest of digested samples; the relative areas of the different ovalbumin signals can be determined from the corresponding ^{1}H NMR spectrum, free of lipids. This enables one to subtract the area of the signals overlapping with those of lipid components in the spectra of the extracts obtained from the digested oil samples taking as a reference the signals that do not overlap with one another (see Figure S1 in the supplementary material of manuscript 7). It is worth noticing that while some signals coming from ovalbumin also overlap with those of *bis*-allylic protons (signals "H+G"), their area is very small in relation to that of the latter, and so can be ignored.

4.3.4. Concerning γ-T

The concentration of γ-T was estimated from signal "g" (see Table S1 of manuscript 7), in the same way described in section 4.3.3. of the experimental design of objective 3.1.

5. Study by SPME-GC/MS of the headspace composition of the digestates

5.1. SPME procedure

The extraction of the volatile components of the several digestates was made in the same conditions and following the same procedure as that described in section 5.1 of the experimental design of objective 3.1.

5.2. GC/MS study

5.2.1. Operating conditions

The GC/MS operating conditions were the same as those above stated in section 5.1 of the experimental design of objective 3.1.

5.2.2. Identification of the compounds present in the headspace of the samples

Most of the components were identified by using commercial standards, acquired from Sigma-Aldrich. See section 5.2. of the experimental design of objective 3.1.

5.2.3. Semi-quantification of the compounds present in the headspace of the samples

The semi-quantification of the components was performed as indicated in section 5.3 of the experimental design of objective 3.1

6. Statistical analysis

The significance of the differences on the several determinations made among the samples were determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM).

Objective 3.3 - Manuscript 8: ^{1}H NMR study of the *in vitro* digestion of highly oxidized soybean oil, focusing on the bioaccessibility of major lipid nutrients and of some oxidation compounds. Effect of the presence of ovalbumin.

1. Samples subject of study

The samples subject of study were two soybean oils, one virgin and the other one refined, both in an advanced degree of oxidation, containing both primary and a wide variety of secondary oxidation products. In order to obtain these samples, 10 g of both the virgin and the refined oils were weighed in glass Petri dishes of 80 mm diameter and submitted to an accelerated storage process at 70 ºC in a convection oven for 8 and 9 days, respectively.

In addition, samples were prepared by mixing each one of the highly oxidized oils with ovalbumin, acquired from a protein manufacturer. Two different proportions of ovalbumin were tested: 0.26 g of ovalbumin per g of oil and 2.6 g of ovalbumin per g of oil. Food grade ovalbumin was acquired from a protein manufacturer (Apasa SA).

2. *In vitro* gastrointestinal digestion

All the samples above mentioned (0.5 g of oil in all cases) were digested as described in section 2. of the experimental design of objective 3.1.

3. Lipid extraction of the digestates

The lipid extraction of all the samples was performed in the same way as stated in section 3. of the experimental design of objective 3.2.

4. Analysis by ^{1}H NMR

4.1. Operating conditions

The ^{1}H NMR spectra of all the samples were acquired in quadruplicate using a Bruker Avance 400 spectrometer as indicated in section 4.1 of the experimental design of objective 3.1.

4.2. Identification of some compounds

The identification of the different types of oxidation products present in the various samples was carried out on the basis of the proton signal assignments shown in Table S2 (see supplementary material of manuscript 8), made from bibliographic data and with the aid of several standard compounds, also given in the supplementary material.

4.3. Quantification from ^{1}H NMR spectral data

4.3.1. Concerning the various types of glycerides

It was made as indicated in section 4.3.1. of the experimental section of objective 3.1.

4.3.2. Concerning lipid composition

It was made as indicated in section 4.3.2. of the experimental section of objective 3.2.

4.3.3. Concerning oxidation compounds

The concentrations of the different types of oxidation compounds present both in the starting oils and in the extracts of the digested samples, expressed in mmol/mol AG+FA, were also estimated as in a previous study (Nieva-Echevarría et al., 2017a) in the same way described in section 4.3.3. of the experimental section of objective 3.2.

5. Statistical analysis

The significance of the differences on the several determinations made among the samples was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM).

RESULTS AND DISCUSSION

Manuscript 1

ASSESSMENT OF SOYBEAN OIL OXIDATIVE STABILITY FROM RAPID ANALYSIS OF ITS MINOR COMPONENT PROFILE

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ABSTRACT

In this work the effect of minor soybean oil components on its oxidative stability is tackled. To this aim, two soybean oils, one virgin and the other refined, both with very similar compositions in acyl groups, but differing in their minor component profile, were subjected to an accelerated storage process at 70 ºC. They were characterized by means of ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and Direct Immersion Solid-Phase Microextraction coupled to Gas Chromatography-Mass Spectrometry (DI SPME-GC/MS), while their evolution under oxidative conditions was monitored by ^{1}H NMR. The lower levels of tocols and sterols found in the virgin oil, together with its higher free fatty acid content, result in a lower oxidative stability when compared to the refined one. This is deduced from slower degradation of acyl groups and later generation of hydroperoxides, epoxides and aldehydes in the former oil. These findings reveal that virgin soybean oil quality is not necessarily higher than that of the refined type, and that a simple, fast analysis of minor components by DI SPME-GC/MS would enable one to establish quality levels within oils of the same botanical origin both in terms of composition in potentially bioactive compounds and of oxidative stability.

KEYWORDS: soybean oil, oxidative stability, ^{1}H nuclear magnetic resonance, direct immersion SPME, minor components, hydroperoxides, epoxides, aldehydes

1. INTRODUCTION

As is well known, highly unsaturated vegetable oils are particularly prone to oxidation; this process not only entails the loss of essential lipidic nutrients and the generation of potentially toxic compounds, but also the development of undesirable flavours which may provoke consumer rejection (Esterbauer, 1993; Guillén and Goicoechea, 2008). For a long time this has motivated the search for different types of strategies which avoid, or at least reduce, oxidative reactions in this type of foodstuffs. Related to this, it is widely recognized that minor oil component composition depends on botanical origin (Alberdi-Cedeño et al., 2017a) and on processing (Jung et al., 1989), both of which have a crucial effect on oxidative stability (Chen et al., 2011; Choe and Min, 2006; Kamal-Eldin, 2006). Thus, the simplistic assumption that oils of a higher unsaturation degree are more prone to oxidation may lead to erroneous conclusions if the influence of minor components is not taken into account (Nagy et al., 2016).

Minor oil components include not only several types of compounds with antioxidant ability like tocopherols, tocotrienols and other phenolic compounds, squalene, sterols or cyclic dipeptides (Alberdi-Cedeño et al., 2017b; Dessi et al., 2002; Kamal-Eldin and Appelqvist, 1996; Seppanen et al., 2010; Siger et al., 2008; Yoshida and Niki, 2003), but also other compounds reported to increase the susceptibility of oils to oxidation, such as free fatty acids (Choe, 2008). In this context, it must be pointed out that although most seed oils are usually subjected to a refining process before being destined for human consumption, some can also be consumed without being refined, which is to say as either virgin or cold-pressed oils. Therefore, bearing in mind that oil refining processes can reduce the concentration of tocopherols, tocotrienols, sterols, carotenoids and free fatty acids (Ferrari et al., 1996; Jung et al., 1989; Verleyen et al., 2002), it is possible to find commercial vegetable oils with very similar compositions in main components (acyl groups) but which differ in minor ones. Despite this, the possibility of considering the minor component profile as an indicator of the oxidative stability of edible oils seems to have been little exploited. Regarding this issue, some studies have been conducted in very recent years aimed at finding relationships between the composition of a wide range of vegetable oils and their resistence to oxidation (Ayyildiz et al., 2015; Bozan and Temelli, 2008; Castelo-Branco et al., 2016; Redondo-Cuevas et al., 2018; Yang et al., 2013). In these works, in general, attention is paid to both fatty acid composition and minor components like polyphenols, sterols, tocols, β-carotene,

lutein or chlorophyll, and oxidative stability is assessed by determining induction periods through the Rancimat method at temperatures of 110 or 120 ºC. However, in the study carried out by Yang et al. (2013) composition in main components was not analyzed, even though oils of the same botanical origin can display significant variations in their acyl groups profile due for example to climatic and cultivar effects (Brignoli et al., 1976; Zhang et al., 2012), which can also affect their oxidative stability.

Of the various classes of minor oil components, tocopherols deserve special attention due to their complex effect on oxidative stability of lipids (Dolde and Wang, 2011; Frankel et al., 1959; Kamal-Eldin and Appelqvist, 1996); this could contribute to explaining the lack of correlation between the abundance of this type of compounds and the oxidative stability of oils observed in some studies (Ayyildiz et al., 2015; Bozan and Temelli, 2008). In this respect, Zaunschirm et al. (2018) reported that more elevated ratios between the sum of γ- and δ-tocopherol concentrations and the concentration of α-tocopherol seemed to be related to a lower oxidation rate. To date, notwithstanding, understanding the effect of tocopherols on the oxidative stability of edible oils remains a challenge.

Another subject of further consideration when analyzing the above-mentioned studies should be the selection of the Rancimat test to assess oil oxidative stability, since the limitations of this method were already discussed some years ago by Frankel (1993). Thus, although according to some authors (Farhoosh and Moosavi, 2007), it can be useful to act as a "screening" test to identify oils with lower stability under frying conditions, the only oxidation products considered are volatile organic acids, mainly formic acid; this represents an extreme over-simplification of the oxidation process, during which myriads of compounds, many of them non-volatile, can be generated (Frankel et al., 1977a, b). In addition, two additional factors must also be taken into account when performing oxidative stability tests at high temperatures: on the one hand, that the reactions taking place under these conditions may be different from those occurring at lower temperatures, as during the storage of oils, and on the other, that the action mechanism of antioxidants can also vary depending on temperature (Kamal-Eldin, 2006). In fact, Nagy et al. (2016) reported that incorporation into correlation models of data regarding lipid oxidation progress under accelerated storage conditions at 60 ºC (coming from peroxide value, conjugated dienes and trienes, TBARs and *p*-anisidine values analyses) yielded somewhat higher correlations than those obtained by

determining only the oil stability index (OSI) at 120 ºC. Moreover, measurement of the induction period led to highly surprising conclusions concerning the stability of corn and soybean oils (Castelo-Branco et al., 2016), which was found to be similar to that of cold-pressed olive oil (Läubi and Bruttel, 1986), even though the oxidative stability of this latter has been shown to be considerably higher than that of the former oils, both under accelerated storage conditions (Guillén and Ruiz, 2005a, b) and at frying temperature (Guillén and Ruiz, 2008).

In the light of all the above, this work aims to study the influence of the composition in minor components of two commercially available soybean oils, one virgin and the other refined, on their oxidative stability. For this purpose, the composition in acyl groups of the two selected oils was analyzed by means of ^{1}H Nuclear Magnetic Resonance (^{1}H NMR). As far as the study of the minor oil component profile was concerned, this was accomplished by means of Direct Immersion Solid-Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (DI-SPME-CGMS). This methodology has the extraordinary advantage of providing information about different groups of minor components simultaneously and rapidly with no modification of the sample, unlike the several types of laborious methodologies and analytical techniques employed in other studies to obtain data about the various kinds of minor components. Next, the oils subject of study were submitted to an accelerated storage (AS) process at 70 ºC and their respective behaviours were studied by means of ^{1}H NMR. To this aim, the evolution throughout the AS process of the different types of oil acyl groups together with the generation of various groups of specific oxidation products, both primary and secondary, were monitored.

Given that a detailed analysis of the oxidation products that are generated in soybean oil subjected to AS conditions can be found in previous papers (Martin-Rubio et al., 2018a, b), this study focuses its interest on only certain groups of oxidation products that can be considered representative enough to achieve the main goal of this work: hydroperoxides, epoxides and aldehydes. For the same reason, the differences between the evolutions of both types of oils will be the main subject of discussion.

2. MATERIALS AND METHODS

2.1. Samples

The samples subject of study were two commercial soybean oils, one of them virgin (VSO), and the other one refined (RSO).

2.2. Characterization of the oils subject of study

2.2.1. Analysis of the oil main components (acyl groups)

The molar percentages of the different types of oil acyl groups were determined by ^{1}H NMR, as in previous works (Guillén and Ruiz, 2003; Guillén and Uriarte, 2009, 2012).

2.2.2. Analysis of the minor oil components

Extraction of the minor oil components was performed by means of Solid-Phase Microextraction, following the methodology described by Alberdi-Cedeño et al. (2017a). To this aim, a fiber of 65 μm StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB), acquired from Supelco (Bellefonte, PA, U.S.), was immersed directly into 6 ml of edible oil at room temperature for 45 minutes. The thermal desorption process of the extracted oil components and their subsequent separation was carried out in a gas chromatograph equipped with a mass spectrometry detector (GC/MS) in the same way described in the above mentioned work.

Identification of most of the extracted components was made by comparison of their retention times and mass spectra with those of commercial standards acquired from Sigma-Aldrich (St. Louis, MO, USA) and Larodan Fine Chemicals AB (Malmo, Sweden). Others were identified by matching of their mass spectra with spectra from a commercial library by more than 85% (W9N08, Wiley ver. 9.0 and NIST ver. 8.0 library) and also with those obtained from the literature.

Semi-quantification was based on arbitrary units of the base peak ion area counts divided by 10^5. The base peaks of the several compounds identified, together with their respective molecular weights, are displayed in Table S1 (see supplementary material). All the determinations were carried out in duplicate in order to obtain a mean value with the corresponding standard deviation for each of the components studied.

2.3. Accelerated storage (AS) process

10 g portions of each oil sample were poured into plastic Petri dishes of 80 mm diameter for each of the days monitored throughout the AS process. These were heated at 70º C in a convection oven with circulating air but without forced convection,

simulating AS conditions. The evolution of the samples was followed by ^{1}H NMR until their total polymerization, when it was no longer possible to take an aliquot sufficiently fluid to be analyzed. The AS process was carried out in duplicate with all the studied samples.

2.4. Monitoring by ^{1}H NMR of the evolution of VSO and RSO throughout the AS process

2.4.1. Operating conditions

The ^{1}H NMR spectra of all the samples taken throughout the AS process were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz, the weight of each sample being approximately 0.16 g. These were mixed in a 5 mm diameter tube with 400 µl of deuterated chloroform containing 0.2% of non deuterated chloroform and a small amount (0.03%) of tetramethylsilane as internal reference. The acquisition parameters used were the same as in previous studies (Guillén and Ruiz, 2003, 2005a). The experiments were carried out at 25 ºC.

2.4.2. Identification of some components

The identification of the oil acyl groups and of the products formed throughout the AS process was carried out on the basis of the signal assignment shown in Table S2 (see supplementary material), made from bibliographic data and with the aid of several standard compounds. These were: (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E,E*)-2,4-hexadienal, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, 4,5-epoxy-(*E*)-2-decenal and 12,13-epoxy-9(*Z*)-octadecenoic acid methyl ester (isoleukotoxin methyl ester), acquired from Sigma-Aldrich, 4-hydroxy-(*E*)-2-nonenal, 4-hydroperoxy-(*E*)-2-nonenal, *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, purchased from Cayman Chemical (Ann Arbor, MI, USA) and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica (Barcelona, Spain).

2.4.3. Quantitative data derived from ^{1}H NMR spectra

The molar percentages of the several kinds of oil acyl groups were estimated throughout the AS process as in previous studies (Guillén and Uriarte, 2012), by means of the following equations: $Ln\% = 100(A_H/3A_I)$; $L\% = 100(2A_G/3A_I)$; $O\%$ (or $MU\%$) = $100(A_E/3A_I)$-$Ln\%$-$L\%$, where A_H and A_G are the areas of the signals of *bis*-allylic protons of linolenic and linoleic groups, respectively (signals "H" and "G" in Table S2); given that their respective signals overlap to a certain extent, the total area corresponding to each of them was calculated using pure trilinolein and trilinolenin

(Sigma-Aldrich) as references. A_I, in turn, is the area of the signal of the protons at *sn*-1 and *sn*-3 positions in the glycerol backbone of triglycerides, while A_E corresponds to that of mono-allylic protons (signals "I" and "E", respectively, in Table S2). The molar percentage of saturated acyl groups can be obtained by difference.

The concentrations of the different types of oxidation products were estimated as millimoles per mol of triglyceride (mmol/mol TG). The general equation to carry out this determination was the following: $[OP] = [(A_{OP}/n)/(A_I/4)]*1000$, where A_{OP} is the area of the signal selected for the quantification of each oxidation product (OP) and n the number of protons that generate the signal. It must be pointed out that for the determination of the so-called major epoxides (see section 3.2.2.2), signals between 2.87 and 3.17 ppm approximately have been considered together. As Table S2 shows, some of the compounds that could give these signals contribute with two protons (signals "e1", "e2", "e3" and "f1") but others with only one (signals "e4", "e5", "e6", "f2" and "f3"). However, given that the conditions of this study make it impossible to elucidate exactly which ones of all these types of compounds are present, it is assumed that the signal at approximately 2.9 ppm corresponds mainly to epoxides contributing with two protons and the one at 3.1 ppm to epoxy-compounds contributing with only one. It must also be noticed that to estimate the area of the epoxy-compounds giving signal at 2.9 ppm, it is necessary to subtract the area corresponding to the side-band of the *bis*-allylic protons signal (signals "H" and "G" in Table S2).

3. RESULTS AND DISCUSSION

3.1. Characterization of VSO and RSO

3.1.1. Composition in main components determined by ^{1}H NMR

The molar percentages of the different kinds of acyl groups in the two types of soybean oil were the following: 5.3 ± 0.7 for linolenic, 43.7 ± 0.5 for linoleic, 31.9 ± 0.7 for oleic and 19.1 ± 0.5 for saturated groups in the case of VSO, and 4.8 ± 0.1 for linolenic, 45.8 ± 0.8 for linoleic, 32.2 ± 0.5 for oleic and 17.2 ± 1.2 for saturated groups in the case of RSO.

These data reveal that both types of soybean oils display very similar proportions of acyl groups, so it is reasonable to assume that the differences in their respective oxidative stabilities will be due to their composition in minor components.

3.1.2. Composition in minor components determined by DI-SPME-GC/MS

The methodology used here for the determination of minor oil components allows one to compare the amounts and relative proportions of several types of compounds in different oils. Figure 1 shows the abundances of the main minor components that are expected to influence the oxidative stability of soybean oil. In addition to tocopherols (α-, β-, γ- and δ-), other components that might also have an impact on the resistance of soybean oil to oxidation are, on the one hand, γ-tocotrienol, squalene and sterols due to their potential antioxidant abilities (Dessi et al., 2002; Kamal-Eldin and Appelqvist, 1996; Seppanen et al., 2010; Singh, 2013; Yoshida and Niki, 2003), and on the other free fatty acids, which unlike the previously mentioned compounds, could favour lipid oxidation (Choe, 2008).

Data in Figure 1 show that, contrary to what might be expected bearing in mind the denomination of the studied oils, the refined one (RSO) contains higher concentrations of practically all the compounds considered to have antioxidant ability than the virgin does. Thus, RSO exhibits a higher level of tocols, among which tocopherols account for the highest proportion by far. Nonetheless, the tocopherol profile is the same in both cases, γ-tocopherol being most abundant, followed by δ- and α-tocopherols, in line with data reported in other studies (Cerretani et al., 2009). It is worth noticing the presence of γ-tocotrienol, also attributed antioxidant ability (Seppanen et al., 2010), not detected in other studies concerning soybean oil (Cerretani et al., 2009; Rao and Perkins, 1972), as well as of α- and γ-tocomonoenols; this type of compounds has been found in very few vegetable oils, especially palm oil (Ng et al., 2004) but also in roasted pumpkin seed oil (Butinar et al., 2011).

Regarding sterol content, including desmethylsterols but also less commonly found *4,4'*-dimethyl sterols like α- and β-amyrin, this is also higher in RSO than in VSO, β-sitosterol being the most abundant, in agreement with the findings of other authors (Phillips et al., 2002). Squalene concentration, instead, is basically the same in both oils. Despite these results appearing somewhat surprising, considering that refining of soybean oil can deplete the level of certain minor components (Jung et al., 1989), it must be taken into account that several factors other than the refining process can also affect the minor oil component composition. Thus, as pointed out by Chu and Lin (1993), the tocopherol content of soybean oil can also be influenced by the state of the soybean or its content in damaged beans, together with storage conditions and length. In addition, soybean variety and climatic factors can also affect soybean oil composition

(Erickson, 2015) Moreover, regarding the refining process, it is worth mentioning that alternatives have more recently been sought to reduce as far as possible the impact of refining on the beneficial components of oils (Fine et al., 2016).

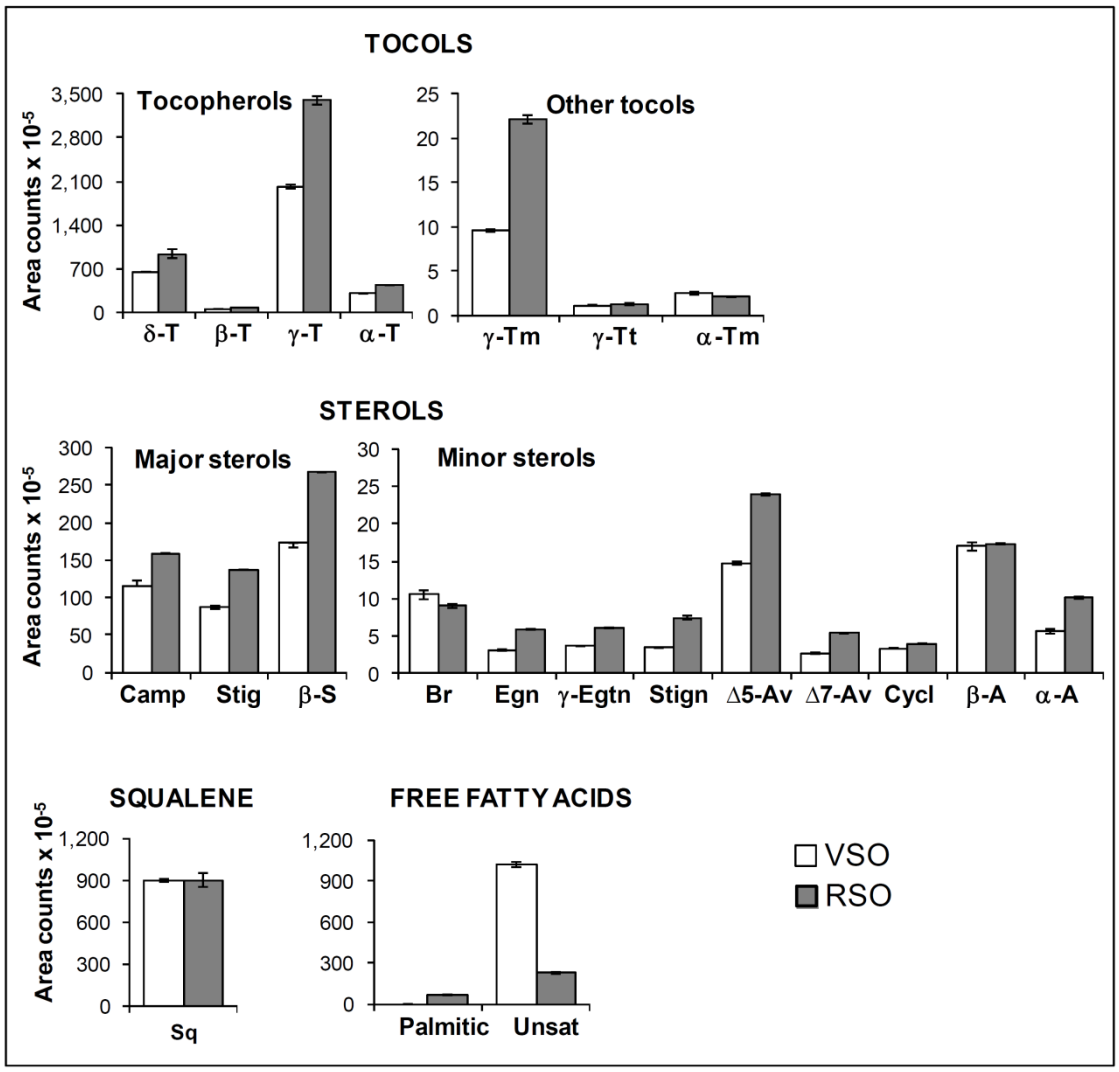

Figure 1. Bar graphics representing the abundance, expressed as arbitrary area units of the base peak of each compound (see Table S1) extracted from the total ion chromatograms obtained by DI-SPME/GC-MS, divided by 10^5, in VSO and RSO of: tocols, sterols, squalene and free fatty acids. All the figures reported are mean values. T: tocopherol; Tm: tocomonoenol; Ttr: tocotrienol; Camp: campesterol; Stig: stigmasterol; S: sitosterol; Br: brassicasterol; Egn: ergostanol; Egtn: ergostenol; Stign: stigmastanol; Av: avenasterol; Cycl: cycloartenol; A: amyrin; Sq: squalene; Unsat: sum of oleic, linoleic and linolenic acids.

By contrast, Figure 1 shows that the amount of free fatty acids, both saturated and unsaturated, is higher in ASV than in RSO, which is in agreement with the removal of this type of minor oil components during the refining process (Jung et al., 1989).

With regard to phenolic compounds other than the tocols above mentioned, which are considered important antioxidants in other plant-derived foodstuffs, their content in soybean oil is low (Castello-Branco et al., 2016; Siger et al., 2008; Tuberoso et al., 2007), so although data about this type of compounds are not provided, it is not expected that they greatly affect the oxidative stability of this tpe of oil.

3.2. Study by ^{1}H NMR of the evolution under AS conditions of VSO and RSO

As stated above, in order to assess the influence of the composition in minor components on the evolution of the studied oils, the degradation of the various types of oil acyl groups, together with the generation of different groups of oxidation products were monitored by ^{1}H NMR. The evolution of some spectral regions where changes occur throughout the AS process of RSO can be observed in Figure 2.

3.2.1. Evolution of the different types of oil acyl groups

The evolutions of the different kinds of oil acyl groups (linolenic, linoleic and diunsaturated ω-6, monounsaturated and saturated+modified), expressed in molar percentages, are represented *versus* time in days in Figure 3A. This graph shows that the molar percentages of all the types of unsaturated groups, especially those of the polyunsaturated ones (linolenic and linoleic), decrease with time both in VSO and in RSO, this diminution being slow during a first stage, but very quick afterwards. In consequence, the molar percentage of saturated+modified (S+M) groups increases accordingly. When comparing the evolution of the two studied soybean oils, it is observed that the first phase of acyl group degradation is longer in RSO than in VSO, since S+M groups molar percentage does not begin to increase at the fastest rate until day 8 in RSO *versus* day 6 in VSO. This results in a slower degradation of the former and in a longer total oil polymerization process (13 days against 10 in VSO).

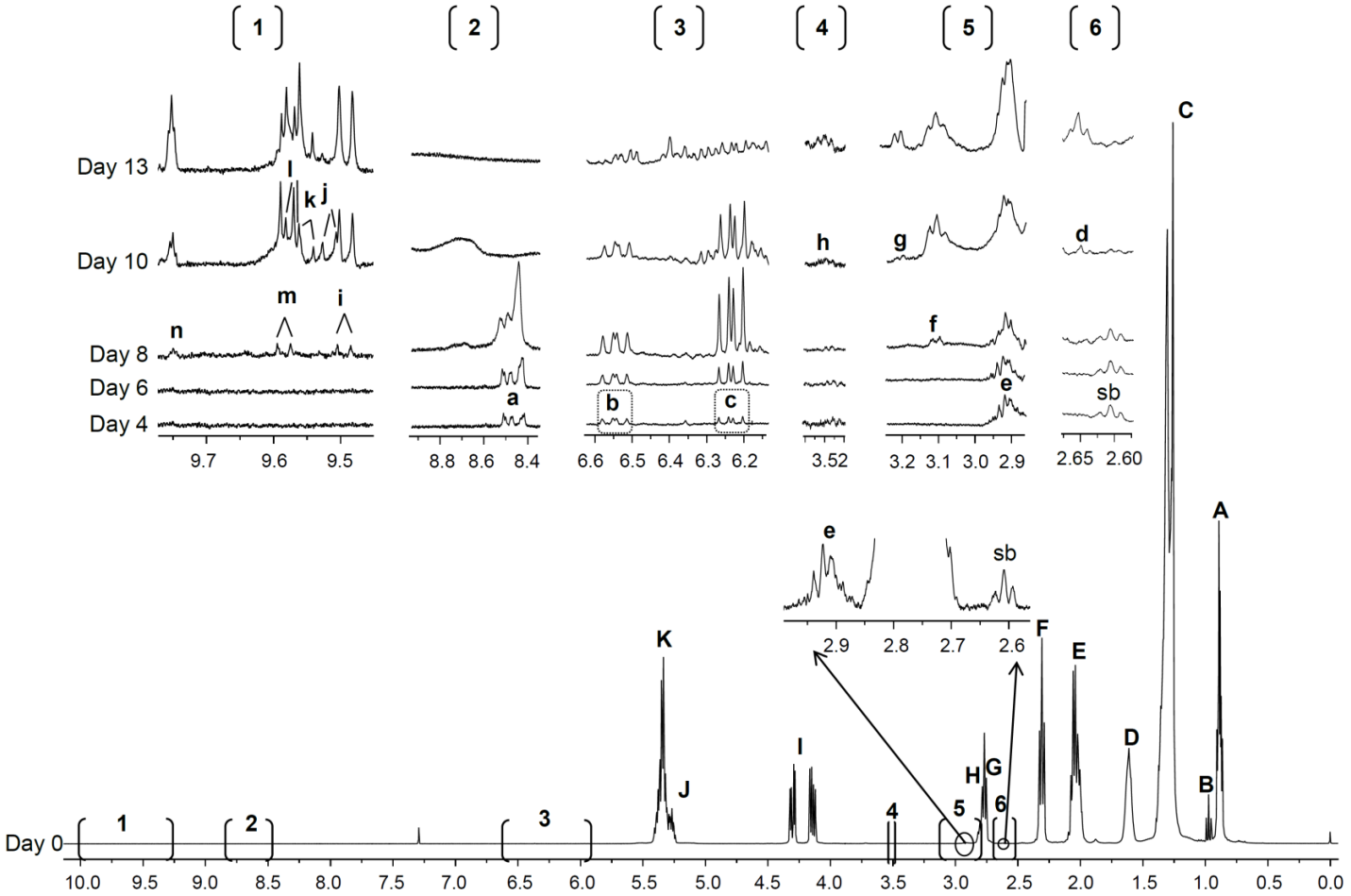

Figure 2. ^{1}H NMR spectrum of sample RSO before being subjected to the oxidation process, together with the enlargements of some spectral regions where changes occur throughout time. Letters agree with those in Table S2, considering that "e" includes signals "e1-e6", "f" signals "f1-f3" and "h" signals "h1+h2". The plots corresponding to the same ^{1}H NMR spectral region are presented at a fixed value of absolute intensity, for them to be valid for comparative purposes.

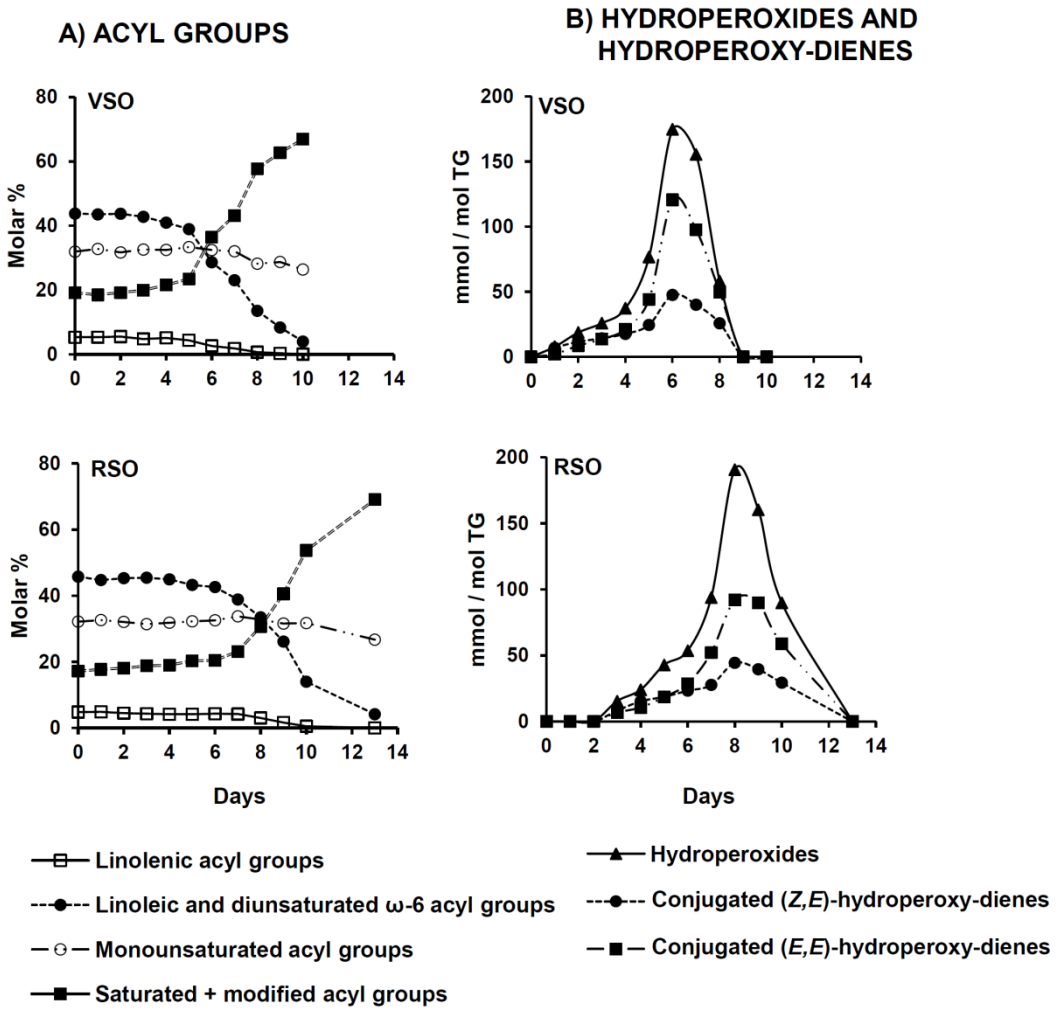

Figure 3. Evolution throughout the accelerated storage process in VSO and RSO of: A) the molar percentages of linolenic, linoleic and diunsaturated ω-6, monounsaturated and saturated+modified acyl groups; and B) the concentrations, in mmol/mol TG, of hydroperoxides and their associated conjugated (*Z*,*E*)- and (*E*,*E*)-dienes. All the figures reported are mean values.

3.2.2 Formation and evolution of oxidation products

3.2.2.1. Evolution of the concentration of hydroperoxides and of their associated conjugated (*Z*,*E*)- and (*E*,*E*)-hydroperoxy-dienes

^{1}H NMR analysis allows one to determine both total hydroperoxides and those with (*Z*,*E*)- and (*E*,*E*)-conjugated dienes. The evolution of all of them will be discussed below.

The evolution of the concentration of total hydroperoxides, which can be estimated from signal “a” (see Table S2 and Figure 2), is shown in Figure 3B. The first noticeable

feature is that in VSO hydroperoxides are already detectable after the first day under AS conditions, while in RSO these are not observed until day 2, indicating lower oxidative stability of the virgin oil. In line with acyl group evolution, hydroperoxide concentration in both oils increase slowly over 4 and 6 days in VSO and RSO, respectively, but then the growing rate becomes much higher until the maximum is reached after 6 days in VSO and 8 days in RSO. In view of these results, it becomes clear that oxidation proceeds faster in VSO than in RSO.

Hydroperoxides generated in oils under AS conditions usually support conjugated diene systems with either (*Z*,*E*)- and (*E*,*E*)-isomerism that, as indicated above, can be monitored separately by measuring their corresponding signals (see Table S2, signals "b" and "c", respectively). The evolution of these signals throughout the AS process can be observed in Figure 2, while the progress of their estimated concentrations in the two oils studied is shown in Figure 3B. This latter reveals that, in line with total hydroperoxide evolution, the emergence of both types of CD-OOH occurs earlier in VSO than in RSO. In addition, a similar trend is observed in both cases, (*Z*,*E*)- and (*E*,*E*)-CD-OOH being detected in similar proportions during the first days of the AS process; then the generation of (*E*,*E*)-isomers predominates over that of the (*Z*,*E*) until the maximum level of both types of CD-OOH is reached, coinciding with the peak level of total hydroperoxides.

3.2.2.2. Epoxides

Epoxides constitute a relevant group of secondary lipid oxidation products, due to their notable concentration (Goicoechea and Guillén, 2010; Martin-Rubio et al., 2018a, b) and to their potential toxicity (Greene et al., 2000; Liu et al., 2018), despite which they receive little attention in oxidation studies.

Table S2, which compiles some of these compounds, shows that only (*E*)-epoxystearates (letter "d") and (*E*)-epoxy-keto-enes (letters "g" and "h") generate signals isolated from those of the rest of epoxides. This makes identification and determination of specific types of epoxides by ^{1}H NMR difficult. Despite this, an estimation of their overall amount can be made, which can be useful to assess the contribution of this type of oxidation products to the total of compounds generated. The evolution of their corresponding signals with time can be observed in Figure 2 (letters "d", "e", "f", "g" and "h"), while the progress of their estimated concentrations throughout the AS process is displayed in Figure 4, in mmol/mol TG. As pointed out in

section 2.4.3, most of the potential epoxides considered, designated as "major epoxides", give their signals between 2.87 and 3.17 ppm approximately (see Table S2 and Figure 2) and, according to the data compiled in Table S2, they are supposed to include (*Z*)-epoxystearates derived from oleic groups, as well as different types of epoxy-compounds coming from polyunsaturated groups; some of these latter possibly support other functional groups like, for example, hydroperoxy- or hydroxy-ones.

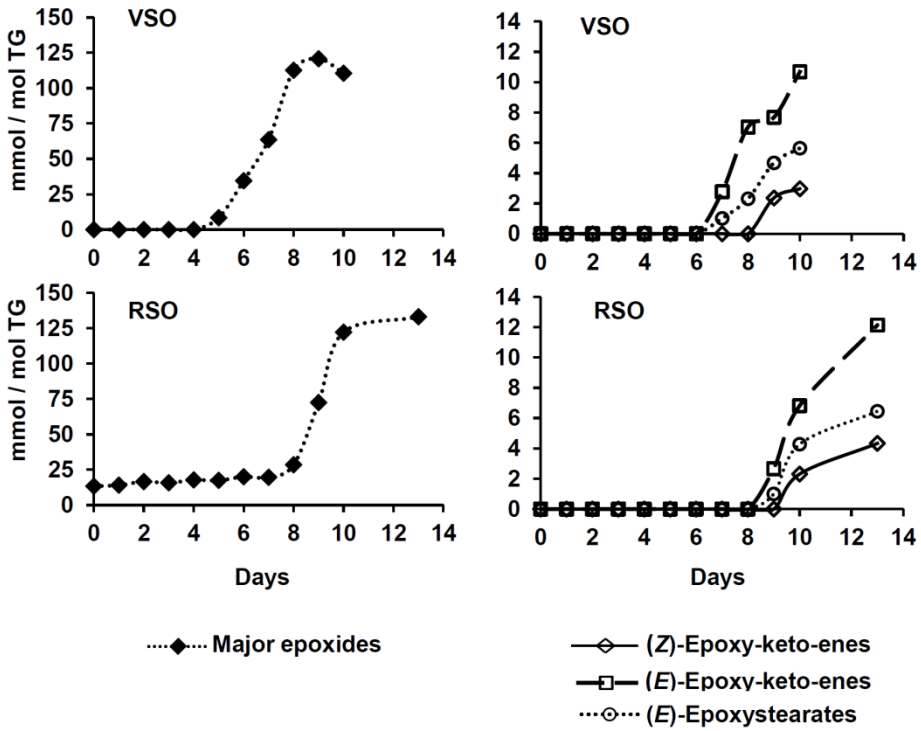

Figure 4. Evolution throughout the accelerated storage process of the concentrations, in mmol/mol TG, of the different types of epoxides in VSO and RSO. All the figures reported are mean values.

With regard to major epoxides it is worth noticing that a part of this type of epoxides, possibly monoepoxides from linolenic and/or linoleic groups, were present in the fresh refined soybean oil, this is before being subjected to the heating process. This is deduced from the observation of signal "e" in Figure 2. Thus, in the absence of epoxides this signal resembles that of the other side-band (see signal "sb"); however, in the case of RSO it is clear that there are additional compounds contributing to signal "e", since its intensity is higher than that of the side-band.

Figure 4A reveals that, as might be expected from the evolution of hydroperoxides in the two oils studied, the appearance of major epoxides in VSO occurs before their concentration begins to increase in RSO.

Apart from the aforementioned major epoxides, other types of epoxides, present in considerable lower concentrations, have also been tentatively identified; these are epoxy-keto-enes and (*E*)-epoxystearates, derived from polyunsaturated and oleic groups, respectively; their corresponding evolutions are shown in Figure 4B. As in the case of major epoxides, it can be observed that both types of compounds are detected later in RSO than in VSO, the pattern of generation being the same in the two oils. Regarding epoxy-keto-enes, these compounds appear later than the major epoxides which suggests that they might derive from other oxidation products generated at an earlier stage and not directly from hydroperoxides. As far as (*E*)-epoxystearates are concerned, their evolution is in line with that of monounsaturated groups (see Figure 3A), whose degradation takes longer than that of the polyunsaturated ones.

3.2.2.3. Aldehydes

Due to the reactivity and toxicity of some aldehydes, such as oxygenated α,β-unsaturated (Guillén and Goicoechea, 2008), this type of compounds constitute an important group of oxidation products. However, the estimation of their concentration is usually based on the determination of only a few of them, being malondialdehyde, measured through the TBARs assay, the sole target of many studies (Barriuso et al., 2013; Shahidi and Zhong, 2005). The progress of the ^{1}H NMR signals of the different kinds of aldehydes throughout the AS process in the refined oil can be observed in Figure 2 (signals “i” to “n”), and the evolutions of their respective concentrations in the two studied oils, in mmol/mol TG, in Figure 5. As shown in this figure, aldehydes are also generated earlier in VSO than in RSO (after 6 and 8 days under AS conditions, respectively). Moreover, while all the different kinds of aldehydes are detected at the same time in VSO, the aldehyde appearance process in RSO is staggered in such a way that 4-hydroxy-(*E*)-2-alkenals, 4,5-epoxy-2-alkenals and (*E*,*E*)-2,4-alkadienals are detected one day later than the first ones detected (n-alkanals, (*E*)-2-alkenals and 4-hydroperoxy-(*E*)-2-alkenals). This indicates a lower generation rate of this type of oxidation products in RSO. With regard to the evolution with time of the several groups of aldehydes, this is the same in both oils.

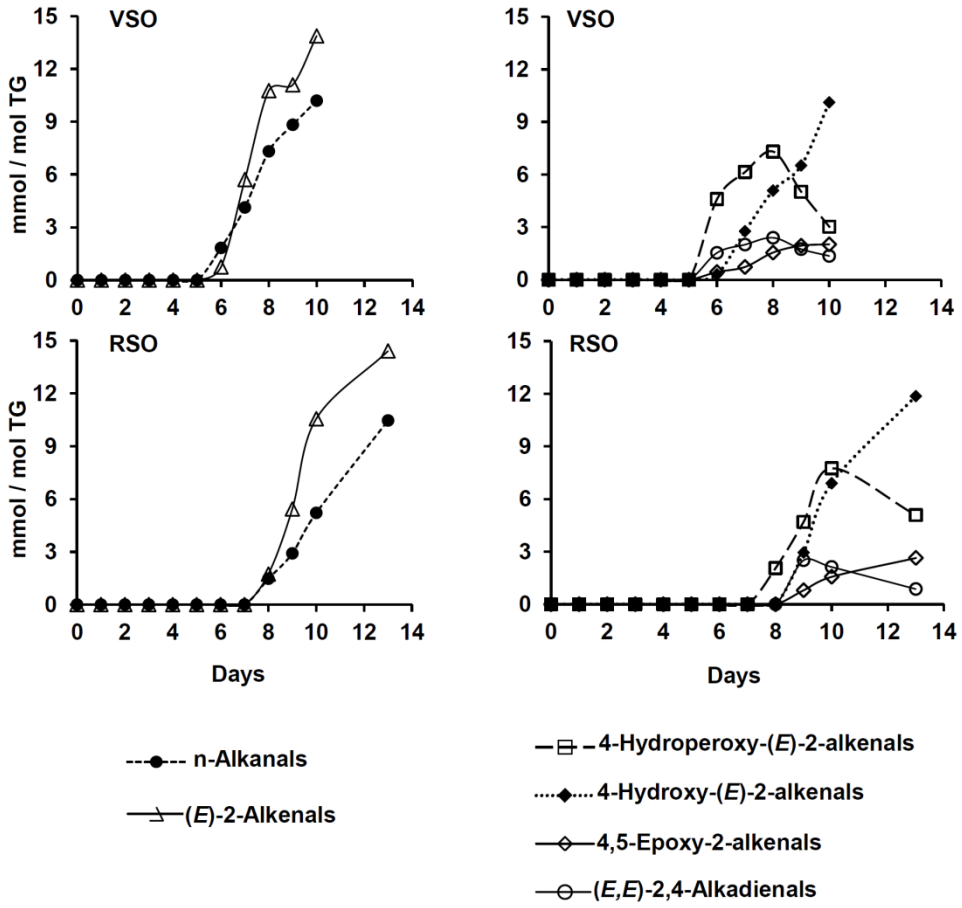

Figure 5. Evolution throughout the accelerated storage process of the concentrations, in mmol/mol TG, of the different types of aldehydes in VSO and RSO. All the figures reported are mean values.

3.3. Final remarks

The analysis of the evolution of the two oils studied evidences the lower oxidative stability of the virgin soybean oil when compared to the refined one. Therefore, although it has been considered as a general rule that virgin oils show longer induction periods than the refined ones due to their higher concentrations of minor antioxidant compounds (Chaiyasit et al., 2007), this observation should not be linked to the designation of the oil as virgin or refined, but will only depend on its composition in both acyl groups and minor components. In fact, in similar results to these, Redondo-Cuevas et al. (2018) found that refined sunflower and rapeseed oils showed greater resistence to oxidation than did their respective virgin cold-pressed counterparts on the basis of longer induction periods determined by the Rancimat method. In the same vein, Wroniak et al. (2008) also reported that cold-pressed oils were less stable in Rancimat

test in comparison with oils after full refining. However, the factors contributing to these observations were not discussed in detail.

It is also worth noticing that, although a higher concentration of tocopherols seems to contribute to a higher oxidative stability in the soybean oils here studied, this apparently logical relationship should not be directly extrapolated to other types of vegetable oils with a tocopherol profile where α-tocopherol and not γ-tocopherol, was the major one. This assertion is based on the conclusions of many works which suggest that increasing concentrations of α-tocopherol might result in a lower oxidative stability (Dolde and Wang, 2011; Frankel et al., 1959; Jung and Min, 1990). Indeed, Redondo-Cuevas et al. (2018) found that the concentration of total tocopherols showed a negative correlation with oxidative stability.

4. CONCLUSIONS

The findings of this study reveal to what extent the composition in minor components of two soybean oils with very similar compositions in acyl groups can affect their oxidative stability and evolution under AS conditions. In this respect, it has been shown that oxidation proceeds more slowly in the oil which has the highest concentrations of tocols and sterols, and is poorer in free fatty acids. This is deduced from a slower degradation of oil acyl groups and from a later and, in some cases, more gradual generation of oxidation products.

Contrary to what the denomination of the two soybean oils studied might suggest, the virgin one is lower in tocopherol and sterol content. Therefore, it should not be generally assumed that the content of virgin soybean oils in minor components considered to be beneficial for human health is higher than that of the refined ones, thus confirming recent findings of other authors. However, the higher concentration of components with antioxidant ability in the refined oil seems to contrast with the initial presence of epoxides in the same oil; this arises the convenience of analyzing the causes of the occurrence of this type of oxidation products in fresh oils.

Finally, this work shows that a simple analysis of the composition in minor components of commercial oils by means of direct immersion SPME followed by GC/MS would make it possible to establish different categories or quality levels within oils of the same botanical origin, in terms not only of composition in potentially bioactive compounds but also of oxidative stability. Thus, the establishment of

parameters indicative of this quality, not considered until now, might be valuable for oil producers, who could add value to their products, for consumers andt also for food industry, which would have a means to identify oils with different oxidative stabilities.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Alberdi-Cedeño, J., Ibargoitia, M. L., Cristillo, G., Sopelana, P., Guillén, M. D. (2017a). A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components. *Food Chemistry, 221*, 1135-1144.

Alberdi-Cedeño, J., Ibargoitia, M. L., Guillén, M. D. (2017b). Bioactive compounds detected for the first time in corn oil: Cyclic dipeptides and other nitrogenated compounds. *Journal of Food Composition and Analysis, 62*, 197-204.

Ayyildiz, H. F., Topkafa, M., Kara, H., Sherazi, S. T. H. (2015). Evaluation of fatty acid composition, tocols profile, and oxidative stability of some fully refined edible oils. *International Journal of Food Properties, 18*, 2064-2076.

Barriuso, B., Astiasarán, I., Ansorena, D. (2013). A review of analytical methods measuring lipid oxidation status in foods: a challenging task. *European Food Research and Technology, 236*, 1-15.

Bozan, B., Temelli, F. (2008). Chemical composition and oxidative stability of flax, safflower and poppy seed and seed oils. *Bioresource Technology, 99*, 6354-6359.

Brignoli, C. A., Kinsella, J. E., Weihrauch, J. L. (1976). Comprehensive evaluation of fatty acids in foods. V. Unhydrogenated fats and oils. *Journal of the American Dietetic Association, 68*, 224-229.

Butinar, B., Bučar-Miklavčič, M., Mariani, C., Raspor, P. (2011). New vitamin E isomers (gamma-tocomonoenol and alpha-tocomonoenol) in seeds, roasted seeds and roasted seed oil from the Slovenian pumpkin variety 'Slovenska golica'. *Food Chemistry, 128*, 505-512.

Castelo-Branco, V. N., Santana, I., Di-Sarli, V. O., Freitas, S. P., Torres, A. G. (2016). Antioxidant capacity is a surrogate measure of the quality and stability of vegetable oils. *European Journal of Lipid Science and Technology, 118*, 224-235.

Cerretani, L., Lerma-García, M. J., Herrero-Martínez, J. M., Gallina-Toschi, T., Simó-Alfonso, E. F. (2009). Determination of tocopherols and tocotrienols in vegetable oils by nanoliquid chromatography with ultraviolet-visible detection using a silica monolithic column. *Journal of Agricultural and Food Chemistry, 58*, 757-761.

Chaiyasit, W., Elias, R. J., McClements, D. J., Decker, E. A. (2007). Role of physical structures in bulk oils on lipid oxidation. *Critical Reviews in Food Science and Nutrition, 47*, 299-317.

Chen, B., McClements, D. J., Decker, E. A. (2011). Minor components in food oils: a critical review of their roles on lipid oxidation chemistry in bulk oils and emulsions. *Critical Reviews in Food Science and Nutrition, 51*, 901-916.

Choe, E. (2008). Effects and mechanisms of minor compounds in oil on lipid oxidation. In C. C. Akoh, & D. B. Min (Eds.), *Food lipids: chemistry, nutrition, and biotechnology*, (pp. 449-474). Boca Raton: CRC Press.

Choe, E., Min, D. B. (2006). Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety, 5*, 169-186.

Chu, Y. H., Lin, J. Y. (1993). Factors affecting the content of tocopherol in soybean oil. *Journal of the American Oil Chemists' Society, 70*, 1263-1268.

Dessi, M. A., Deiana, M., Day, B. W., Rosa, A., Banni, S., Corongiu, F. P. (2002). Oxidative stability of polyunsaturated fatty acids: effect of squalene. *European Journal of Lipid Science and Technology, 104*, 506-512.

Dolde, D., Wang, T. (2011). Oxidation of corn oils with spiked tocols. *Journal of the American Oil Chemists' Society, 88*, 1759-1765.

Erickson, D. R. (2015). *Practical handbook of soybean processing and utilization*. Elsevier.

Esterbauer, H. (1993). Cytotoxicity and genotoxicity of lipid-oxidation products. *The American Journal of Clinical Nutrition, 57*, 779S–785S.

Farhoosh, R., Moosavi, S. M. R. (2007). Rancimat test for the assessment of used frying oils quality. *Journal of Food Lipids, 14*, 263-271.

Ferrari, R. A., Schulte, E., Esteves, W., Brühl, L., Mukherjee, K. D. (1996). Minor constituents of vegetable oils during industrial processing. *Journal of the American Oil Chemists' Society, 73*, 587-592.

Fine, F., Brochet, C., Gaud, M., Carre, P., Simon, N., Ramli, F., Joffre, F. (2016). Micronutrients in vegetable oils: the impact of crushing and refining processes on vitamins and antioxidants in sunflower, rapeseed, and soybean oils. *European Journal of Lipid Science and Technology, 118*, 680-697.

Frankel, E. N. (1993). In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends in Food Science & Technology, 4*, 220-225.

Frankel, E. N., Cooney, P. M., Moser, H. A., Cowan, J. C., Evans, C. D. (1959). Effect of antioxidants and metal inactivators in tocopherol-free soybean oil. *Fette, Seifen, Anstrichmittel, 61*, 1036-1039.

Frankel, E. N., Neff, W. E., Rohwedder, W. K., Khambay, B. P. S., Garwood, R. F., Weedon, B. C. L. (1977a). Analysis of autoxidized fats by gas chromatography-mass spectrometry: II. Methyl linoleate. *Lipids, 12*, 908-913.

Frankel, E. N., Neff, W. E., Rohwedder, W. K., Khambay, B. P. S., Garwood, R. F., Weedon, B. C. L. (1977b). Analysis of autoxidized fats by gas chromatography-mass spectrometry: III. Methyl linolenate. *Lipids, 12*, 1055-1061.

Goicoechea, E., Guillén, M. D. (2010). Analysis of hydroperoxides, aldehydes and epoxides by ^{1}H nuclear magnetic resonance in sunflower oil oxidized at 70 and 100 ºC. *Journal of Agricultural and Food Chemistry, 58*, 6234-6245.

Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau, C., Hammoc, B. D. (2000). Metabolism of monoepoxides of methyl linoleate: bioactivation and detoxification. *Archives of Biochemistry and Biophysics, 376*, 420-432.

Guillén, M. D., Goicoechea, E. (2008). Toxic oxygenated α,β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition, 48*, 119-136.

Guillén, M. D., Ruiz, A. (2003). Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups. *European Journal of Lipid Science and Technology, 105*, 688–696.

Guillén, M. D., Ruiz, A. (2005a). Oxidation process of oils with high content of linoleic acyl groups and formation of toxic hydroperoxy- and hydroxyalkenals. A study by ^{1}H nuclear magnetic resonance. *Journal of the Science of Food and Agriculture, 85*, 2413-2420.

Guillén, M. D., Ruiz, A. (2005b). Study by proton nuclear magnetic resonance of the thermal oxidation of oils rich in oleic acyl groups. *Journal of the American Oil Chemists' Society, 82*, 349-355.

Guillén, M. D., Ruiz, A. (2008). Monitoring of heat-induced degradation of edible oils by proton NMR. *European Journal of Lipid Science and Technology, 110*, 52-60.

Guillén, M. D., Uriarte, P. S. (2009). Contribution to further understanding of the evolution of sunflower oil submitted to frying temperature in a domestic fryer: study by ^{1}H Nuclear Magnetic Resonance. *Journal of Agricultural and Food Chemistry, 57*, 7790-7799.

Guillén, M. D., Uriarte, P. S. (2012). Study by ^{1}H NMR spectroscopy of the evolution of extra virgin olive oil composition submitted to frying temperature in an industrial fryer for a prolonged period of time. *Food Chemistry, 134*, 162-172.

Jung, M. Y., Min, D. B. (1990). Effects of α-, γ-, and δ-tocopherols on oxidative stability of soybean oil. *Journal of Food Science, 55*, 1464-1465.

Jung, M. Y., Yoon, S. H., Min, D. B. (1989). Effects of processing steps on the contents of minor compounds and oxidation of soybean oil. *Journal of the American Oil Chemists' Society, 66*, 118-120.

Kamal-Eldin, A. (2006). Effect of fatty acids and tocopherols on the oxidative stability of vegetable oils. *European Journal of Lipid Science and Technology, 108*, 1051-1061.

Kamal-Eldin, A., Appelqvist, L. Å. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids, 31*, 671-701.

Läubi, M. W., Bruttel, P. A. (1986). Determination of oxidative stability of fat and oils: comparison between the active oxygen method (AOCS Cd 12-57) and the Rancimat method. *Journal of the American Oil Chemists' Society, 63*, 792-795.

Liu, Y., Cheng, Y., Li, J., Wang, Y., Liu, Y. (2018). Epoxy stearic acid, an oxidative product derived from oleic acid, induces cytotoxicity, oxidative stress, and apoptosis in HepG2 Cells. *Journal of Agricultural and Food Chemistry, 66*, 5237-5246.

Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L., Guillén, M. D. (2018). Prooxidant effect of α-tocopherol on soybean oil. Global monitoring of its oxidation process under accelerated storage conditions by ^{1}H Nuclear Magnetic Resonance. *Food Chemistry, 245*, 312-323.

Martin-Rubio, A. S., Sopelana, P., Guillén, M. D. (2018). A thorough insight into the complex effect of gamma-tocopherol on the oxidation process of soybean oil by means of ^{1}H Nuclear Magnetic Resonance. Comparison with alpha-tocopherol. *Food Research International, 114*, 230-239.

Nagy, K., Kerrihard, A. L., Beggio, M., Craft, B. D., Pegg, R. B. (2016). Modeling the impact of residual fat-soluble vitamin (FSV) contents on the oxidative stability of commercially refined vegetable oils. *Food Research International, 84*, 26-32.

Ng, M. H., Choo, Y. M., Ma, A. N., Chuah, C. H., Hashim, M. A. (2004). Separation of vitamin E (tocopherol, tocotrienol, and tocomonoenol) in palm oil. *Lipids, 39*, 1031-1035.

Phillips, K. M., Ruggio, D. M., Toivo, J. I., Swank, M. A., Simpkins, A. H. (2002). Free and esterified sterol composition of edible oils and fats. *Journal of Food Composition and Analysis, 15*, 123-142.

Rao, M. K., Perkins, E. G. (1972). Identification and estimation of tocopherols and tocotrienols in vegetable oils using gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry, 20*, 240-245.

Redondo-Cuevas, L., Castellano, G., Torrens, F., Raikos, V. (2018). Revealing the relationship between vegetable oil composition and oxidative stability: A multifactorial approach. *Journal of Food Composition and Analysis, 66*, 221-229.

Seppanen, C. M., Song, Q., Csallany, A. S. (2010). The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *Journal of the American Oil Chemists' Society, 87*, 469-481.

Shahidi, F., & Zhong, Y. (2005). Lipid oxidation: measurement methods. In F. Shaidi (Ed.), *Bailey's Industrial Oil and Fat Products. Volume 1: Edible Oil & Fat Products: Chemistry, Properties, and Health Effects*. John Wiley & Sons, Inc.

Siger, A., Nogala-Kalucka, M., Lampart-Szczapa, E. (2008). The content and antioxidant activity of phenolic compounds in cold-pressed plant oils. *Journal of Food Lipids, 15*, 137-149.

Singh, A. (2013). Sitosterol as an antioxidant in frying oils. *Food Chemistry, 137*, 62-67.

Tuberoso, C. I., Kowalczyk, A., Sarritzu, E., Cabras, P. (2007). Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. *Food Chemistry, 103*, 1494-1501.

Verleyen, T., Sosinska, U., Ioannidou, S., Verhé, R., Dewettinck, K., Huyghebaert, A., De Greyt, W. (2002). Influence of the vegetable oil refining process on free and esterified sterols. *Journal of the American Oil Chemists' Society, 79*, 947-953.

Wroniak, M., Krygier, K., Kaczmarczyk, M. (2008). Comparison of the quality of cold pressed and virgin rapeseed oils with industrially obtained oils. *Polish Journal of Food and Nutrition Sciences, 58*, 85-89.

Yang, M., Huang, F., Liu, C., Zheng, C., Zhou, Q., Wang, H. (2013). Influence of microwave treatment of rapeseed on minor components content and oxidative stability of oil. *Food and Bioprocess Technology, 6*, 3206-3216.

Yoshida, Y., Niki, E. (2003). Antioxidant effects of phytosterol and its components. *Journal of Nutritional Science and Vitaminology, 49*, 277-280.

Zaunschirm, M., Pignitter, M., Kienesberger, J., Hernler, N., Riegger, C., Eggersdorfer, M., Somoza, V. (2018). Contribution of the ratio of tocopherol homologs to the oxidative stability of commercial vegetable oils. *Molecules, 23*, 206.

Zhang, X., Gao, B., Shi, H., Slavin, M., Huang, H., Whent, M., Sheng, Y., Yu, L. (2012). Chemical composition of 13 commercial soybean samples and their antioxidant and anti-inflammatory properties. *Journal of Agricultural and Food Chemistry, 60*, 10027-10034.

SUPPLEMENTARY MATERIAL OF MANUSCRIPT 1

ASSESSMENT OF SOYBEAN OIL OXIDATIVE STABILITY FROM RAPID ANALYSIS OF ITS MINOR COMPONENT PROFILE

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Table S1. Minor components found in the soybean oils studied together with their respective molecular weight (MW) and their base peak (BP). The asterisked compounds were acquired commercially and used as standards for identification purposes.

Compound	**MW**	**BP**
Tocols		
δ-Tocopherol *	402	402
β-Tocopherol *	416	416
γ-Tocopherol *	416	416
γ-tocomonoenol	414	414
α-Tocopherol *	430	165
γ-tocotrienol *	410	151
α-tocomonoenol	428	428
Sterols		
Brassicasterol *	398	314
Campesterol *	400	400
Ergostanol	402	215
Stigmasterol *	412	412
γ-Ergostenol	400	400
β-Sitosterol *	414	414
Stigmastanol *	416	416
Δ5-Avenasterol *	412	314
Δ7-Avenasterol	412	285
Cycloartenol	426	69
β-Amyrin	426	218
α-Amyrin	426	218
Squalene *	410	69
Free fatty acids		
Palmitic acid *	256	73
§Linoleic acid *	280	55
§Oleic Acid *	282	55
§Linolenic acid *	278	55

§Linoleic, oleic and linolenic acids peaks overlapped in the chromatograms. Thus, in order to quantify these compounds altogether, ion 55 was taken since it is common to all these unsaturated fatty acids.

Table S2. Chemical shifts, multiplicities and assignments of the ^{1}H NMR signals in $CDCl_3$ of the main types of triglyceride (TG) protons, and of some oxidation compounds, present in the different soybean oil samples, before and throughout the oxidation process.

Signal	Chemical shift (ppm)	Multi-plicity	Functional group	
			Type of protons	Compound
Main acyl groups[a]				
A	0.88	t	-C**H**$_3$	saturated and monounsaturated ω-9 acyl groups
	0.89	t	-C**H**$_3$	linoleic acyl groups
B	0.97	t	-C**H**$_3$	linolenic acyl groups
C	1.19-1.42	m*	-(C**H**$_2$)$_n$-	acyl groups
D	1.61	m	-OCO-CH$_2$-C**H**$_2$-	acyl groups in TG
E	1.94-2.14	m**	-C**H**$_2$-CH=CH-	unsaturated acyl groups
F	2.26-2.36	dt	-OCO-C**H**$_2$-	acyl groups in TG
G	2.77	t	=HC-C**H**$_2$-CH=	linoleic acyl groups
H	2.80	t	=HC-C**H**$_2$-CH=	linolenic acyl groups
I	4.22	dd,dd	ROC**H**$_2$-CH(OR')-C**H**$_2$OR''	glyceryl groups
J	5.27	m	ROCH$_2$-C**H**(OR')-CH$_2$OR''	glyceryl groups
K	5.28-5.46	m	-C**H**=C**H**-	acyl groups
Oxidation compounds				
Hydroperoxides[b]				
a	8.3-9.0	bs	-OO**H**	monohydroperoxide group
Conjugated dienic systems[b]				
-	5.47	ddm	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroperoxy group in octadecadienoic acyl groups[c]
-	5.76	dtm		
-	6.06	ddtd		
b	6.27	ddm		
-	5.51	dtm	-C**H**=C**H**-C**H**=C**H**-	(*E*,*E*)-conjugated double bonds associated with
-	5.56	ddm		
-	6.00	ddtd		

c	6.58	dddd		hydroperoxy group in octadecadienoic acyl groups

Epoxides

Epoxy-derivatives

d	2.63[d]	m	-C**H**O**H**C-	(*E*)-9,10-epoxystearate
e1	2.88[d]	m	-C**H**O**H**C-	(*Z*)-9,10-epoxystearate
e2	2.9[e]	m	-C**H**O**H**C-	monoepoxy-octadecenoate groups
			-C**H**OHC-CH_2-CHO**H**C-	diepoxides
e3	2.94***	m	-C**H**O**H**C-	(*Z*)-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid
f1	3.10[e]	m	-CHO**H**C-CH_2-C**H**OHC-	diepoxides

Epoxy-keto-derivatives

e4	2.89[f]/2.90[g]	td[h]/m[i]	-CO-CH=CH-CHO**H**C-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
e5	2.91[f]	td	-C**H**OHC-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
g	3.20[f,g]	dd	-CO-CH=CH-C**H**OHC-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
			-CHO**H**C-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-C**H**OHC-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-CO-CH=CH-CHO**H**C-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
h1	3.52[f]	dd	-CHO**H**C-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
h2	3.53[f]	dd	-CO-CH=CH-C**H**OHC-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate

Epoxy-hydroxy-derivatives

e6	2.93[h]	dt	-C**H**OHC-CHOH-CH=CH-	*threo*-11-hydroxy-(*E*)-12,13-epoxy-

				(*Z*)-9-octadecenoate
f2	3.09[i]/3.097[j]	dd	-CHO**H**C-CH=CH-CHOH-	9-hydroxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate
Epoxy-hydroperoxy-derivatives				
f3	3.11[i]	dd	-CHO**H**C-CH=CH-CHOOH-	9-hydroperoxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate[k]
Aldehydes				
i	9.49[l]	d	−C**H**O	(*E*)-2-alkenals
j	9.52[l]	d	−C**H**O	(*E,E)*-2,4-alkadienals
k	9.55[l]	d	−C**H**O	4,5-epoxy-2-alkenals
l	9.57[l]	d	−C**H**O	4-hydroxy-(*E*)-2-alkenals
m	9.58[l]	d	−C**H**O	4-hydroperoxy-(*E*)-2-alkenals
n	9.75[l]	t	-C**H**O	n-alkanals

t: triplet; m: mutiplet; d: doublet; bs: broad signal; *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***Assignment made with the aid of standard compounds

[a]Assignments taken from Guillén, M. D., Ruiz, A. (2003). *Journal of the Science of Food and Agriculture, 83*, 338–346.

[b]Data taken from Goicoechea, E.,Guillén, M. D. (2010). *Journal of Agricultural and Food Chemistry, 58*, 6234-6245.

[c]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Chan, H. W. S., Levett, G. (1977). *Lipids, 12*, 99-104.

[d]Data taken from Du, G., Tekin, A., Hammond, E. G., Woo, L. K. (2004). *Journal of the American Oil Chemists' Society*, *81*, 477–80.

[e]Data taken from Aerts, H. A. J., Jacobs, P. A. (2004). *Journal of the American Oil Chemists' Society, 81*, 841-846.

[f]Data taken from Lin, D., Zhang, J., Sayre, L. M. (2007). *The Journal of Organic Chemistry, 72*, 9471-9480.

[g]Data taken from Gardner, H. W., Kleiman, R., Weisleder, D. (1974). *Lipids, 9*, 696-706.

[h]Data taken from Garssen, G. J., Veldink, G. A., Vliegenthart, J. F., Boldingh, J. (1976). *The FEBS Journal, 62*, 33-36.

[i]Data taken from Gardner, H. W., Weisleder, D., Kleiman, R. (1978). *Lipids, 13*, 246-252.

[j]Data taken from Van Os Cornelis, P. A., Vliegenthart, J. F. G., Crawford, C. G.,Gardner, H. W. (1982). *Biochimica et Biophysica Acta, 713*, 173-176.

[k]δ-Ketols (hydroxy-keto-derivatives) could also contribute to this signal (Gardner et al., 1974).

[l]Data taken from Guillén, M. D., Ruiz, A. (2004). *European Journal of Lipid Science and Technology*, *106*, 680–687.

Manuscript 2

PROOXIDANT EFFECT OF α-TOCOPHEROL ON SOYBEAN OIL. GLOBAL MONITORING OF ITS OXIDATION PROCESS UNDER ACCELERATED STORAGE CONDITIONS BY ^{1}H NUCLEAR MAGNETIC RESONANCE

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ABSTRACT

The effect of adding α-tocopherol in proportions ranging from 0.002 to 5% in weight on the oxidative stability of soybean oil was studied. For the first time, the oxidation process under accelerated storage conditions including evolution of the molar percentages of the several types of oil acyl groups, and formation and evolution of various kinds of oxidation products comprising hydroperoxides, hydroxy-dienes and other alcohols, epoxides, aldehydes and keto-dienes, was followed by ^{1}H nuclear magnetic resonance. It is proved that, except in the lowest proportion, α-tocopherol not only exerts a prooxidant effect on soybean oil but also modifies its oxidation pathway, affecting the oxidation products generation rate, their nature, relative proportions and concentrations. It is noticeable that the highest α-tocopherol concentrations induce the generation of some toxic compounds at earlier stages of the thermoxidation process and sometimes in higher concentration, such as certain oxygenated α,β-unsaturated aldehydes and monoepoxides derived from linoleic groups.

Chemical compounds studied in this article: α-Tocopherol (PubChem CID: 2116); (*E*)-2-Hexenal (PubChem CID: 2116); (*E*,*E*)-2,4-Decadienal (PubChem CID: 5283349); 4-Hydroxy-(*E*)-2-nonenal (PubChem CID: 1693); (*E*)-9,10-epoxystearate (PubChem CID: 12235226)

KEYWORDS: α-tocopherol, soybean oil, ^{1}H nuclear magnetic resonance, oxidation, prooxidant, hydroperoxides, conjugated hydroperoxydienes, conjugated hydroxy-dienes, epoxides, aldehydes, keto-dienes, alcohols

1. INTRODUCTION

Vitamin E, in particular α-tocopherol (α-T), is employed by industry due to its classically attributed antioxidant properties and beneficial effects on human health. As far as vegetable oils are concerned, European legislation allows the addition of α-T on the basis of the *quantum satis* principle (Commission Regulation 1129/2011), this is without an established limit, to refined oils, except for refined olive oils. This contrasts with the body of knowledge provided by many scientific studies, where controversial results have been obtained concerning not only the antioxidant ability of α-T (Seppanen, Song & Csallany, 2010), but also its effect on human health (Brigelius-Flohé, 2009).

Many studies have been carried out on the effect of α-T on the oxidative stability of lipids, but dissenting results are observed depending on the test system, the α-T concentration, the temperature and the methodology chosen to assess the antioxidant ability, among other factors (Seppanen et al., 2010). Thus, whereas some authors have reported an antioxidant effect of α-T in methyl linoleate (Mäkinen, Kamal-Eldin, Lampi & Hopia, 2000), in rapeseed oil triacylglycerols (Lampi, Kataja, Kamal-Eldin & Vieno, 1999; Ohm, Stöckmann & Schwarz, 2005) or in olive oil (Wagner & Elmadfa, 2000), others have observed a prooxidant action of α-T, for example, on purified rapeseed oil (Isnardy, Wagner & Elmadfa, 2003) and on linoleic acid (Cillard, Cillard, Cormier & Girre, 1980). Moreover, other works have shown both an antioxidant and a prooxidant effect depending on the α-T concentration in linoleic acid (Koskas, Cillard & Cillard, 1984), in purified soybean oil (Jung & Min, 1990) and in stripped corn oil (Dolde & Wang, 2011).

Some of these studies are based on the performance of non-specific classical methods, such as the peroxide value or the spectrophotometrical measurement of conjugated dienes (Cillard et al., 1980; Dolde & Wang, 2011; Isnardy et al., 2003; Jung & Min, 1990, Lampi et al., 1999), or the *p*-anisidine value to measure the concentration of certain aldehydes altogether (Isnardy et al., 2003; Lampi et al., 1999). Other works, by contrast, deal with the determination, by means of high performance liquid chromatography (HPLC), of specific oxidation compounds such as certain individual hydroperoxides, hydroxy-dienes or keto-dienes (Banni et al., 1996; Koskas et al., 1984; Mäkinen et al., 2000). Aldehydes have also been the subject of research but, in general, only a few of them have been considered, or even only hexanal (Ohm et al., 2005). Therefore, the information provided by these studies is very limited and, in most of the

cases, excludes some of the most toxic oxidation products such as oxygenated α,β-unsaturated aldehydes (Guillén & Goicoechea, 2008).

Controversial results about the antioxidative effect of α-T can also be found at the biological level. In this respect, the dual effect (anti- and pro-oxidant) of α-T has been shown, for example, in LDL (Schneider, 2005). Moreover, α-T has also been associated with enhanced tumor formation *in vivo* when taken in high doses (Toth & Patil, 1983).

All the above mentioned serves to evidence the importance of making an assessment of the effect of α-T on the oxidative stability of food lipids as accurate and complete as possible. Therefore, taking all this into account, the goal of this work was to monitor, by means of ^{1}H nuclear magnetic resonance (^{1}H NMR), the effect of the addition of different amounts of α-T on the entire thermoxidative process of soybean oil carried out at 70º C, simulating accelerated storage conditions. ^{1}H NMR, which has been proved to be extremely useful for the study of the behaviour of lipids under diverse oxidative conditions (Martínez-Yusta, Goicoechea & Guillén, 2014), allows one to follow both the evolution of the different types of oil acyl groups and the formation of a wide range of oxidation products. Thus, this work covers the monitoring both of hydroperoxides and their associated conjugated dienes as well as of other types of oxidation compounds including conjugated hydroxy-dienes and keto-dienes, epoxides, aldehydes and alcohols, some of which are toxic (Greene, Williamson, Newman, Morisseau & Hammoc, 2000; Guillén & Goicoechea, 2008). The analysis of all these data will contribute to elucidate to what extent α-T in the proportions tested affects the oxidation pathway of soybean oil.

2. MATERIALS AND METHODS

2.1. Samples

The starting oil was a soybean oil (SO) purchased from a local supermarket. The average concentrations of the four tocopherols, in mg/kg, estimated by using a SPME-CG/MS methodology previously developed in our laboratory (Alberdi-Cedeño, Ibargoitia, Cristillo, Sopelana & Guillén, 2017) were the following: 130.0 for α-T, 781.7 for γ-T, 316.1 for δ-T and 21.9 for β-T.

Subsequently several samples of this oil enriched with α-T were prepared by adding 0.002, 0.02, 0.2, 2 and 5% in weight of α-T to the oil, and given the following

designations: SO+0.002, SO+0.02, SO+0.2, SO+2 and SO+5, respectively. α-Tocopherol with a purity of 98.2% was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The molar percentages of the different types of oil acyl groups were determined by ^{1}H NMR, as in previous works (Guillén & Ruiz, 2003; Guillén & Uriarte, 2009, 2012). These were 6.6 ± 0.4 for linolenic, 42.8 ± 0.4 for linoleic, 32.4 ± 1.0 for oleic and 18.2 ± 1.9 for saturated groups.

2.2. Thermal treatment

10 g portions of each SO sample were poured into plastic Petri dishes of 80 mm diameter. These were placed in a convection oven and heated at 70º C with circulating air but without forced convection, simulating accelerated storage conditions. Aliquots were taken every day for their study by ^{1}H NMR. The evolution of the samples was monitored until their total polymerization, when it is no longer possible to take a sufficiently fluid sample to be analyzed. The heating experiments were carried out in duplicate with all the studied samples.

2.3. Monitoring by ^{1}H NMR of the evolution of SO and of the SO enriched with different proportions of α-T

2.3.1. Operating conditions

The ^{1}H NMR spectra of the starting oils and of the corresponding aliquots taken throughout the accelerated storage conditions were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz. The weight of each aliquot was approximately 0.16 g. These were mixed in a 5 mm diameter tube with 400 µl of deuterated chloroform that contained 0.2% of non deuterated chloroform and a small amount (0.03%) of tetramethylsilane as internal references. The acquisition parameters used were: spectral width 5000 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.744 s and pulse width 90º, with a total acquisition time of 8 min 55 s. The relaxation delay and acquisition time selected allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making possible their use for quantitative purposes. The experiments were carried out at 25 ºC, as in previous works (Guillén & Ruiz, 2003, 2005). Each sample was analyzed in duplicate, in order to obtain a mean value for the concentration of each of the studied components. In Figures 1 and 3, the plots corresponding to the same ^{1}H NMR spectral region in each

sample have been drawn at a fixed value of absolute intensity to be valid for comparative purposes.

2.3.2. Identification of oil components

The identification of the various oil components was made on the basis of the signals assignments shown in Table S1. For this purpose, several standard compounds were used; these were (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E,E*)-2,4-hexadienal, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, (*E*)-2-penten-1-ol and 1-hexanol, acquired from Sigma-Aldrich (St. Louis, MO, USA), and 4-hydroperoxy-(*E*)-2-nonenal, 4-hydroxy-(*E*)-2-nonenal and 4,5-epoxy-(*E*)-2-decenal, purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.3.3. Quantitative data estimated from ^{1}H NMR spectra

The molar percentages of the several kinds of acyl groups throughout the oils thermodegradative process were estimated as in previous studies (Guillén & Uriarte, 2012). For this purpose, trilinolein and trilinolenin, acquired from Sigma-Aldrich, were used. It must be noticed that, mainly in samples SO+2 and SO+5, the signals of the α-T added are detectable in their ^{1}H NMR spectra during part of the accelerated storage period. Taking into account that some of these signals (Baker & Myers, 1991) partially overlap with some signals of triglycerides, their contribution was subtracted when determining the molar percentages of oil acyl groups.

The concentrations of the different types of oxidation products generated were estimated as millimol per mol of triglyceride (mmol/mol TG). The general equation to carry out this determination was the following: $[OP] = [(A_{OP}/n)/(A_{TG}/4)]*1000$, where A_{OP} is the area of the signal selected for the quantification of each oxidation product (OP), n the number of protons that generate the signal and A_{TG} the area of the protons at *sn*-1 and *sn*-3 positions in the glycerol backbone of TG (signal "I" in Table S1).

2.4. Statistic and kinetic studies

The Microsoft Office Excel 2007 software was used to find equations that fit heating time and the concentrations of saturated+modified acyl groups, of hydroperoxides and of the conjugated dienes associated to them, in all the studied samples.

3. RESULTS AND DISCUSSION

The evolution of the thermoxidation process of soybean oil, enriched or not with different levels of α-T, was followed from the beginning of the process to its total polymerization. Attention was focused both on acyl group degradation and on new compound formation and evolution.

It must be pointed out that the oxidation process of the sample with 0.002% of α-T added (SO+0.002) was the same as that of the non-enriched oil (SO), so this enrichment level does not modify the oxidative stability of the original soybean oil. This could be explained because this addition level involves only a small increase in the α-T concentration of the oil (of approximately 20 mg/kg against the 130.0 mg/kg already existing). For this reason, this sample will not be taken into account when discussing the effect of the α-T addition to SO.

3.1. Effect of α-T on the evolution of the different types of oil acyl groups

As it is known, accelerated storage conditions produce the degradation of the acyl groups of edible oils (Guillén & Ruiz, 2005), and the most unsaturated ones are the most affected, almost eventually disappearing at the advanced stages of the process. Figure 1 shows, as example, the ^{1}H NMR spectrum of SO oil before being subjected to the accelerated storage process (day 0), together with the enlargements of some spectral regions where changes occur throughout the thermoxidation process and their evolution with time. It can be observed that, after 10 days under accelerated storage conditions, signal "H", due to *bis*-allylic protons of linolenic groups (see Table S1), has completely disappeared and that the intensity of signal "G", due to *bis*-allylic protons of linoleic and diunsaturated ω-6 groups, is very low.

The molar percentages of the different kinds of acyl groups (linolenic, linoleic and diunsaturated ω-6, monounsaturated and saturated+modified) are represented *versus* time in days in Figure 2a. It can be observed that, as a consequence of the degradation of the unsaturated groups, the molar percentage of saturated+modified (S+M) groups increases in all the samples and, to some extent, the evolution of this latter parameter globally summarizes the evolution of the degradation rate of acyl groups in the accelerated storage process of the several samples.

Although the thermodegradation processes of all the samples evolve overall as above mentioned, clear differences are observed among them. These concern the time at which acyl groups begin to degrade and the rate at which the degradation proceeds once it has started. The first is associated with the oxidative stability of the sample, and the second with the evolution of its oxidation process.

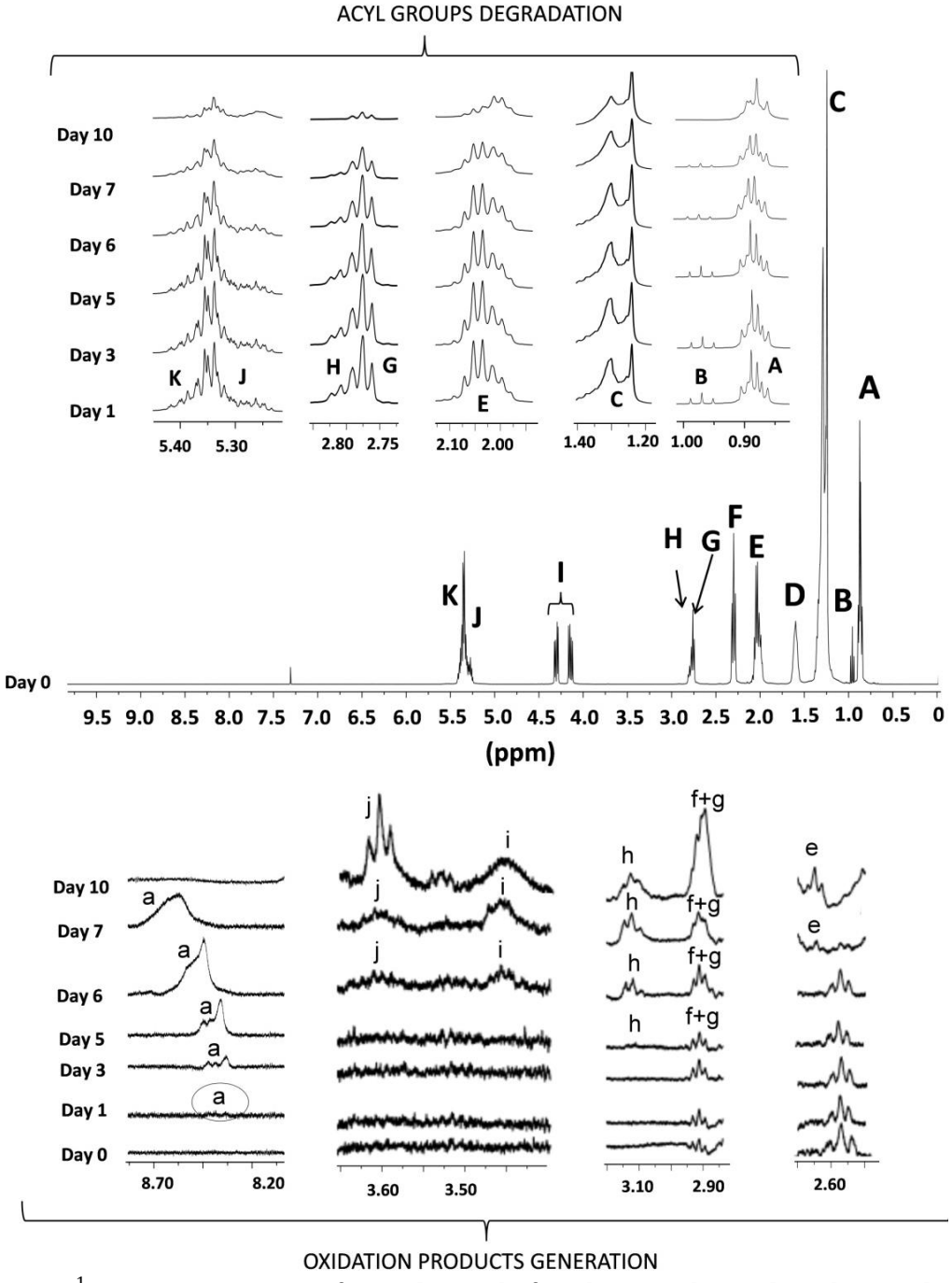

Figure 1. ^{1}H NMR spectrum of sample SO before being subjected to the accelerated storage process, together with the enlargements of some spectral regions where changes occur throughout the accelerated storage process and their evolution with time. Letters agree with those in Table S1.

3.1.1. Time at which the degradation of acyl groups begins

This information can be obtained from the detailed analysis of the evolution of the molar percentage of the S+M groups *versus* time in the several samples, represented in Figure 2a. Table 1 shows the estimated rates at which the molar percentage of these groups increases in each sample during different periods of time under the above mentioned accelerated storage conditions. These rates d(S+M)%/dt are given by the slopes of the straight parts of the lines that fit S+M molar percentage and heating time; likewise, the correlation coefficients (R) between the experimental data and those of the corresponding fitted equations are also given.

Table 1 evidences that in SO sample the molar percentage of S+M remains constant during the first day under accelerated storage conditions. However, in the samples enriched with α-T, the molar percentage of S+M increases since the beginning of the accelerated storage time due to the degradation of some of the polyunsaturated acyl groups (see Figure 2a). These results prove that the enrichment of the oil with α-T in the proportions before mentioned (0.02, 0.2, 2 and 5%), provokes a reduction of its oxidative stability; that is, α-T acts as prooxidant under the conditions of this study.

3.1.2. Evolution of the oxidation process once it has begun

Regarding the evolution of the oxidation process once it has been initiated, data in Table 1 indicate that its rate also depends on the α-T enrichment level. As this table shows, in all samples the process can be approximately divided into two periods with different degradation rates until total polymerization is reached. The first one is very short for SO, (from days 1 to 4) and the rate of degradation (0.74%/day) is also the lowest of all. As the level of α-T enrichment of the sample increases, the duration of this period becomes longer and the rate of the process is faster, ranging from 0.93%/day in SO+0.02 to 3.96%/day in SO+5. This means that in the sample having the highest level of α-T the initial rate of oxidation is considerably higher than in the samples with lower α-T concentrations.

The second period has a similar duration in most of the samples, except for the most α-T-enriched one (SO+5), where it is longer. Moreover, in the samples with the highest α-T concentrations (SO+2 and SO+5) total polymerization is reached after 12 and 20 days, respectively. It must be noticed that in this second period SO sample exhibits the

highest degradation rate (8.91%/day), whereas SO+5 sample has the lowest one (2.39 %/day), this being the reason why this latter reaches total polymerization after 20 days.

In summary, the total polymerization of the samples, which entails almost the total disappearance of the polyunsaturated acyl groups, is produced faster in SO and in the samples having lower α-T levels, than in those richer in α-T. However, the onset of oxidation is faster in the α-T-enriched samples than in SO, and the degradation rate in the first period of the oxidation process increases with α-T concentration.

3.2. Formation and evolution of oxidation products. Influence of the α-T enrichment.

3.2.1. Hydroperoxides

The thermodegradation of unsaturated acyl groups gives rise to the formation of hydroperoxides in all the samples studied. This can be observed by the appearance in their ^{1}H NMR spectra of signals due to the proton of the hydroperoxy group (signal "a" in Figure 1 and Table S1) and to the protons of conjugated dienes associated to them, which can exhibit either (*Z*,*E*)- or (*E*,*E*)-conjugated isomerism (signals "d" and "c", respectively, in Figure 3a and Table S1). Figure 2b shows the evolutions of the molar concentrations of total hydroperoxides and of those having (*Z*,*E*)- and (*E*,*E*)-conjugated dienes separately, expressed in mmol/mol TG.

3.2.1.1. Evolution of the concentration of total hydroperoxides

Figures 1 (signal "a") and 2b reveal that, in SO, hydroperoxides are detected after one day under accelerated storage conditions and their concentration rises with time until it reaches a maximum on the 7th day, after which it decreases sharply. Figure 2b also shows that the increasing rate of hydroperoxides concentration is not constant over time, but two periods with different formation rates can be noticed, that of the first one (from days 1 to 4) being lower. These periods, expressed in days, and the corresponding paces of increase of hydroperoxides concentration are shown in Table S2.

In the α-T-enriched samples, hydroperoxides are detected at the same time as in SO (see Figure 2b); however, as Table S2 shows, their concentration increases at a higher rate as α-T concentration rises (from 6.03 mmol.mol TG^{-1}/day in SO to 67.05 mmol.mol TG^{-1}/day in SO+5). Actually, both Figure 2b and Table S2 reveal that, in the samples with the lowest α-T enrichment levels (0.02 and 0.2%), two periods with different hydroperoxides concentration increasing rates are noticed, while in those with higher α-

T levels the pace at which hydroperoxides concentration grows can be considered constant over time. Moreover, the maximum concentration reached is also higher as more elevated the α-T enrichment degree is (from 134.36 mmol/mol TG in sample SO to 285.08 mmol/mol TG in SO+5). These findings confirm that the addition of α-T reduces the oxidative stability of the SO oil, thus exhibiting a prooxidant effect. An increase in the concentration of hydroperoxides due to the addition of α-T has also been reported by other authors in the oxidation processes of purified soybean oil (Jung & Min, 1990) and of purified rapeseed oil triacylglycerols (Isnardy et al., 2003).

The amount of α-T added not only influences the generation kinetics of hydroperoxides and their maximum concentration, but also their decomposition since, as Figure 2b shows, this begins earlier in the α-T-enriched oils. It is noticeable that, in SO+5 sample, the concentration of hydroperoxides remains high between days 4 and 7. This seems to agree with the hydroperoxide stabilization observed by Mäkinen, Kamal-Eldin, Lampi and Hopia (2001) during the oxidation of methyl linoleate in the presence of α-T.

3.2.1.2. Evolution of conjugated (*Z,E*)- and (*E,E*)-hydroperoxydienes

All the above commented refers to (*Z,E*)- and (*E,E*)-hydroperoxides jointly. However, the evolution of both kinds of hydroperoxides, which can be measured from signals "d" and "c", respectively (see Figure 3a and Table S1), is not the same (see Figure 2b).

As Figure 2b shows, in SO and SO+0.02, the formation rates of conjugated (*Z,E*)- and (*E,E*)-hydroperoxydienes ((*Z,E*)- and (*E,E*)-CD-OOH, respectively) are initially similar for a short period of time, after which the concentration of (*E,E*)-CD-OOH becomes higher than that of the (*Z,E*)-ones. Notwithstanding, the two kinds of hydroperoxides reach their maximum concentration at the same time in both samples (after 7 days under accelerated storage conditions). The growing rates of the molar concentrations of both (*Z,E*)- and (*E,E*)-CD-OOH in all the studied samples are given in Table S3.

It can be seen in Figure 2b and Table S3 that, as the α-T concentration increases, so does the formation rate of (*Z,E*)-CD-OOH in comparison with that of the (*E,E*)-ones, reaching their maximum earlier; this is considerably higher in the samples with the highest α-T concentrations (SO+2 and SO+5). Moreover, in these latter, both a diminution in the formation rate of (*E,E*)-CD-OOH and a decrease in their maximum

concentration are observed in comparison with the samples with lower α-T levels, the more pronounced the higher the α-T concentration is. These results, which coincide with those observed by other authors in methyl linoleate in the presence of high α-T concentrations (Mäkinen & Hopia, 2000; Mäkinen et al., 2001), could be explained by an inhibition of the isomerization of (*Z*,*E*)- to (*E*,*E*)-hydroperoxides, due to the ability of α-T to donate a hydrogen atom to (*Z*,*E*)-peroxyl radicals before they have time to rearrange to the more thermodynamically stable (*E*,*E*)-hydroperoxides (Banni et al., 1996; Mäkinen & Hopia, 2000).

3.2.2. Formation of conjugated (*Z*,*E*)-hydroxy-dienes

The formation of conjugated (*Z*,*E*)-hydroxy-dienes has been observed by ^{1}H NMR in the oxidation process of sunflower oil at room temperature with limited oxygen (Guillén & Goicoechea, 2009), but not in the thermodegradative processes of vegetable oils carried out at 70 ºC (Martínez-Yusta et al., 2014), probably due to their low concentration. However, a detailed analysis of the ^{1}H NMR spectra of SO sample throughout the accelerated storage process, allows one to notice the appearance of very small signals that could be assigned to (*Z*,*E*)-hydroxy-dienes ((*Z*,*E*)-CD-OH) (signal "b" in Figure 3a and Table S1). The intensity of these signals clearly increases in the samples with the highest α-T concentration (SO+2 and SO+5). According to some authors, this increase in the concentration of (*Z*,*E*)-CD-OH seems to be due to the ability of α-T to donate an hydrogen atom to alkoxyl radicals (Hopia, Huang & Frankel, 1996).

The enlargements of Figure 2b, which show the evolution of the molar concentration of these compounds in all the studied samples, in mmol/mol TG, reveal that, although the addition of α-T to SO anticipates the detection of (*Z*,*E*)-CD-OH from day 3 to day 2, their evolution with time is hardly affected in the samples with lower proportions of α-T added (SO+0.02 and SO+0.2). However, both the maximum concentration of (*Z*,*E*)-CD-OH and their existence period increase as the α-T amount rises.

It is worth pointing out that the increase in the concentration of hydroxy-dienes could have health implications, since this type of compounds have not only been related with some pathologies such as atherosclerosis (Ramsden et al., 2012), but have also been considered potential precursors of reactive cytotoxic aldehydes in biological systems (Schneider, Porter & Brash, 2004).

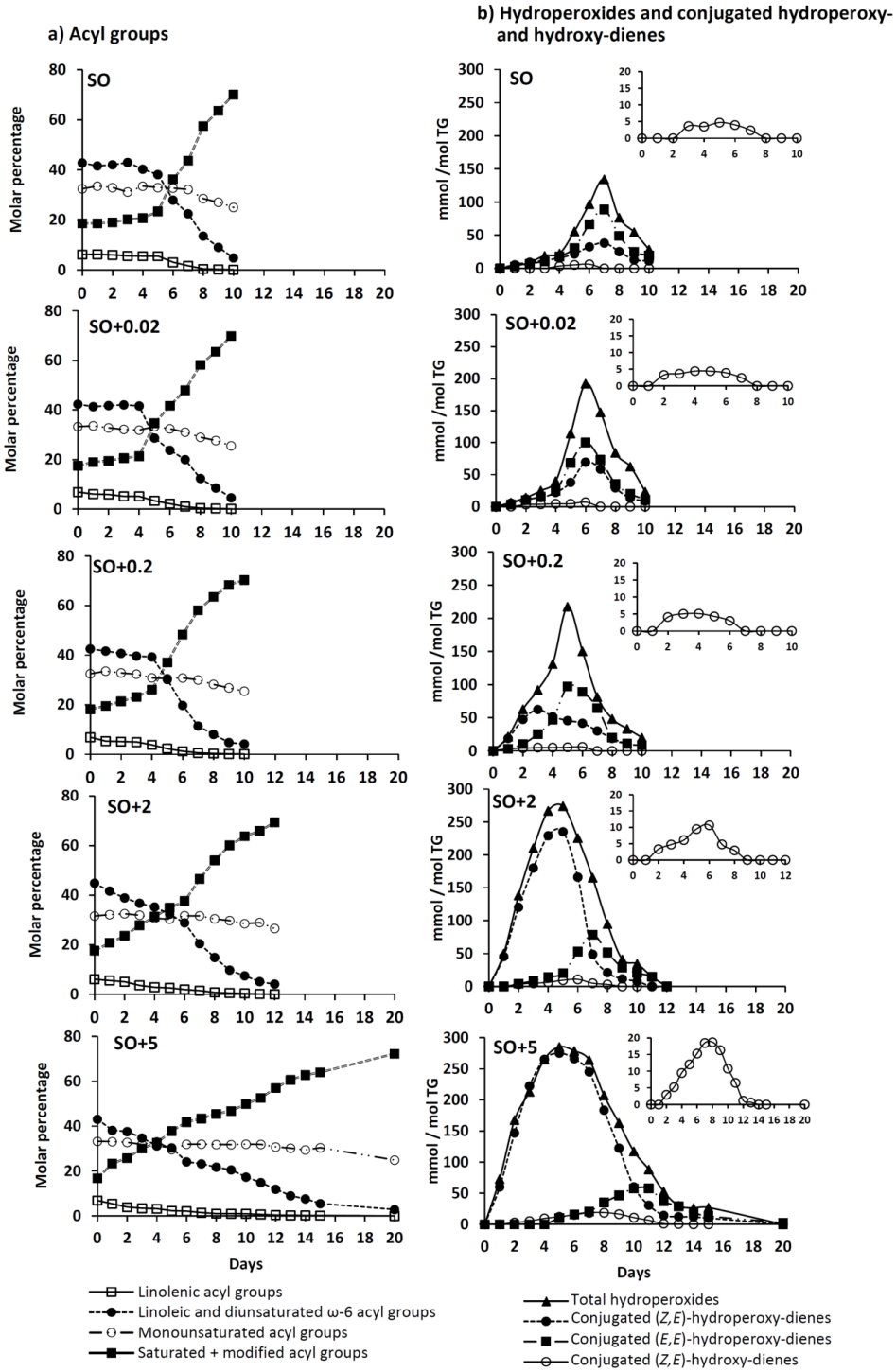

Figure 2. Evolution throughout the accelerated storage process, in each one of the studied soybean oil samples, of: a) the molar percentages oflinolenic, linoleic and diunsaturated ω-6, monounsaturated and saturated + modified acyl groups; b) the concentrations, in mmol/mol TG, of hydroperoxides and their associated conjugated (*Z*,*E*)- and (*E*,*E*)-dienes, and of conjugated (*Z*,*E*)-hydroxy-dienes. All the figures reported are mean values.

3.2.3. Formation of epoxides

As was the case of other vegetable oils subjected to the same conditions as in this study (Martínez-Yusta et al., 2014), the formation of mono- and possibly di-epoxides was observed in the samples here studied. The appearance and further evolution with time of the signals due to this type of compounds in the oil without α-T added (SO) can be observed in Figure 1 (see signals "e", "f"+g" and "h"). Figure 4a, in turn, shows the evolution of the concentrations of the various types of epoxides detected in the different samples, in mmol/mol TG.

3.2.3.1. Monoepoxides other than (*E*)-epoxystearates

This type of monoepoxides, which give signals around 2.9 ppm (see Table S1), comprises monoepoxides coming from linoleic and linolenic acyl groups (signal "g"), as well as (*Z*)-epoxystearates (signal "f"), derived from oleic groups. However, considering that polyunsaturated groups (linoleic and linolenic) are, by far, those which degrade at a faster rate (see Figure 2a), it can be assumed, at least during the first stages of the accelerated storage process, that the monoepoxides formed are those coming from these groups.

Figure 4a shows that the evolution of the concentration of this type of monoepoxides is very similar in all the samples, in the sense that it always exhibits an increase with heating time. However, some differences are observed in the samples with α-T added when compared with SO.

Overall, as the α-T concentration gets higher, the time at which these monoepoxides are detected decreases (from day 5 in SO to day 2 in SO+5), and their initial concentration increases. Furthermore, in the samples with the highest α-T amounts (SO+2 and SO+5) monoepoxides seem to stabilize, to a certain extent (between days 5 and 7 in SO+2 and between 7 and 10 in SO+5), to rise again until the end of the accelerated storage process. An increase in the maximum concentration reached at the end of the accelerated storage process is also observed in the most enriched samples (SO+2 and SO+5).

3.2.3.2. (*E*)-Epoxystearates

These monoepoxides derive specifically from oleic groups and, as can be observed in Figure 1 (see signal "e"), they are detected in SO after 7 days under accelerated storage conditions, later than the rest of monoepoxides.

The addition of α-T to SO affects the evolution of the concentration of (*E*)-epoxystearates differently depending on the α-T amount. Actually, as Figure 4a shows, the formation rate of (*E*)-epoxystearates increases with α-T concentration until a certain enrichment level (0.2%), above which the generation of this type of compounds becomes slower. The maximum concentrations, reached in all cases at the end of the accelerated storage process, are quite similar in all the α-T-enriched samples (see Figure 4a). However, if the same point of the process is considered for all the samples, the concentration of (*E*)-epoxystearates is always lower in those having the highest α-T levels (SO+2 and SO+5). It is also worth noticing that, in these two latter, the emergence of (*E*)-epoxystearates and the subsequent increase in their concentration almost parallels both the decrease in the molar percentage of oleic groups (see Figure 2a) and the second rise in the concentration of the other type of monoepoxides mentioned in section 3.2.3.1. This could suggest that the late increase observed in the concentration of monoepoxides other than (*E*)-epoxystearates could be related to the generation of (*Z*)-epoxystearates, also derived from the degradation of oleic groups.

3.2.3.3. Diepoxides

In previous works, the determination of diepoxides has been made on the basis of the signal assignement made by Aerts and Jacobs (2004), according to whom this type of compounds give signals at 2.9 and 3.1 ppm. However, recent findings of our research group (unpublished results) show that, under certain conditions, the signal at 3.1 ppm is not accompanied by another one of the same intensity at 2.9 ppm. In addition, the signals observed in the ^{1}H NMR spectra recorded throughout the accelerated storage process of the studied soybean oil neither match exactly the ones reported by other authors for diepoxides. Thus, according to Xia, Budge and Lumsden (2016), diepoxides separated by a methylene group give three multiplets at 3.00, 3.09 and 3.14 ppm. However, the one appearing at 3.00 ppm has not been observed either in this study or in any of those performed previously under the same oxidative conditions (Goicoechea & Guillén, 2010). This lack of agreement might be due to the fact that diepoxides formed under the conditions of our study could be different from those reported in the literature, and therefore, give different signals. It must be noticed that, as Xia and coworkers themselves (2016) have commented on, the epoxides formed during the oxidation process of vegetable oils and those resulting from epoxidation reactions using hydrogen peroxide and formic acid, could lead to the formation of different types of epoxides.

Actually, some researchers (Byrdwell and Neff, 2001) have indicated that epoxides can form at different points of the acyl group moiety, leading to the formation of a wide range of mono- and di-epoxides, whose chemical shifts, as far as we know, have not been described yet. Therefore, we will tentatively assign the signal at 3.1 ppm to diepoxides, even though the contribution of other oxidation products supporting epoxy groups should not be discarded (see Table S1).

In SO sample, as Figure 4a shows, diepoxides are detected at the same time as monoepoxides other than (*E*)-epoxystearates (after 5 days under accelerated storage conditions), and in similar concentration, measured from signal "h" (see Figure 1 and Table S1). These reach a maximum the 9^{th} day, to decrease thereafter.

In the α-T-enriched samples, in general, the formation of diepoxides takes place earlier than in SO (see Figure 4a). However, their maximum concentration, quite similar in all the samples, is reached more quickly in the oils with lower α-T concentrations than in those with higher α-T levels. This means that, once diepoxides have emerged, the increasing rate of their concentration tends to diminish with α-T amount.

In summary, it could be said that the addition of α-T to SO, above all when the enrichment level is high, induces an earlier formation of both diepoxides and monoepoxides other than (*E*)-epoxystearates. However, as the α-T concentration increases, the generation of the latter seems to be favoured over that of the former. This fact deserves particular attention since some monoepoxides derived from linoleic groups, such as leukotoxin and isoleukotoxin, are well-known toxic compounds, precursors of toxic diols (Greene et al., 2000).

The formation of higher concentrations of certain epoxides in the samples with the highest α-T concentrations could be related to an enhancement of some reactions mediated by alkoxyl radicals, such as cyclization to epoxides (Schaich, 2013).

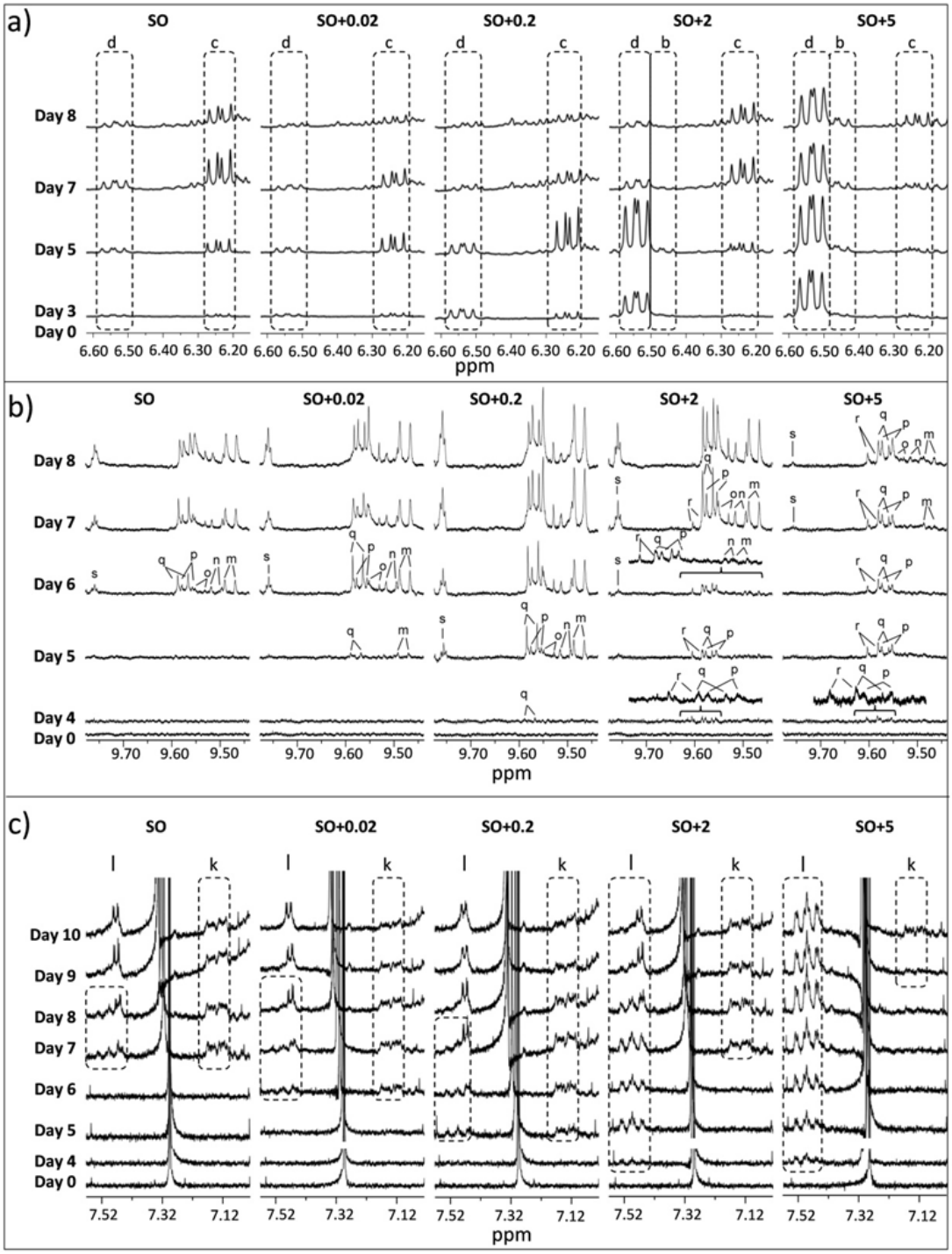

Figure 3. Evolution throughout several days of the accelerated storage process, in all the studied samples, of the ^{1}H NMR spectral signals of: a) conjugated (*Z,E*)- and (*E,E*)-hydroperoxydienes (signals "d" and "c", respectively) and conjugated (*Z,E*)-hydroxy-dienes (signal "b"); b) aldehydes: *n*-alkanals (signal "s"), 4-hydroperoxy-(*E*)-2-alkenals (signal "q"), 4-hydroxy-(*E*)-2-alkenals (signal "p"), 4,5-epoxy-2-alkenals (signal "o"), (*E,E*)-2,4-alkadienals (signal "n"), (*E*)-2-alkenals (signal "m"), and (*Z,E*)-2,4-alkadienals (signal "r"); and c) conjugated (*Z,E*)- and (*E,E*)-keto-dienes (signals "l" and "k", respectively). Letters agree with those in Table S1.

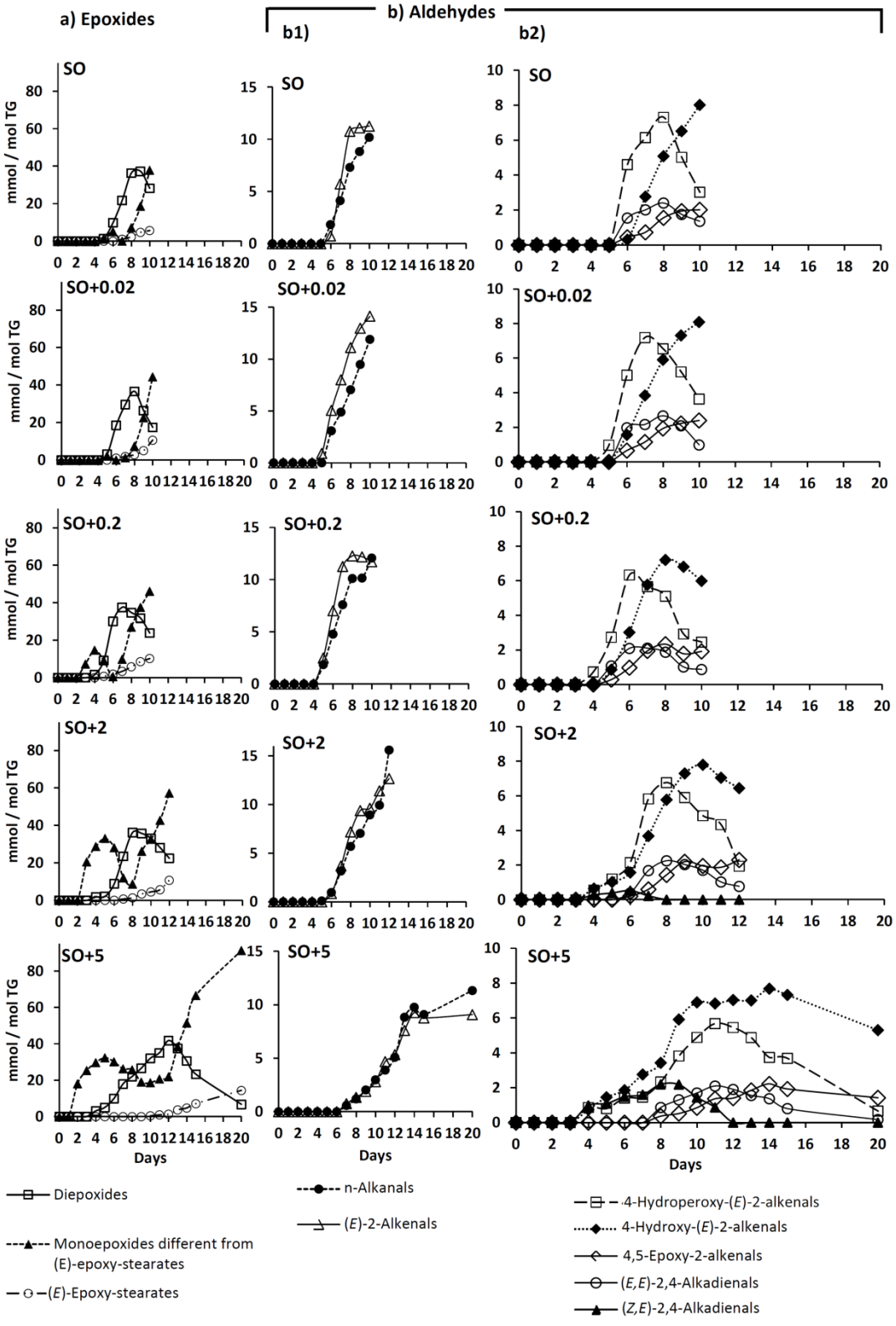

Figure 4. Evolution throughout the accelerated storage process, in each one of the studied soybean oil samples, of the concentrations, expressed in mmol/mol TG, of the different types of: a) epoxides; and b) aldehydes. All the figures reported are mean values

3.2.4. Formation of aldehydes

Aldehydes constitute an important group of secondary oxidation products, since some of them, especially the oxygenated α,β-unsaturated ones, are well-known for their reactivity and toxicity (Guillén & Goicoechea, 2008). Figure 3b shows the appearance and evolution throughout the accelerated storage process (up to 8 days) of aldehydic signals (signals "m-s" in Table S1) in the ^{1}H NMR spectra of the several samples. The evolution of the concentrations of the different kinds of aldehydes throughout the accelerated storage process, in mmol/mol TG, can be observed in Figure 4b.

As both Figures 3b and 4b show, in SO all the various kinds of aldehydes are detected after 6 days of heating. As the concentration of α-T increases, the formation of some kinds of aldehydes begins earlier than in SO, that of others is delayed and the generation of aldehydes which are not detected in SO takes place.

Among the several kinds of aldehydes detected, n-alkanals and (*E*)-2-alkenals are the most abundantly generated in all the samples (see Figure 4b1), and their concentrations, in general, increase over time. The appearance time of these two types of aldehydes (between day 5 in sample SO+0.2 and day 7 in SO+5) and their maximum concentrations are not greatly affected by the level of α-T enrichment. However, these latter are reached later in the samples with the greatest α-T levels (SO+2 and SO+5). These findings could explain the results obtained by Huang, Frankel and German (1994), who found that, despite increasing α-T concentrations caused a rise in hydroperoxide generation, the ability to inhibit hexanal (n-alkanal) formation improved.

(*E*,*E*)-2,4-Alkadienals are formed in lower concentrations than the previous ones, which agrees with observations made regarding sunflower oil subjected to the same conditions as in this work (Goicoechea & Guillén, 2010; Guillén & Ruiz, 2005). As in the case of n-alkanals and (*E*)-2-alkenals, the increase in the amount of added α-T up to a proportion of 2% does not exert a marked effect either on the onset or on the maximum concentration of this type of aldehydes. However, in sample SO+5, although the maximum concentration is also very similar to that observed in the rest of the samples, both a delay in the time at which these aldehydes are detected (day 8) and an increase in the time needed to reach the maximum concentration can be observed in Figure 4b2.

Among the different types of aldehydes detected, the oxygenated α,β-unsaturated ones (4,5-epoxy-alkenals, 4-hydroxy-(*E*)-2-alkenals and 4-hydroperoxy-(*E*)-2-alkenals)

deserve special attention due to their toxicity, as pointed out above. 4,5-epoxy-alkenals are considered to derive from (*E*,*E*)-2,4-alkadienals (Gassenmeier & Schieberle, 1994) and, in fact, the concentration of the former is initially lower than that of the latter in all the samples (see Figures 3b and 4b2); however, as the concentration of 4,5-epoxy-alkenals approximates its maximum, that of (*E*,*E*)-2,4-alkadienals drops.

It is worth highlighting that the formation of 4-hydroperoxy- and 4-hydroxy-(*E*)-2-alkenals is greatly affected by the α-T concentration, in such a way that, as Figures 3b and 4b2 show, the higher the α-T enrichment level, the earlier their appearance. It must also be noticed that in the samples with the highest α-T concentrations (SO+2 and SO+5), these two kinds of aldehydes are formed at the same time; this contrasts with the rest of the samples and with the mechanism for the generation of 4-hydroxy-(*E*)-2-alkenals proposed by Gardner and Hamberg (1993), according to whom these latter can proceed from 4-hydroperoxy-(*E*)-2-alkenals. Moreover, a slight increase in the concentration of 4-hydroxy- relative to 4-hydroperoxy-(*E*)-2-alkenals can be observed in the most α-T-enriched samples, especially in SO+5.

Finally, the appearance of **(***Z*,*E*)-2,4-alkadienals together with 4-hydroperoxy- and 4-hydroxy-(*E*)-2-alkenals, in the samples with the highest α-T concentrations (see signal "r" in Figure 3b) must be noted. This type of aldehydes had not been detected previously by ^{1}H NMR in vegetable oils subjected to the same heating conditions as in this study (Guillén & Ruiz, 2005; Martínez-Yusta et al., 2014). An increase in the concentration of volatile unsaturated aldehydes with (*Z*,*E*)-configuration due to the addition of α-T was also reported by Kulas, Olsen and Ackman (2002) in fish oil stored at 30 ºC.

3.2.5. Formation of conjugated keto-dienes

The thermoxidation of the soybean oil here studied also gives rise to the formation of oxidation products tentatively identified as conjugated keto-dienes, which can exhibit both (*Z*,*E*)- and (*E*,*E*)-isomerism (Banni et al., 1996). As can be observed in Table S1, (*Z*,*E*)-keto-octadecadienoic derivatives give signals centered between 7.43 and 7.50 ppm and the (*E*,*E*)-ones at 7.13 ppm. The evolution of these signals throughout the accelerated storage process in the different samples can be observed in Figure 3c and the progress of their corresponding concentrations, in mmol/mol TG, in Figure 5a. Figure 3c reveals that, in sample SO, both (*Z*,*E*)- and (*E*,*E*)-keto-dienes are detected after 7 days of heating (signals "l" and "k", respectively). Unlike the signals of (*E*,*E*)-

keto-dienes, those of the (*Z,E*)-ones disappear quickly with time. These findings agree with those of Mäkinen and Hopia (2000), who also found that (*E,E*)-keto-dienes were those predominantly formed during the decomposition of methyl linoleate hydroperoxides.

The enrichment of SO oil with α-T affects the evolutions of both types of keto-dienes differently. With regard to (*Z,E*)-keto-dienes, Figures 3c and 5a show that, as the α-T concentration increases, this type of compounds appears earlier. However, in the case of (*E,E*)-keto-dienes, their formation begins sooner as the α-T concentration increases up to a determined α-T addition level (0.2%), above which their onset is considerably delayed in comparison with that of (*Z,E*)-keto-dienes, in such a way that, in SO+5, (*E,E*)-keto-dienes are not detected until the 9^{th} day.

In relation to the maximum concentration reached in the different samples, it can be said that while that of (*E,E*)-keto-dienes is scarcely affected by the α-T enrichment level, that of the (*Z,E*)-ones increases with the α-T amount, to the point that, unlike the rest of the samples, the concentration of (*Z,E*)-keto-dienes in SO+5 remarkably exceeds that of the (*E,E*)-ones. This trend agrees with the findings of Mäkinen and Hopia (2000), who also observed a prevalent formation of (*Z,E*)-keto-dienes during the decomposition of methyl linoleate hydroperoxides in presence of α-T.

3.2.6. Formation of alcohols

The formation of other types of alcohols than the hydroxy-dienes described in section 3.2.2 is observed at a more advanced stage of the accelerated storage process in all the studied samples; these include primary alcohols as well as certain diols, tentatively identified as leukotoxin- and/or isoleukotoxin-diols. These latter, which derive from linoleic groups monoepoxides, deserve special attention since they have been shown to have negative effects *in vitro* and in animal studies (Markaverich et al, 2005).

The evolution of the ^{1}H NMR signals of these two kinds of alcohols throughout the accelerated storage process of sample SO can be observed in Figure 1 (signals “i” and “j”), while the evolution of their concentrations in all the studied samples is shown in Figure 5b, in mmol/mol TG. This reveals that both primary alcohols and diols are detected simultaneously in all the samples, but their increasing concentration rate is always higher for the latter.

As Figure 5b shows, the enrichment of SO oil with α-T slightly accelerates the onset of this type of compounds in the samples with a proportion of added α-T higher than 0.02% (from day 6 to 5). However, in the oils with the highest α-T levels (SO+2 and SO+5), a clear reduction in the increasing rate of the concentration of diols and, above all, of primary alcohols is observed, especially in SO+5. In this latter, a slightly higher proportion of diols relative to primary alcohols than in the rest of the samples is also noticed.

3.2.7. Relationship between the evolution of hydroperoxides and those of other oxidation products in the different samples studied

In the samples with the lowest α-T enrichment levels (SO+0.02 and SO+0.2), an acceleration in the generation of both (*Z*,*E*)- and (*E*,*E*)-hydroperoxides in relation to SO occurs (see Figure 2b). This is reflected in the evolution of epoxides, aldehydes, keto-dienes and alcohols, which are generally detected earlier than in SO.

However, when the α-T concentrations are higher, (*Z*,*E*)- and (*E*,*E*)-hydroperoxides are affected differently and not all the oxidation products evolve similarly. This is especially noticeable in the evolutions both of (*Z*,*E*)- and (*E*,*E*)-keto-dienes, and of (*Z*,*E*)-hydroxy-dienes, which go in line with that of their precursor hydroperoxides, but it can also be observed in the case of aldehydes.

On the one hand, both the earlier formation of 4-hydroperoxy- and 4-hydroxy-(*E*)-2-alkenals, and the appearance of (*Z*,*E*)-2,4-alkadienals in the samples with the highest α-T concentration coincide with a high concentration of (*Z*,*E*)-hydroperoxides. Moreover, in the case of (*Z*,*E*)-2,4-alkadienals, their evolution with time also goes in parallel to that of (*Z*,*E*)-hydroperoxides (see Figure 2b). On the other hand, the delay in the generation of n-alkanals, (*E*)-2-alkenals, (*E*,*E*)-2,4-alkadienals and 4,5-epoxy-2-alkenals, and the slower increase of their concentration in comparison with the samples having lower α-T levels, seem to go in accordance with the later formation and increase of (*E*,*E*)-hydroperoxides observed in these samples.

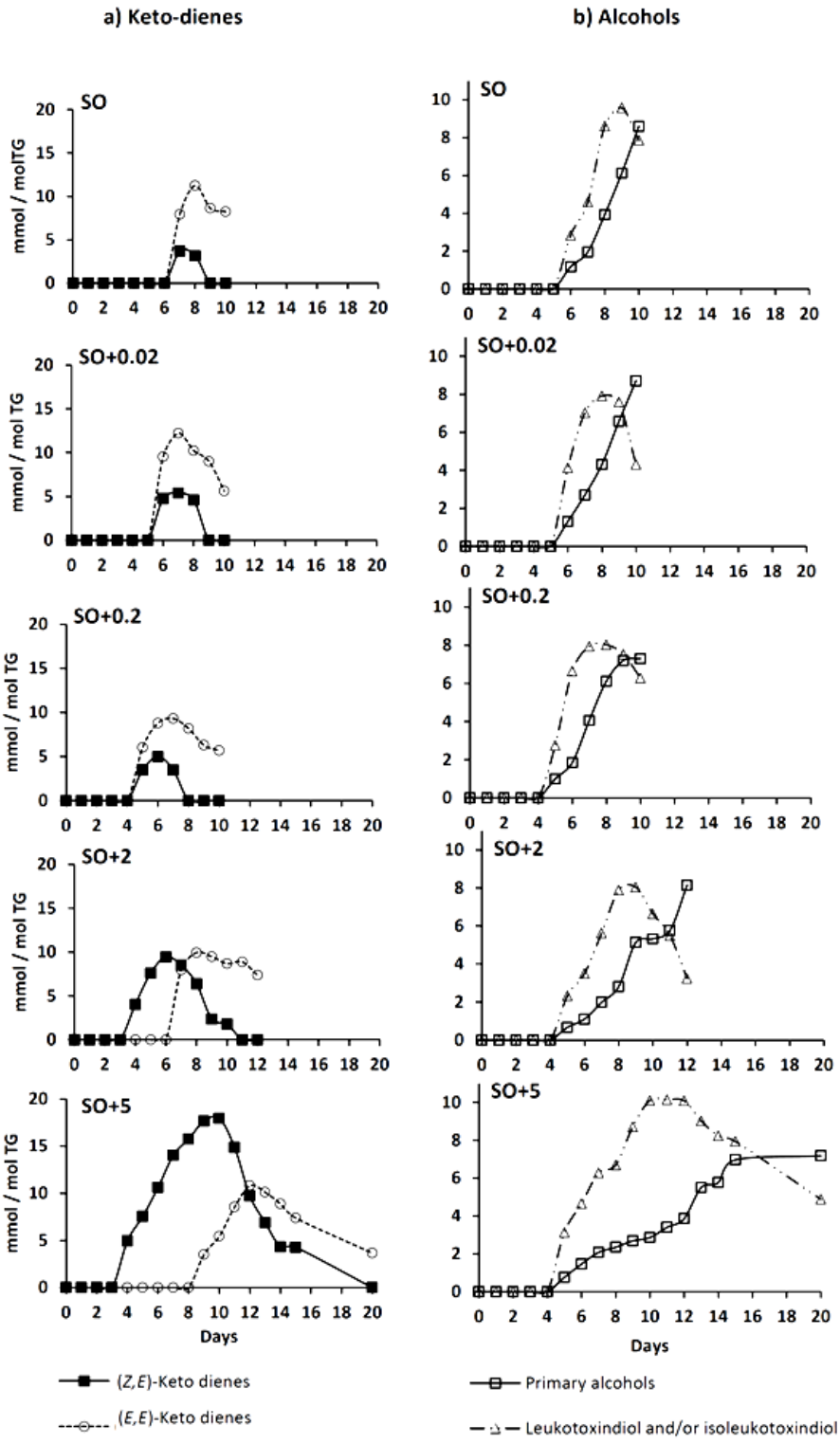

Figure 5. Evolution throughout the accelerated storage process, in each one of the studied soybean oil samples, of the concentrations, expressed in mmol/mol TG, of the different types of: a) conjugated keto-dienes, and; b) alcohols. All the figures reported are mean values.

4. CONCLUSIONS

As far as we know, this is the first time that a so exhaustive study on the influence of a broad range of α-T enrichment levels on the entire oxidation process occurring in a vegetable oil under accelerated storage conditions, involving the simultaneous monitoring of a wide variety of oxidation products, has been made.

Under the conditions of this study, the addition of α-T to soybean oil in all the proportions here tested, except that of 0.002%, which causes no change detectable by ^{1}H NMR, provokes an earlier start of the oxidation process, reducing the oxidative stability of the oil and thus showing a clear prooxidant effect. However, the time needed to reach total polymerization becomes longer in the samples with the highest α-T concentrations.

It has also been proved that the enrichment of SO with α-T affects differently the formation of the several oxidation products depending on the α-T level, modifying the oxidation pathway at high doses. Actually, high proportions of α-T markedly promote the generation of (*Z*,*E*)-hydroperoxides and of other oxidation products with (*Z*,*E*)-configuration, such as (*Z*,*E*)-hydroxy-dienes, (*Z*,*E*)-keto-dienes and (*Z*,*E*)-2,4-alkadienals, these latter not usually detected in the oxidation process of edible oils under the same conditions as in this study.

In particular, attention should be paid to those toxic compounds whose formation occurs earlier and, in some cases, more profusely, the higher the α-T concentration, such as some toxic oxygenated α,β-unsaturated aldehydes and monoepoxides derived from linoleic groups.

It is also worth noticing that, despite epoxides are not usually considered in oxidation processes, they markedly contribute to the total of products formed.

These results evidence that assessing the performance of α-T as antioxidant or prooxidant using only one compound as oxidation marker, or by means of the classical nonspecific methodologies used in many studies, can lead to erroneous conclusions. In addition, this type of studies should be conducted with real oil samples, already containing varying concentrations of tocopherols and other minor components that undoubtedly will affect the behaviour of α-T. Otherwise, the results obtained could hardly be considered of practical interest.

Finally, the findings of this work highlight the need to revise the European regulations to limit the addition of α-T to products intended for human consumption since, as in the vegetable oil here studied, the supplementation with α-T can have a negative effect both on the oxidative stability of the oil and even potentially on human health.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aerts, H. A., & Jacobs, P. A. (2004). Epoxide yield determination of oils and fatty acid methyl esters using ^{1} H NMR. *Journal of the American Oil Chemists' Society*, *81*, 841-846.

Alberdi-Cedeño, J., Ibargoitia, M. L., Cristillo, G., Sopelana, P., & Guillén, M. D. (2017). A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components. *Food Chemistry*, *221*, 1135-1144.

Baker, J. K., & Myers, C. W. (1991). One-dimensional and two-dimensional ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) analysis of vitamin E raw materials or analytical reference standards. *Pharmaceutical Research*, *8*, 763-770.

Banni, S., Contini, M. S., Angioni, E., Deiana, M., Dessi, M. A., Melis, M. P., & Corongiu, F. P. (1996). A novel approach to study linoleic acid autoxidation: importance of simultaneous detection of the substrate and its derivative oxidation products. *Free Radical Research*, *25*, 43-53.

Brigelius-Flohé, R. (2009). Vitamin E: the shrew waiting to be tamed. *Free Radical Biology and Medicine*, *46*, 543-554.

Byrdwell, W. C., & Neff., W. E. (2001). Autoxidation products of normal and genetically modified canola oil varieties determined using liquid chromatography with mass spectrometric detection. *Journal of Chromatography A, 905*, 85-102.

Cillard, J., Cillard, P., Cormier, M., & Girre, L. (1980). α-Tocopherol prooxidant effect in aqueous media: increased autoxidation rate of linoleic acid. *Journal of the American Oil Chemists' Society*, *57*, 252-255.

Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union*, 12.11.2011, L 295/1-177.

Dolde, D., & Wang, T. (2011). Oxidation of corn oils with spiked tocols. *Journal of the American Oil Chemists'* Society, 88, 1759-1765.

Gardner, H. W., & Hamberg, M. (1993). Oxygenation of (3Z)-nonenal to (2E)-4-hydroxy-2-nonenal in the broad bean (*Vicia faba* L.). *Journal of Biological Chemistry, 268*, 6971–6977.

Gassenmeier, K., & Schieberle, P. (1994). Formation of the intense flavor compound trans-4,5-epoxy-(*E*)-2-decenal in thermally treated fats. *Journal of the American Oil Chemists' Society, 71*, 1315–1319.

Goicoechea, E., & Guillén, M. D. (2010). Analysis of hydroperoxides, aldehydes and epoxides by ^{1}H nuclear magnetic resonance in sunflower oil oxidized at 70 and 100 C. *Journal of Agricultural and Food Chemistry*, *58*, 6234-6245.

Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau, C., & Hammoc, B. D. (2000). Metabolism of monoepoxides of methyl linoleate: Bioactivation and detoxification. *Archives of Biochemistry and Biophysics, 376*, 420-432.

Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α, β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition*, *48*, 119-136.

Guillén, M. D., & Goicoechea, E. (2009). Oxidation of corn oil at room temperature: Primary and secondary oxidation products and determination of their concentration in the oil liquid matrix from ^{1}H nuclear magnetic resonance data. *Food Chemistry*, *116*, 183-192.

Guillén, M. D., & Ruiz, A. (2003). Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups. *European Journal of Lipid Science and Technology, 105*, 688–696.

Guillén, M. D., & Ruiz, A. (2005). Oxidation process of oils with high content of linoleic acyl groups and formation of toxic hydroperoxy- and hydroxyalkenals. A study by ^{1}H nuclear magnetic resonance. *Journal of the Science of Food and Agriculture, 85*, 2413–2420.

Guillén, M. D., & Uriarte, P. S. (2009). Contribution to further understanding of the evolution of sunflower oil submitted to frying temperature in a domestic fryer: Study by ^{1}H nuclear magnetic resonance. *Journal of Agricultural and Food Chemistry, 57*, 7790–7799.

Guillén, M. D., & Uriarte, P. S. (2012). Study by ^{1}H NMR spectroscopy of the evolution of extra virgin olive oil composition submitted to frying temperature in an industrial fryer for a prolonged period of time. *Food Chemistry, 134*, 162-172.

Hopia, A., Huang, S. W., & Frankel, E. N. (1996). Effect of α-tocopherol and Trolox on the decomposition of methyl linoleate hydroperoxides. *Lipids, 31*, 357-365.

Huang, S. W., Frankel, E. N., & German, J. B. (1994). Antioxidant activity of alpha-and gamma-tocopherols in bulk oils and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 42*, 2108-2114.

Isnardy, B., Wagner, K.-H., & Elmadfa, I. (2003). Effects of α-, γ-, and δ- tocopherols on the autoxidation of purified rapeseed oil triacylglycerols in a system containing low oxygen. *Journal of Agricultural and Food Chemistry, 51*, 7775–7780.

Jung, M.Y., & Min, D.B. (1990). Effects of α-, γ-, and δ-tocopherols on oxidative stability of soybean oil. *Journal of Food Science, 55*, 1464-1465.

Koskas, J. P., Cillard, J., & Cillard, P. (1984). Autoxidation of linoleic acid and behavior of its hydroperoxides with and without tocopherols. *Journal of the American Oil Chemists' Society, 61*, 1466-1469.

Kulas, E., Olsen, E., & Ackman, R. G. (2002). Effect of α-, γ-, and δ-tocopherol on the distribution of volatile secondary oxidation products in fish oil. *European Journal of Lipid Science and Technology, 104*, 520-529.

Lampi, A.-M., Kataja, L., Kamal-Eldin, A., & Vieno, P. (1999). Antioxidant activities of α-and γ-tocopherols in the oxidation of rapeseed oil triacylglycerols. *Journal of the American Oil Chemists' Society, 76*, 749-755.

Mäkinen, E. M., & Hopia, A. I. (2000). Effects of α-tocopherol and ascorbyl palmitate on the isomerization and decomposition of methyl linoleate hydroperoxides. *Lipids, 35*, 1215-1223.

Mäkinen, M., Kamal-Eldin, A., Lampi, A. M., & Hopia, A. (2000). Effects of α- and γ-tocopherols on formation of hydroperoxides and two decomposition products from methyl linoleate. *Journal of the American Oil Chemists' Society, 77*, 801-806.

Mäkinen, M., Kamal-Eldin, A., Lampi, A. M., & Hopia, A. (2001). α-, γ- and δ-Tocopherols as inhibitors of isomerization and decomposition of cis, trans methyl linoleate hydroperoxides. *European Journal of Lipid Science and Technology, 103*, 286-291.

Markaverich, B. M., Crowley, J. R., Alejandro, M. A., Shoulars, K., Casajuna, N., Mani, S., & Sharp, J. (2005). Leukotoxin diols from ground corncob bedding disrupt estrous cyclicity in rats and stimulate MCF-7 breast cancer cell proliferation. *Environmental Health Perspectives, 113*, 1698-1704.

Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). A review of thermo-oxidative degradation of food lipids studied by ^{1}H NMR spectroscopy: Influence of degradative conditions and food lipid nature. *Comprehensive Reviews in Food Science and Food Safety, 13*, 838-859.

Ohm, V. A.; Stöckmann, H., & Schwarz, K. (2005). The more-The better? Estimating the inhibitory activity of alpha-tocopherol towards lipid oxidation. *Journal of Plant Physiology, 162*, 785-789.

Ramsden, C. E., Ringel, A., Feldstein, A. E., Tahae, A. Y., MacIntosh, B. A., Hibbelna, J. R., Majchrzak-Hong, S. F., Faurot, K. R., Rapoport, S. I., Cheone, Y., Chung, Y.-M., Berkd, M., & Manng, J. D. (2012). Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 87*, 135-141.

Schaich, K. M. (2013). Challenges in elucidating lipid oxidation mechanisms: When, where, and how do products arise?. In A. Logan, U. Nienaber, & X. (S) Pan (Eds.), Lipid Oxidation. Challenges in Food Systems (pp. 1-52). Urbana: AOCS Press.

Schneider, C. (2005). Chemistry and biology of vitamin E. *Molecular Nutrition & Food Research, 49*, 7-30.

Schneider, C., Porter N. A., & Brash, A. R. (2004). Autoxidative transformation of chiral omega-6 hydroxy linoleic and arachidonic acids to chiral 4-hydroxy-2*E*-nonenal. *Chemical Research in Toxicology, 17*, 937–941.

Seppanen, C. M., Song, Q., & Csallany, A. S. (2010). The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *Journal of the American Oil Chemists' Society*, *87*, 469-481.

Toth, B., & Patil, K. (1983). Enhancing effect of vitamin E on murine intestinal tumerogenesis by 1,2-dimethylhydrazine dihydrochloride. *Journal of the National Cancer Institute, 70*, 1107-1111.

Wagner, K. H., & Elmadfa, I. (2000). Effects of tocopherols and their mixtures on the oxidative stability of olive oil and linseed oil under heating. *European Journal of Lipid Science and Technology, 102*, 624-629.

Xia, W., Budge, S. M., & Lumsden, M. D. (2016). ^{1}H-NMR characterization of epoxides derived from polyunsaturated fatty acids. *Journal of the American Oil Chemists' Society, 93*, 467-478.

SUPPLEMENTARY MATERIAL OF MANUSCRIPT 2

PROOXIDANT EFFECT OF α-TOCOPHEROL ON SOYBEAN OIL. GLOBAL MONITORING OF ITS OXIDATION PROCESS UNDER ACCELERATED STORAGE CONDITIONS BY ^{1}H NUCLEAR MAGNETIC RESONANCE

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Table S1. Chemical shifts, multiplicities and assignments of the ^{1}H NMR signals in $CDCl_3$ of the main types of triglycerides (TG) protons, and of some oxidation compounds, present in the different soybean oil samples, before and throughout the accelerated storage process. The signal letters agree with those given in Figures 1 and 3.

Signal	Chemical shift (ppm)	Multi-plicity	Functional group	
			Type of protons	Compound
Main acyl groups[a]				
A	0.88	t	-C**H**$_3$	saturated and monounsaturated ω-9 acyl groups
	0.89	t	-C**H**$_3$	linoleic acyl groups
B	0.97	t	-C**H**$_3$	linolenic acyl groups
C	1.19-1.42	m*	-(C**H**$_2$)$_n$-	acyl groups
D	1.61	m	-OCO-CH$_2$-C**H**$_2$-	acyl groups in TG
E	1.94-2.14	m**	-C**H**$_2$-CH=CH-	unsaturated acyl groups
F	2.26-2.36	dt	-OCO-C**H**$_2$-	acyl groups in TG
G	2.77	t	=HC-C**H**$_2$-CH=	linoleic acyl groups
H	2.80	t	=HC-C**H**$_2$-CH=	linolenic acyl groups
I	4.22	dd,dd	ROC**H**$_2$-CH(OR')-C**H**$_2$OR''	glyceryl groups
J	5.27	m	ROCH$_2$-C**H**(OR')-CH$_2$OR''	glyceryl groups
K	5.28-5.46	m	-C**H**=C**H**-	acyl groups
Oxidation compounds				
Hydroperoxides[b]				
a	8.3-8.9	bs	-OO**H**	monohydroperoxide group
Conjugated dienic systems[b,c]				
-	5.44	ddd	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroxy group in octadecadienoic acyl groups[d]
-	5.66	dd		
-	5.97	t		
b	6.49	dd		
-	5.47	ddm	-C**H**=C**H**-C**H**=C**H**-	(*E*,*E*)-conjugated double bonds associated with hydroperoxy group
-	5.76	dtm		
-	6.06	ddtd		
c	6.27	ddm		

				in octadecadienoic acyl groups
-	5.51	dtm	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroperoxy group in octadecadienoic acyl groups[e]
-	5.56	ddm		
-	6.00	ddtd		
d	6.58	dddd		
Epoxides				
e	2.63[f]	m	−C**H**O**H**C−	(*E*)-9,10-epoxystearate
f	2.88[f]	m	-C**H**O**H**C−	(*Z*)-9,10-epoxystearate
g	2.9[g,h]	m	-C**H**O**H**C-	monoepoxy-octadecenoate/-octadecadienoate groups
h	3.1[i,j]	m[i]/dd[j]	-C**H**O**H**C-	diepoxides / (*E*)-12,13-epoxy-9-hydroperoxy-(*E*)-10-octadecenoate
Alcohols				
i	3.45[k]	m	−C**H**OH−C**H**OH−	9,10-dihydroxy-12-octadecenoate (leukotoxindiol) 12,13-dihydroxy-9-octadecenoate (isoleukotoxindiol)
j	3.62	t	−C**H**$_2$OH−	primary alcohols***
Keto-dienes				
k	7.13[l]	dm	-CO−CH=C**H**−CH=CH-	(*E*,*E*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
l	7.50[l]/7.43[m]	dd[l]/ddd[m]	-CO−CH=C**H**−CH=CH-	(*Z*,*E*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
	7.49[l]/7.47[m]	ddd	-CO−CH=C**H**−CH=CH-	(*E*,*Z*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups

Aldehydes

m	9.49[n]	d	−C**H**O	(*E*)-2-alkenals
n	9.52[n]	d	−C**H**O	(*E,E)*-2,4-alkadienals
o	9.55[n]	d	−C**H**O	4,5-epoxy-2-alkenals
p	9.57[n]	d	−C**H**O	4-hydroxy-(*E*)-2-alkenals
q	9.58[n]	d	−C**H**O	4-hydroperoxy-(*E*)-2-alkenals
r	9.60[o]	d	-C**H**O	(*Z,E*)-2,4-alkadienals
s	9.75[n]	t	-C**H**O	n-alkanals

t: triplet; m: mutiplet; d: doublet; bs: broad signal; *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***Assignment made on the basis of standard compounds

[a]Assignments taken from Guillén, M. D., & Ruiz, A. (2003). *Journal of the Science of Food and Agriculture, 83*, 338–346.

[b]Data taken from Goicoechea, E., & Guillén, M. D. (2010). *Journal of Agricultural and Food Chemistry, 58*, 6234-6245 (hydroperoxides and conjugated hydroperoxydienes).

[c]Data taken from Dong, M., Oda, Y., & Hirota, M. (2000). *Bioscience, Biotechnology, and Biochemistry, 64*, 882-886 (conjugated (*Z,E*)-hydroxydienes).

[d]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Kuklev, D. V., Christie, W. W., Durand, T., Rossi, J. C., Vidal, J. P., Kasyanov, S. P., Akulin, V. N., & Bezuglov, V. V. (1997). *Chemistry and Physics of Lipids, 85*, 125-134.

[e]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Chan, H. W.-S., & Levett, G. (1977). *Lipids, 12*, 99-104.

[f]Data taken from Du, G., Tekin, A., Hammond, E. G., & Woo, L. K. (2004). *Journal of the American Oil Chemists' Society, 81*, 477–80.

[g]Data taken from Aerts, H. A. J., & Jacobs, P. A. (2004). *Journal of the American Oil Chemists' Society, 81*, 841-846.

[h]Data taken from Cui, P. H., Duke, R. K., & Duke, C. C. (2008). *Chemistry and Physics of Lipids, 152*, 122-130.

[i]Xia, W., Budge, S. M., & Lumsden, M. D. (2016). *Journal of the American Oil Chemists' Society, 93*, 467-478.

[j]Gardner, H. W., Weisleder, D., & Kleiman, R. (1978). *Lipids, 13*, 246-252.

[k]Data taken from Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau C., & Hammoc B. D. (2000). *Archives of Biochemistry and Biophysics, 376*, 420-43.

[l]Data taken from Dufour, C., & Loonis, M. (2005). *Chemistry and Physics of Lipids, 138*, 60-68.

[m]Data taken from Kuklev et al. (1997).

[n]Data taken from Guillén, M. D., & Ruiz, A. (2004). *European Journal of Lipid Science and Technology, 106*, 680–687.

[o]Data taken from Guillén, M. D., & Uriarte, P. S. (2012). *Food Chemistry, 134*, 162-172.

Table S2. Rates of increase of hydroperoxides concentration in the different samples throughout the accelerated storage process, given by the slopes of the straight parts of the lines in Figure 2b, expressed in mmol.mol TG^{-1}/day, together with the correlation coefficients between the experimental data and those of the fitted equations, the maximum concentration observed in each sample, in mmol/mol TG, and the day when it is reached.

Sample	Days	slope	Days	slope	Maximum Day - Concentration
SO	0-4	6.0250 (R=0.9837)	4-7	37.7342 (R=0.9992)	7 – 134.36
SO+0.02	0-4	9.8089 (R=0.9852)	4-6	76.1630 (R=0.9999)	6 – 192.15
SO+0.2	0-4	33.2942 (R=0.9960)	4-5	86.1699 (R=1.0000)	5 – 217.50
SO+2	0-4	59.8299 (R=0.9807)			5 – 274.16
SO+5	0-4	67.0543 (R=0.9908)			5 – 285.08

Table S3. Rates of increase of the concentrations of conjugated (*Z*,*E*)- and (*E*,*E*)-dienes associated to hydroperoxides in the different samples throughout the accelerated storage process, given by the slopes of the straight parts of the lines in Figure 2b, expressed in mmol.mol TG^{-1}/day, together with the correlation coefficients between the experimental data and those of the fitted equations, the maximum concentration observed in each sample, in mmol/mol TG, and the day when it is reached.

Sample		Days	slope	Days	slope	Maximum Day - Concentration
SO	(*Z*,*E*)	0-4	3.7710 (R=0.9928)	4-7	7.8784 (R=0.9919)	7 – 38.20
	(*E*,*E*)	0-4	4.3806 (R=0.9837)	4-7	24.8250 (R=0.9860)	7 – 88.83
SO+0.02	(*Z*,*E*)	0-4	5.2691 (R=0.9887)	4-6	23.7145 (R=0.9819)	6 – 69.49
	(*E*,*E*)	0-4	6.1410 (R=0.9895)	4-6	37.8748 (R=0.9960)	6 – 100.27
SO+0.2	(*Z*,*E*)	0-3	21.6181 (R=0.9932)			3 – 62.49
	(*E*,*E*)	0-3	8.3716 (R=0.9509)	3-5	35.912 (R=0.9516)	5 – 97.32
SO+2	(*Z*,*E*)	0-4	59.3107 (R=0.9971)			5 – 235.22
	(*E*,*E*)	1-5	5.0653 (R=0.9959)	5-7	29.1958 (R=0.9974)	7 – 78.67
SO+5	(*Z*,*E*)	0-4	69.2604 (R=0.9947)			5 – 275.21
	(*E*,*E*)	4-7	6.7172 (R=0.9823)	7-10	12.2485 (R=0.9972)	11 – 57.81

Manuscript 3

A THOROUGH INSIGHT INTO THE COMPLEX EFFECT OF GAMMA-TOCOPHEROL ON THE OXIDATION PROCESS OF SOYBEAN OIL BY MEANS OF ^{1}H NMR. COMPARISON WITH ALPHA-TOCOPHEROL

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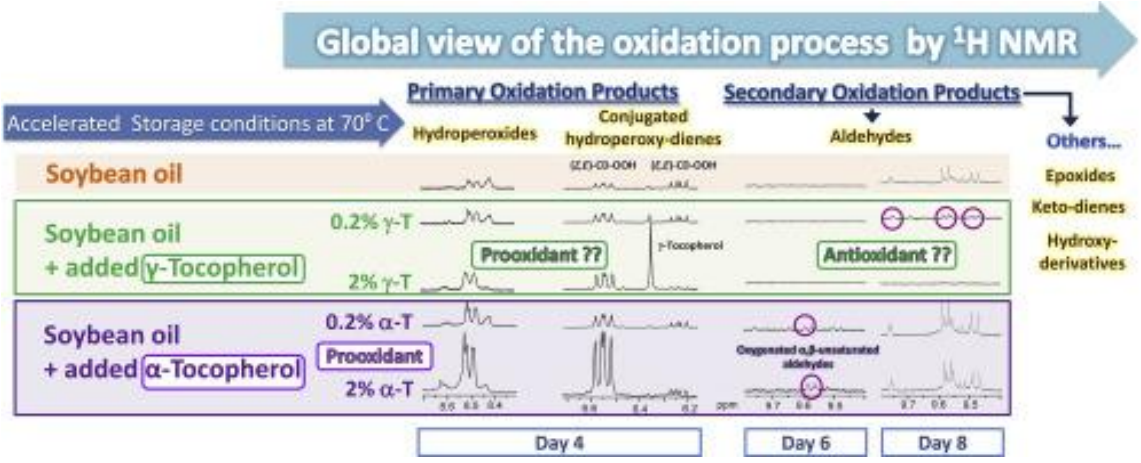

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ABSTRACT

The effect of γ-tocopherol in proportions between 0.02 and 2% by weight on the accelerated storage process of refined soybean oil is studied by ^{1}H NMR, and compared with that of α-tocopherol. Whereas the lowest γ-tocopherol enrichment level does not affect oil evolution, at higher concentrations both γ- and α-tocopherols initially accelerate acyl groups degradation and hydroperoxides generation, more as higher is the tocopherol concentration, this effect being less marked for γ-tocopherol. However, after this initial stage, the rates of acyl groups degradation and hydroperoxides formation decrease with tocopherol concentration. Furthermore, in the case of γ-tocopherol, the higher the enrichment degree, the later hydroperoxides decomposition occurs, so that, unlike α-tocopherol, γ-tocopherol delays the generation of most secondary oxidation products (aldehydes, (*E*,*E*)-keto-dienes, epoxy-keto-enes, (*E*)-epoxystearates and alcohols) with the exception of some epoxides. Similarly to α-tocopherol, γ-tocopherol modifies the oil oxidation pathway at the highest addition level, promoting the formation of compounds with (*Z*,*E*)-isomerism, although less noticeably than α-tocopherol.

Chemical compounds studied in this article: α-Tocopherol (PubChem CID: 2116); γ-Tocopherol (PubChem CID: 92729); (*E*)-2-Hexenal (PubChem CID: 2116); (*E*,*E*)-2,4-Decadienal (PubChem CID: 5283349); 4-Hydroxy-(*E*)-2-nonenal (PubChem CID: 1693); (*E*)-9,10-epoxystearate (PubChem CID: 12235226)

KEYWORDS: γ-tocopherol, α-tocopherol, soybean oil, oxidation evolution, ^{1}H nuclear magnetic resonance, primary oxidation compounds, secondary oxidation compounds.

1. INTRODUCTION

Gamma-tocopherol (γ-T) is the most abundant tocol in certain vegetable oils such as rapeseed, corn, sesame, linseed, camelina or soybean (Alberdi-Cedeño, Ibargoitia, Cristillo, Sopelana & Guillén, 2017; Schwartz, Ollilainen, Piironen & Lampi, 2008). Although it is not considered so biologically active as alpha-tocopherol (α-T) (Schneider, 2005), this compound seems to have certain advantages over α-T with regard to its antioxidant ability. Thus, some studies have shown greater antioxidant efficiency of γ-T in comparison with α-T (Gottstein & Grosch, 1990; Wagner, Isnardy & Elmadfa, 2004) and the susceptibility of γ-T to exhibit a prooxidant action is considered much lower than that of α-T (Gottstein & Grosch, 1990; Huang, Frankel & German, 1994; King, Min & Min, 2011).

The effect of γ-T on the oxidative stability of lipids has been studied not only in linoleic acid and/or methyl linoleate (Gottstein & Grosch, 1990; Koskas, Cillard & Cillard, 1984), but also in more complex matrices such as various types of purified oils like corn (Huang, Frankel & German, 1995), soybean (Jung & Min, 1990), rapeseed (Isnardy, Wagner & Elmadfa, 2003; Lampi, Kataja, Kamal-Eldin & Vieno, 1999) and sunflower (Fuster, Lampi, Hopia & Kamal-Eldin, 1998), oil-in-water emulsions (Heinonen, Haila, Lampi & Piironen, 1997; Huang et al., 1994), olive and linseed oils (Wagner & Elmadfa, 2000), margarine (Azizkhani, Kamkar & Nejad, 2011), fish oil enriched salad dressing (Let, Jacobsen & Meyer, 2007) and lard (King et al., 2011).

Most of these studies reveal an antioxidant action of γ-T across a wide range of enrichment levels (between 0.00015 and 0.2%), at temperatures comprised between 25 and 120 ºC, and the assessment of the γ-T effect has been carried out by using, in general, classical nonspecific methods to evaluate lipid oxidation; among these, headspace oxygen content, conjugated dienes or *p*-anisidine value can be mentioned. Moreover, some authors have determined specific hydroperoxides by HPLC (Koskas et al., 1984) or some volatile aldehydes like hexanal, either by gas chromatography/mass spectrometry (Huang et al., 1994; Isnardy et al., 2003; Let et al., 2007) or by HPLC (Heinonen et al., 1997; Lampi et al., 1999). Among these works, only a few of them have shown a prooxidant effect of γ-T in purified oils like rapeseed (Isnardy et al., 2003), soybean (Jung & Min, 1990) and corn (Huang et al., 1995), as well as in linseed oil (Wagner & Elmadfa, 2000), at concentrations ranging from 0.01 to 0.5%. This contrasts with the wide body of scientific knowledge that has proved the prooxidant

effect of α-T on various types of lipidic matrices (Cillard, Cillard, Cormier & Girre, 1980; Dolde & Wang, 2011; Isnardy et al., 2003; Martin-Rubio, Sopelana, Ibargoitia & Guillén, 2018).

As far as the use of γ-T as an antioxidant additive is concerned, European Union legislation allows its addition to refined vegetable oils on a *quantum satis* basis (Commission Regulation 1129/2011), without an established limit, except for refined olive oils. Therefore, it is also important that studies on the effect of γ-T are performed with real oils, which is to say with all their original components. Otherwise, it would be difficult to extrapolate the results obtained to real practice.

Bearing in mind all the above, the main objective of this work is to study the effect of adding varying proportions of γ-T on the oxidation process of soybean oil both to contribute to understanding the action mechanism of γ-T and compare it with that of α-T, as well as to assess the suitability of γ-T to improve the oxidative stability of commercial oils. For this purpose, the entire oxidation process of a commercial refined soybean oil was carried out under accelerated storage conditions at 70º C and monitored by means of ^{1}H Nuclear Magnetic Resonance (^{1}H NMR), with attention paid to acyl groups degradation and to the formation of a wide range of oxidation products. ^{1}H NMR has been proved to be a powerful technique in the study of oils and fats, and since the early 2000 important progress has been made in the field of lipids due to the employment of this analytical tool. On the one hand, ^{1}H NMR has made it possible to characterize in a simple and fast way different types of food lipids like vegetable and fish oils (Guillén & Ruiz, 2003; Hidalgo & Zamora, 2003; Sacchi et al., 1998; Tyl, Brecker & Wagner, 2008), margarine (Schripsema, 2008; Sopelana, Arizabaleta, Ibargoitia & Guillén, 2013), fish lipids (Vidal, Manzanos, Goicoechea & Guillén, 2012) or milk fat (Belloque & Ramos, 1999), giving qualitative and quantitative information of both main and minor components. Moreover, great progress has been made lately in the analysis of minor compounds of vegetable oils due to the use of ^{1}H NMR in multisupression mode, which provides enhanced sensitivity (Ruiz-Aracama, Goicoechea & Guillén, 2017). On the other hand, ^{1}H NMR has significantly contributed to knowledge of the oxidation processes of oils and fats under very different conditions (Guillén & Ruiz, 2005; Guillén & Uriarte, 2009; Martínez-Yusta, Goicoechea & Guillén, 2014; Nieva-Echevarría, Goicoechea, Manzanos & Guillén, 2016), providing insight not only into the degradation rate of the main oil and/or food lipids components

(acyl groups), but also into the nature and concentration of a wide range of oxidation compounds, both primary and secondary, some of which are not usually determined by the methodologies generally employed to assess lipid oxidation. These studies have revealed that many other products apart from hydroperoxides and aldehydes are generated during lipid oxidation processes and that, among these, epoxides make a great contribution (Martin-Rubio, Sopelana, Ibargoitia & Guillén, 2018). All of which evidences the great ability of ^{1}H NMR to offer a comprehensive view of the oxidation course.

2. MATERIALS AND METHODS

2.1. Samples

The samples subject of study were two refined soybean oils (RSO), of the same brand but from two batches, and those prepared by adding different proportions by weight of γ-T to one oil and of α-T to the other. The designations of the samples were the following: RSO1 (0% of γ-T added), RSO1+0.02γT (0.02%), RSO1+0.2γT (0.2%), RSO1+2γT (2%), RSO2 (0% of α-T added), RSO2+0.2αT (0.2%) and RSO2+2αT (2%). γ-T with a purity ≥ 90% and α-T with a purity of 98.2% were acquired from Eisai Food & Chemical Co. Lltd (Tokyo, Japan) and from Sigma-Aldrich (St. Louis, MO, USA), respectively. Furthermore, γ-T with a purity of 98% was also purchased from Sigma-Aldrich to conduct a complementary assay aimed at checking if γ-T purity could have any influence on the results obtained.

The molar percentages of the different types of acyl groups in the studied oils were determined by ^{1}H NMR, as in previous works (Guillén & Ruiz, 2003; Guillén & Uriarte, 2009, 2012). These were 5.2±0.1 for linolenic, 47.2±0.2 for linoleic, 29.9±0.2 for oleic and 17.7±0.0 for saturated groups in the case of RSO1, and 5.2±0.0 for linolenic, 43.7±0.1 for linoleic, 33.6±0.7 for oleic and 17.1±0.6 for saturated groups in the case of RSO2.

2.2. Accelerated storage (AS) process

10 g portions of each oil sample were poured into plastic Petri dishes of 80 mm diameter for each of the days monitored throughout the AS process. These were heated at 70º C in a convection oven with circulating air but without forced convection, simulating AS conditions. The evolution of the samples was followed by ^{1}H NMR until their total polymerization, when it no longer became possible to take an aliquot that was

sufficiently fluid to be analyzed. The AS process was carried out in duplicate with all the studied samples.

2.3. Monitoring by ^{1}H NMR of the evolution of RSO and of the RSO oil samples enriched with different proportions of either γ-T or α-T

2.3.1. Operating conditions

The ^{1}H NMR spectra of all the samples taken throughout the AS process were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz, the weight of each sample being approximately 0.16 g. These were mixed in a 5 mm diameter tube with 400 µl of deuterated chloroform containing 0.2% of non deuterated chloroform and a small amount (0.03%) of tetramethylsilane as internal references. As in previous studies (Guillén & Ruiz, 2003, 2005), the acquisition parameters used were: spectral width 5000 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.744 s, and pulse width 90º, with a total acquisition time of 12 min 54 s. The relaxation delay and acquisition time selected allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making possible their use for quantitative purposes. The experiments were carried out at 25 ºC.

2.3.2. Identification of some components

The identification of the oil acyl groups and of the products formed throughout the AS process was carried out on the basis of the signal assignment shown in Table S1, made from bibliographic data and with the aid of several standard compounds. These were (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, 4,5-epoxy-(*E*)-2-decenal and 12,13-epoxy-9(*Z*)-octadecenoic acid methyl ester (isoleukotoxin methyl ester), acquired from Sigma-Aldrich, 4-hydroxy-(*E*)-2-nonenal, 4-hydroperoxy-(*E*)-2-nonenal, 9,10-dihydroxy-12(*Z*)-octadecenoic acid (leukotoxin diol), 12,13-dihydroxy-9(*Z*)-octadecenoic acid (isoleukotoxin diol), *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, 9-keto-10(*E*),12(*E*)-octadecadienoic acid and 12R-hydroxy-9(*Z*)-octadecenoic acid methyl ester (ricinoleic acid methyl ester), purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.3.3. Quantitative data derived from ^{1}H NMR spectra

The molar percentages of the several kinds of oil acyl groups were estimated throughout the AS process, as in previous studies (Guillén & Uriarte, 2012), by means of the following equations:

$Ln\% = 100(A_H/3A_I)$,

$L\% = 100(2A_G/3A_I)$,

$O\%$ (or $MU\%$) $= 100(A_E/3A_I)$-$Ln\%$-$L\%$,

where A_H and A_G are the areas of the signals of *bis*-allylic protons of linolenic and linoleic groups, respectively (signals "H" and "G" in Figure 1 and Table S1); given that their respective signals overlap to a certain extent, the total area corresponding to each of these groups was calculated using pure trilinolein and trilinolenin (Sigma-Aldrich) as references. A_I, in turn, is the area of the protons at *sn*-1 and *sn*-3 positions in the glycerol backbone of triglycerides, while A_E is that corresponding to mono-allylic protons (signals "I" and "E", respectively, in Figure 1 and Table S1). The molar percentage of saturated acyl groups can be obtained by difference.

It must be noted that in the samples with the highest tocopherol levels (RSO1+2γT and RSO2+2αT) the signals of γ-T and of α-T are detectable in their ^{1}H NMR spectra during part of the AS period (see Figure 1, signals "u1", "u2", "v" and "w" for γ-T). Considering that some of these signals partially overlap with some of triglycerides (see Table S1), their area was subtracted when determining the molar percentages of oil acyl groups; nevertheless, the results were very similar to those obtained without making this correction.

The concentrations of the different types of oxidation products were estimated as millimol per mol of triglyceride (mmol/mol TG). The general equation to carry out this determination was the following: $[OP] = [(A_{OP}/n)/(A_I/4)]*1000$, where A_{OP} is the area of the signal selected for the quantification of each oxidation product (OP), n the number of protons that generate the signal. It must be pointed out that, for the determination of the so-called major epoxides (see section 3.2.3.1), signals between 2.87 and 3.17 ppm approximately have been considered together. As Table S1 shows, some of the compounds that could give these signals contribute with two protons (signals "f1", "f2" and "g1") but others with only one (signals "f3", "f4", "f5", "g2" and "g3"). However, given that, under the conditions of this study, it is impossible to elucidate exactly which ones of all these types of compounds are present, it has been assumed that the signal at approximately 2.9 ppm corresponds mainly to epoxides contributing with two protons and the one at 3.1 ppm to epoxy-compounds contributing with only one.

2.4. Statistical analysis

The significance of the differences in the concentrations of the different kinds of oxidation products was determined between samples RSO1 and in RSO1+0.02γT by t-student test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

3. RESULTS AND DISCUSSION

As stated above, the study focuses on the effect of adding varying proportions of γ-T on both the evolution of RSO acyl groups and the formation of a wide array of oxidation products under AS conditions, and its comparison with that of α-T. Figure 1, which shows the evolution of the ^{1}H NMR spectrum of RSO1 enriched with 2% of γ-T (RSO1+2γT) throughout the AS process, evidences the diminution with time of the spectral signals specific of unsaturated acyl groups (B, E, G+H and K), as well as the generation and further evolution of new signals corresponding to different types of oxidation products, which will be discussed in detail later.

3.1. Effect of the γ-T enrichment of RSO on the evolution of the different types of oil acyl groups, and comparison with that of α-T

As expected, during the AS process the degradation of polyunsaturated acyl groups (linolenic and linoleic) takes place, linolenic groups completely disappearing by the end of the AS process. By contrast, the molar percentage of monounsaturated groups exhibits only a slight decline, occurring at the most advanced stages of the process. Their estimated evolution can be observed in Figure 2A. As a result of the degradation of unsaturated acyl groups, the molar percentage of the saturated+modified (S+M) ones increases with time, so the evolution of these latter can summarize the overall decrease of the former. Figure 2A reveals that during the first 3 days no noticeable changes in the molar percentage of S+M groups are perceived. However, from this point onwards two main stages can be distinguished, their duration varying from one sample to another

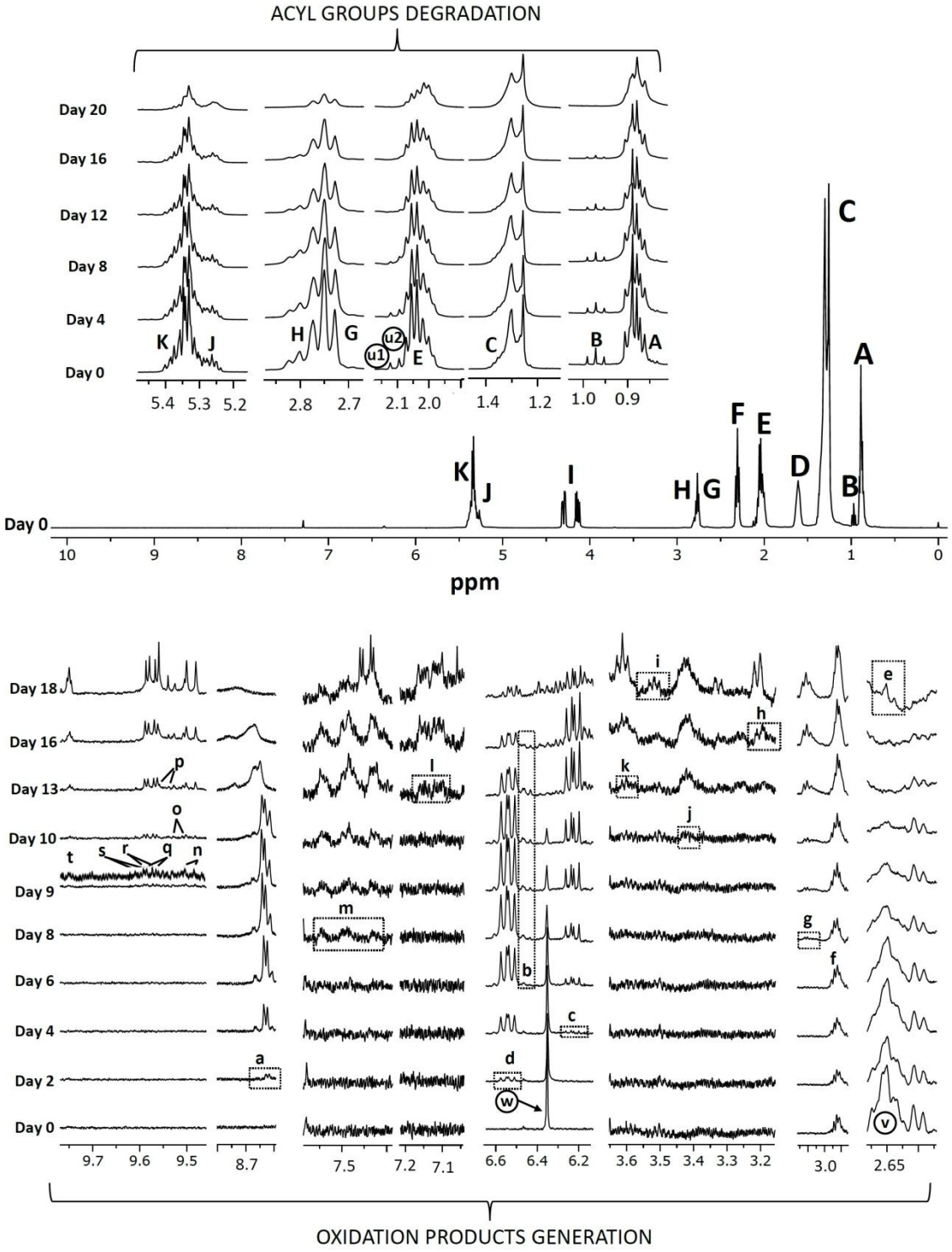

Figure 1. ^{1}H NMR spectrum of sample RSO1+2γT before being subjected to the accelerated storage process, together with the enlargements of some spectral regions where changes occur throughout this process and their evolution with time. Letters agree with those in Table S1, considering that "f" includes signals "f1-f5", "g" signals "g1-g3" and "j" signals "j1+j2". The plots corresponding to the same ^{1}H NMR spectral region are presented at a fixed value of absolute intensity, for them to be valid for comparative purposes.

In sample RSO1 the increasing rate of the molar percentage of S+M groups in the first stage (from days 3 to 7) is considerably lower than in the second one (from day 7 onwards). The addition of a proportion of 0.02% of γ-T has virtually no impact on acyl groups evolution but, as the γ-T concentration increases, the first stage is lengthened and the rising pace of S+M groups is accelerated in comparison with the reference oil. The opposite is observed in the second stage, when the growing rate of S+M groups is reduced compared to the non-enriched oil as γ-T concentration gets higher. This leads to a considerable enlargement of the process in RSO1+2γT and to a delay of its total polymerization.

In agreement with the findings of a previous work (Martin-Rubio et al., 2018), when α-T is added to RSO2 (see Figure 2B), an acceleration in the degradation of polyunsaturated groups is also observed in the earlier stage of the AS process. However, a diminution of the growing rate of S+M groups in relation to the reference oil is noticed during the second stage, the more pronounced the greater the α-T concentration. Therefore, it could be considered that, to a certain extent, the action of both types of tocopherols on RSO acyl groups exhibits a similar tendency, although with different intensities.

3.2. Effect of γ-T on the formation and evolution of oxidation products coming from acyl groups degradation and comparison with α-T

3.2.1. Hydroperoxides (primary oxidation products)

Given that the analysis by ^{1}H NMR allows one to determine both total hydroperoxides and those with (*Z*,*E*)- and (*E*,*E*)-conjugated dienes, the evolution of all of them will be discussed consecutively.

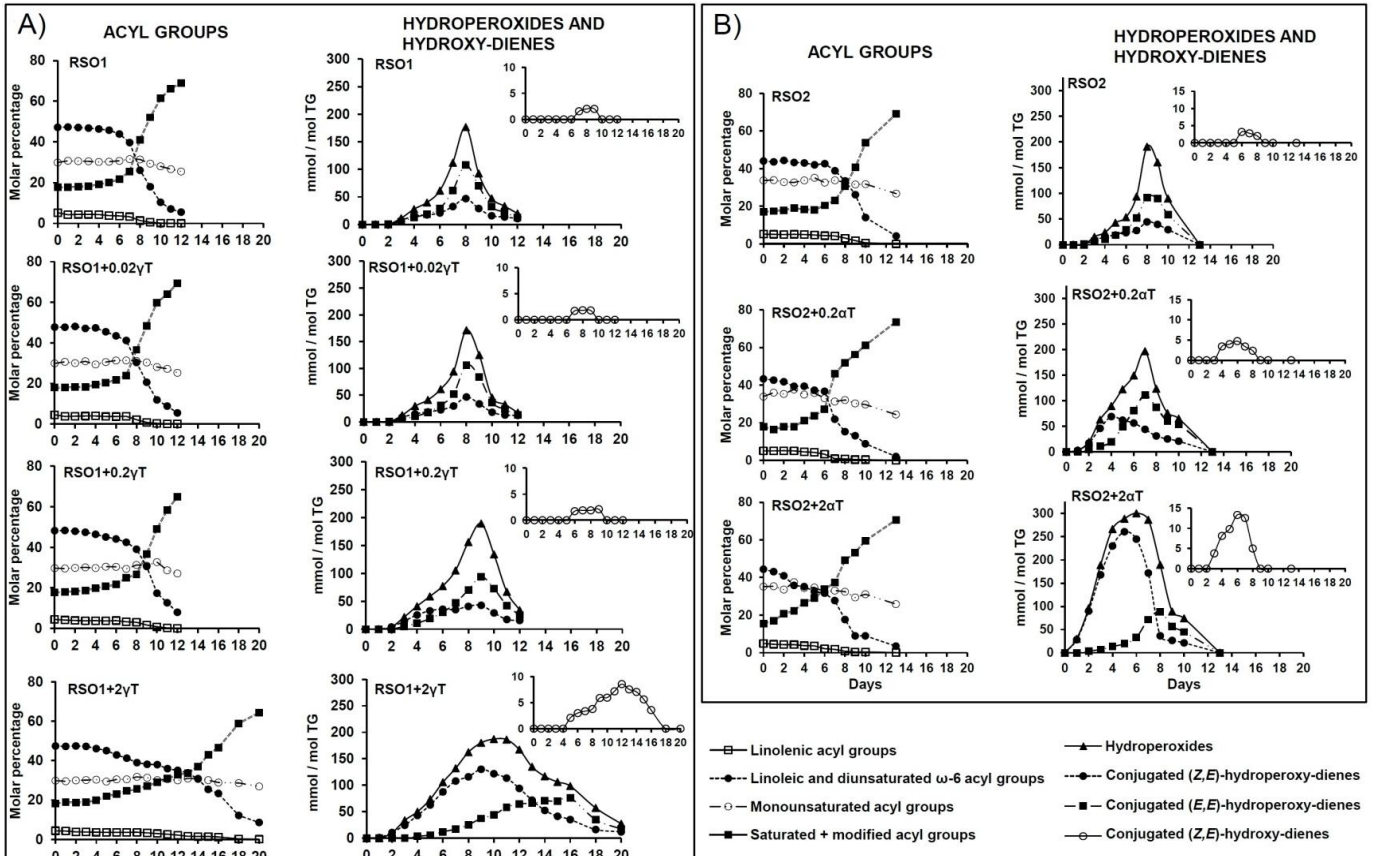

Figure 2. Evolution throughout the accelerated storage process of the molar percentages of linolenic, linoleic and diunsaturated ω-6, monounsaturated and saturated+modified acyl groups, and of the concentrations, in mmol/mol TG, of hydroperoxides and their associated conjugated (*Z,E*)- and (*E,E*)-dienes, as well as of conjugated (*Z,E*)-hydroxy-dienes in: A) the samples with varying proportions of γ-T and their reference oil (RSO1); B) the samples with varying proportions of α-T and their reference oil (RSO2). All the figures reported are mean values.

3.2.1.1. Hydroperoxides giving signals between 8.3 and 9.0 ppm

The progress of the concentration of hydroperoxides, estimated from signal "a" (see Table S1), is shown in Figure 2A. Figure 1, in turn, displays the evolution of hydroperoxides signals over time in sample RSO1+2γT.

Figure 2A shows that, in line with acyl groups evolution, both the original oil and sample RSO1+0.02γT exhibit the same behaviour. Thus, in both cases, hydroperoxides are detected after 3 days under AS conditions and in similar concentrations, and the rates of increase of these throughout time are also comparable. Hydroperoxides concentration rises slowly at first (from days 2 to 6) and then sharply until the maximum is reached.

However, in the samples having higher γ-T concentrations hydroperoxides appear earlier and at first their formation occurs more quickly than in the oils with lower γ-T levels. Therefore, the addition of γ-T in proportions higher than 0.02% slightly reduces the oxidative stability of RSO1; this agrees with the findings of other authors (Huang et al., 1995; Jung & Min, 1990), who also observed that γ-T promoted the formation of hydroperoxides in purified corn and soybean oils under heating conditions (55-60 ºC), at levels of 0.5 and 0.1%, respectively. By contrast, after this first stage of the AS process, the growing pace of hydroperoxides concentration in RSO1+0.2γT and RSO1+2γT samples becomes slower than in the reference oil, in line with increased γ-T level. Actually, in RSO1+2γT hydroperoxides concentration rises practically at the same rate from the beginning until the maximum is reached. Furthermore, hydroperoxides decomposition also takes longer in RSO1+2γT than in the rest of the samples.

Comparison with the evolution in the samples enriched with α-T. As Figure 2B shows, the addition of α-T also accelerates the formation of hydroperoxides and their initial concentration surge, in step with α-T concentration, although to a greater extent than γ-T. In fact, unlike in the case of γ-T, the maximum concentration of hydroperoxides is reached earlier in the α-T-enriched samples than in the reference oil, and it is considerably higher in RSO2+2αT than in the rest of the oils studied. All these findings confirm that, under the conditions of this study, α-T exerts a more prooxidant action on RSO than γ-T.

Regarding the samples with the highest tocopherol enrichment level (2%), it must also be noticed that hydroperoxides decomposition proceeds more slowly for γ-T than

for α-T. This agrees with the results of Kamal-Eldin, Lampi and Hopia (2001), who also observed that γ-T inhibited methyl linoleate hydroperoxides decomposition more efficiently than α-T did.

The differences observed between the effect of both tocopherols on hydroperoxides evolution and concentration could be related to the structural differences existing between them, which make γ-T a less potent hydrogen donor (Gottstein & Grosch, 1990), so less prone to participate in prooxidant reactions than α-T. Actually, whereas α-T signals disappear after 6 days under AS conditions, those of γ-T are still detectable after 12 days, what suggests that α-T reacts and then degrades faster than γ-T.

3.2.1.2. Conjugated (*Z*,*E*)- and (*E*,*E*)-hydroperoxy-dienes ((*Z*,*E*)- and (*E*,*E*)-CD-OOH)

As mentioned above, (*Z*,*E*)- and (*E*,*E*)-hydroperoxides can be monitored separately, by measuring their corresponding signals over time (see Table S1, signals "d" and "c", respectively). The evolution of these latter in sample RSO1+2γT can be observed in Figure 1. Figure 2A, which shows the estimated progress of their concentrations in the γ-T-enriched samples and in their reference oil, reveals that both in RSO1 and in RSO1+0.02γT (*Z*,*E*)- and (*E*,*E*)-CD-OOH are detected at the same time and in similar concentrations. However, as the AS process advances, the (*E*,*E*)-isomers predominate over the (*Z*,*E*)-ones until the maximum of both types of CD-OOH is reached.

As γ-T concentration rises over the 0.02% enrichment level, the generation of (*Z*,*E*)-CD-OOH accelerates and their concentration increases compared with the reference oil, to the extent that in RSO1+2γT exceeds that of (*E*,*E*)-CD-OOH, being considerably higher than in the rest of the samples. In addition, at this high γ-T level, a delay in the generation and subsequent concentration increase of (*E*,*E*)-CD-OOH in relation to the (*Z*,*E*)-ones is observed.

As Figure 2B shows, the addition of α-T to RSO2 also promotes the generation of (*Z*,*E*)-CD-OOH, although to a much higher extent than γ-T. Actually, unlike γ-T, the maximum concentration of (*Z*,*E*)-CD-OOH in the α-T-enriched samples is reached more quickly than in the original oil, and in RSO2+2αT it attains a much higher level than in the rest of the samples studied, including those with γ-T added.

Regarding (*E*,*E*)-CD-OOH, as in RSO1+2γT (see Figure 2A), a delay in the generation of this type of hydroperoxides in relation to the (*Z*,*E*)-ones is noticed in the

sample with the highest α-T concentration. All the results concerning the effect of α-T on hydroperoxides evolution match well with the findings of another previous study (Martin-Rubio et al., 2018).

3.2.2. Conjugated (*Z,E*)-hydroxy-dienes

In addition to hydroperoxy-dienes, the formation of (*Z,E*)-hydroxy-dienes is also observed during the AS process of RSO1 and of the corresponding γ-T-enriched samples. As Figure 2A reveals, this type of compounds is generated at very low levels in the samples with γ-T proportions lower than 2%, although in RSO1+0.2γT these are detected something earlier. However, in RSO1+2γT (see also Figure 1, signal "b") a rise in their concentration and in their existence period (from days 5 to 16) occurs in comparison with the other oils.

According to some authors (Hopia, Huang & Frankel, 1996), the increased formation of (*Z,E*)-hydroxy-dienes in the presence of α-T can be explained by its ability to donate an H atom to alkoxyl radicals, and this could also occur for γ-T.

In agreement with previous findings (Martin-Rubio et al., 2018), the formation of hydroxy-dienes is also favoured when adding α-T to RSO2 (Figure 2B), but this effect is more marked than in the case of γ-T. This seems again to point to a higher hydrogen donating ability of α-T against γ-T. It is worth noticing that, in line with the evolution of (*Z,E*)-CD-OOH, in RSO2+2αT the maximum concentration of (*Z,E*)-OH-dienes is higher and their existence period shorter than in RSO1+2γT.

3.2.3. Epoxides

Epoxides constitute a relevant group of oxidation products due to their concentration and to the potential toxicity of some of them such as leukotoxin and isoleukotoxin, which are monoepoxides derived from linoleic groups (Greene, Williamson, Newman, Morisseau & Hammoc, 2000); despite this, they do not usually receive much attention in oxidation studies. Regarding the monitoring of epoxides formation by ^{1}H NMR, it is worth noticing that, as Table S1 shows, (*E*)-epoxystearates (signal "e"), coming from oleic groups, are the only ones to give a signal clearly distinguishable from those of the rest of epoxides considered. Actually, the identification, and consequently the determination of epoxides by ^{1}H NMR, presents a challenge, since not only can epoxides form at different points of the acyl group chain (Byrdwell & Neff, 2001), but they can also be accompanied by other functional groups like hydroxy-, hydroperoxy-

or keto-, giving rise to a very wide variety of epoxy-compounds, some of which are compiled in Table S1. Taking all this into account, most of the epoxides other than (*E*)-epoxystearates have been considered as a whole, irrespective of the number of epoxy groups or of the presence of other functional groups. Only two types of tentatively identified epoxy-keto-ene compounds (see Table S1) have been determined separately, as described in section 3.2.3.2.

The estimated progress of the concentrations of the different types of epoxides during the AS process in all the γ-T-enriched samples and in their corresponding reference oil is presented in Figure 3A. Figure 1, in turn, shows the evolution of their signals ("e", "f", "g", "h" e "i") in sample RSO1+2γT.

3.2.3.1. Major epoxides (epoxides other than (*E*)-epoxystearates and those tentatively identified as epoxy-keto-enes)

As Figure 3A reveals, in RSO1 and in RSO1+0.02γT, these major epoxides are distinguishable from the 7th day onwards and their concentration progressively increases, reaching very similar concentrations in both samples.

As the γ-T level rises, the formation of these epoxides begins somewhat earlier but at a lower rate, attaining a higher concentration at the end of the AS process due to its longer duration.

3.2.3.2. Epoxy-keto-enes

The concentrations of these tentatively identified compounds, divided into (*E*)- and (*Z*)-isomers, have been estimated assuming that signal "h" (see Figure 1) could be due to protons of the four epoxy-keto-octadecenoates included in Table S1, and that signal "i" is due exclusively to the (*Z*)-isomers (see signals "i1" and "i2" in Table S1). Therefore, the amount of the (*E*)-isomers has been calculated by difference.

As Figure 3A shows, in RSO1 and in RSO1+0.02γT epoxy-keto-enes are generated later and in lower concentrations than the major epoxides mentioned in section 3.2.3.1, especially the (*Z*)-isomers. It is also observed that γ-T in proportions greater than 0.02% delays their formation, more markedly in the oil with the highest γ-T concentration, just the opposite that happens in the formation of the so-called major epoxides. This indicates that the generation routes of both kinds of epoxides are different.

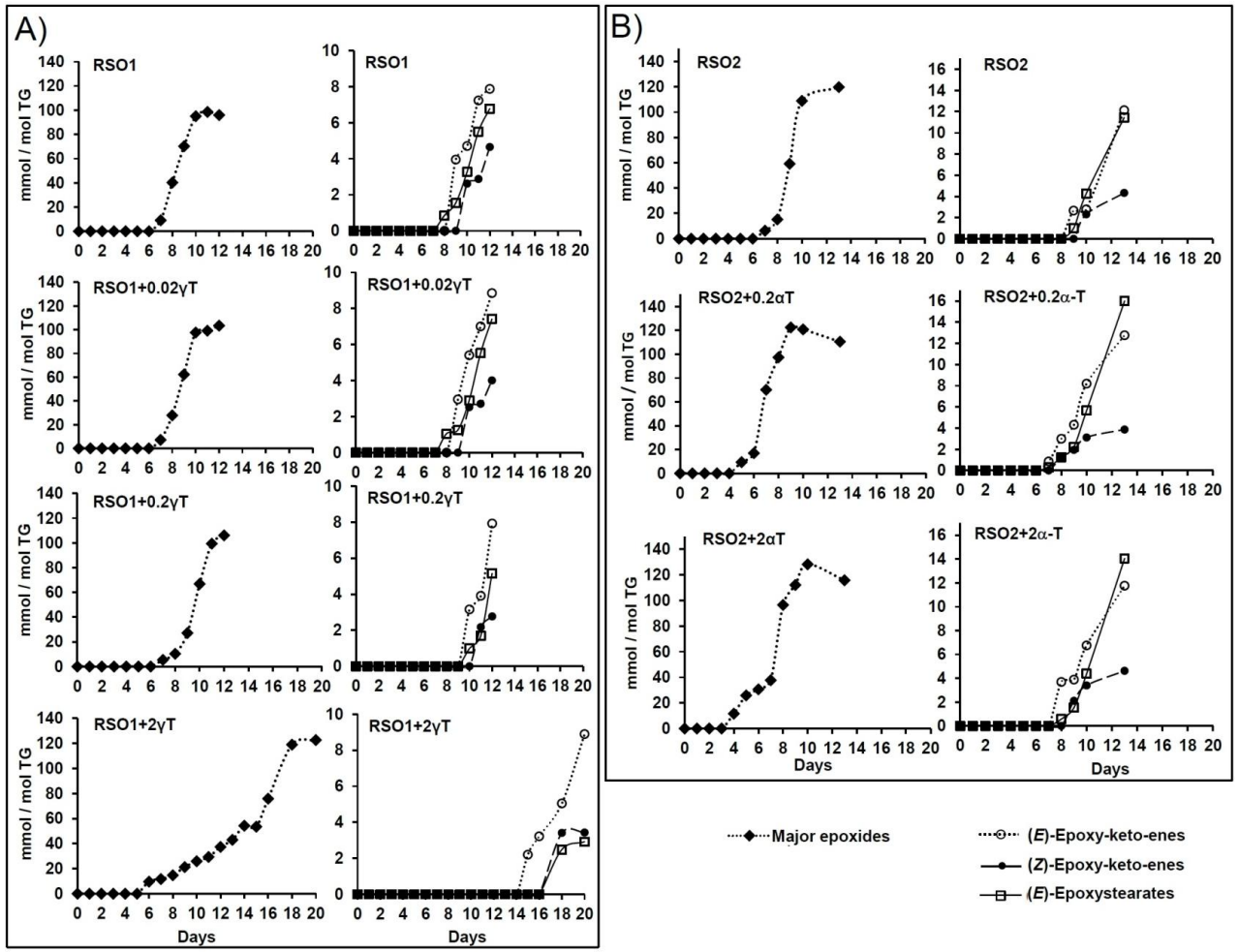

Figure 3. Evolution throughout the accelerated storage process of the concentrations, in mmol/mol TG, of the different types of epoxides in: A) the samples with varying proportions of γ-T and their reference oil (RSO1); B) the samples with varying proportions of α-T and their reference oil (RSO2). All the figures reported are mean values.

3.2.3.3. (*E*)-Epoxystearates

It can be noticed in Figure 3A that (*E*)-epoxystearates, coming from oleic groups, are also detected later than the major epoxides and, in agreement with the evolution of monounsaturated groups (see Figure 2A), γ-T levels above 0.02% delay their generation, above all in the most enriched oil.

3.2.3.4. Comparison with α-T

Regarding major epoxides, as Figure 3B reveals, the addition of α-T speeds up their emergence, in step with greater α-T concentration, and considerably more markedly than in the γ-T-enriched oils, reflecting the different effect of both tocopherols on hydroperoxides generation and decomposition rates in the various samples.

The generation of epoxy-keto-enes and of (*E*)-epoxystearates is also accelerated in the samples with α-T added (see Figure 3B), what contrasts with that observed in the γ-T-enriched samples (see Figure 3A).

3.2.4. Aldehydes

Aldehydes make up a wide and important group of oxidation compounds due to the reactivity and toxicity of some of them, such as the oxygenated α,β-unsaturated ones, considered to be involved in the development of degenerative diseases (Guillén & Goicoechea, 2008). The estimated evolution of the concentrations of the different types of aldehydes detected in all the γ-T-enriched samples and in their reference oil is given in Figure 4A. In addition, Figure 1 shows the generation and evolution of aldehydic signals in sample RSO1+2γT (letters "n" to "t").

Figure 4A demonstrates that aldehydes emerge at the same time in RSO1 and in RSO1+0.02γT but, as the γ-T concentration increases, aldehydes are detected later. This coincides to a certain extent with the findings of Huang and coworkers (1994), who reported a greater ability of γ-T in inhibiting hexanal formation in stripped corn oil at higher concentration. It can also be observed that in RSO1 and in the oils with γ-T-enrichment levels lower than 2% the first aldehydes detected are n-alkanals, (*E*)-2-alkenals and 4-hydroperoxy-(*E*)-2-alkenals, while 4-hydroxy-(*E*)-2-alkenals, (*E*,*E*)-2,4-alkadienals and 4,5-epoxy-2-alkenals are observable the following day. However, in RSO1+2γT 4-hydroxy-(*E*)-2-alkenals also appear in the first place, as well as (*Z*,*E*)-2,4-alkadienals, these latter present in very low concentration (see the enlargement in Figure 4A and Figure 1, signal "s") and absent in the rest of the samples.

The relative abundances and evolution of the different types of aldehydes are, in general, the same as those observed in previous works carried out under these same conditions (Goicoechea & Guillén, 2010; Martin-Rubio et al., 2018). Nevertheless, in the most γ-T-enriched sample the concentration of 4-hydroxy-(*E*)-2-alkenals is very similar to that of 4-hydroperoxy-(*E*)-2-alkenals for a few days after their emergence (from days 9 to 13); this contrasts with the evolution observed in the rest of the samples, where the former are detected later and initially in lower concentration than the latter. (*Z*,*E*)-2,4-alkadienals, in turn, evolve somewhat differently than the rest of aldehydes, given that they disappear before the end of the AS period.

As for the maximum concentrations reached by the several kinds of aldehydes, they seem to be in accordance with both their earlier or later onset and the length of the AS period in each of the samples and, overall, substantial differences are not observed among them.

By contrast with oils enriched with γ-T, α-T accelerates the appearance of aldehydes in relation to the original oil, the higher the α-T concentration gets (see Figure 4B).

Nevertheless, it could be thought that the appearance of different types of aldehydes in the samples enriched with any of the two tested tocopherols is closely related to the concentration in the system of the different kinds of CD-OOH. This can be deduced from the results obtained in the samples with the highest tocopherol enrichment level because in this case the formation rate of (*Z*,*E*)-CD-OOH and (*E*,*E*)-CD-OOH is fairly different, as Figures 2A and 2B show.

Thus, in the case of RSO2+2αT, the first aldehydes detected are the oxygenated α,β-unsaturated 4-hydroxy-(*E*)-2-alkenals and 4-hydroperoxy-(*E*)-2-alkenals, as well as (*Z*,*E*)-2,4-alkadienals. This occurs when (*Z*,*E*)-CD-OOH have reached their maximum concentration (day 5, see Figures 2B and 4B) whereas the level of (*E*,*E*)-CD-OOH is very small. Later, when the (*E*,*E*)-CD-OOH concentration has attained a value near its maximum, n-alkanals and (*E*)-2-alkenals also become visible (day 7, see Figures 2B and 4B). This fact could suggest that the first cited aldehydes might come from (*Z*,*E*)-CD-OOH whereas (*E*,*E*)-CD-OOH might be the precursors of the second ones. These results are in agreement with the findings of a previous work where even a higher enrichment level of α-T was studied (Martin-Rubio et al., 2018).

Likewise, in sample RSO1+2γT 4-hydroxy-(*E*)-2-alkenals, 4-hydroperoxy-(*E*)-2-alkenals and (*Z*,*E*)-2,4-alkadienals are also among the first formed aldehydes. These are

detected when (*Z*,*E*)-CD-OOH have reached their peak concentration (see day 9 in Figures 2A and 4A). At the same time n-alkanals and (*E*)-2-alkenals also appear in the system, coinciding with the time when the (*E*,*E*)-CD-OOH concentration has reached a certain value.

These results suggest that the time at which some of the secondary oxidation products such as aldehydes are formed is determined by the concentration in the system of the CD-OOH from which they come, this being why the appearance of aldehydes occurs in the samples highly enriched with α-T before it does in the oils highly enriched with γ-T.

3.2.5. Compounds with keto-dienic systems

The estimated progress of the concentrations of compounds with (*Z*,*E*)- and (*E*,*E*)-keto-dienes in the γ-T-enriched oils and in their respective reference is shown in Figure 5A; in addition, the evolution of their signals in sample RSO1+2γT can be observed in Figure 1 (signal "m" for (*Z*,*E*) (and *E*,*Z*)- and "l" for (*E*,*E*)-keto-dienes). As Figure 5A reveals, keto-dienes are detected in RSO1 and RSO1+0.02γT one day later than aldehydes, the (*E*,*E*)-ones being in higher concentration than the (*Z*,*E*)-ones throughout the entire AS process. They both reach their maximum concentrations simultaneously but, unlike (*E*,*E*)-keto-dienes, the (*Z*,*E*)-ones almost disappear before the end of the AS process.

In the samples with higher γ-T concentrations keto-dienes evolution is in line with that of (*Z*,*E*)- and (*E*,*E*)-CD-OOH in each of these samples (see Figure 2A). This is especially noticeable in RSO1+2γT, where the concentration of (*Z*,*E*)-keto-dienes reaches considerably higher levels than in the rest of the samples and their duration is longer, while the formation of the (*E*,*E*)-ones is markedly retarded compared to the reference oil.

Regarding the samples enriched with α-T (see Figure 5B), and also in agreement with both (*Z*,*E*)- and (*E*,*E*)-CD-OOH evolutions (see Figure 2B), the higher the α-T concentration, the earlier the generation of (*Z*,*E*)-keto-dienes and the later the generation of the (*E*,*E*)-ones in relation to the former. All these findings agree with those of a previous study (Martin-Rubio et al., 2018).

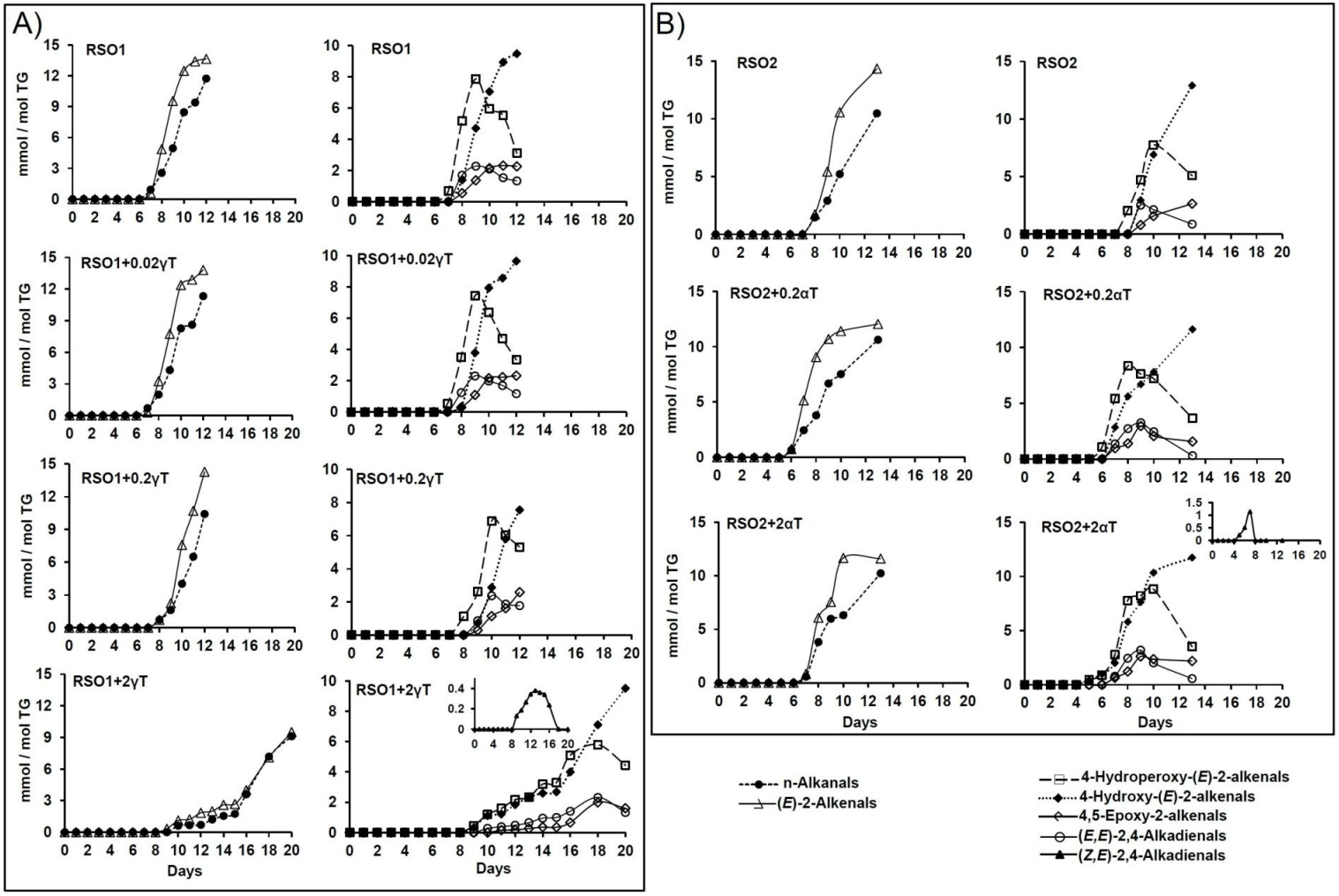

Figure 4. Evolution throughout the accelerated storage process of the concentrations, in mmol/mol TG, of the different types of aldehydes in: A) the samples with varying proportions of γ-T and their reference oil (RSO1); B) the samples with varying proportions of α-T and their reference oil (RSO2). All the figures reported are mean values

3.2.6. Alcohols

Alcohols other than those exhibiting conjugated dienes might also be generated as a consequence of the RSO oxidation progress. These can include compounds with only one hydroxy group, as well as dihydroxy-derivatives; it is worth noticing that the hydrolysis of some of these latter could give leukotoxin- and/or isoleukotoxin-diols, which are considered to have negative effects *in vitro* and in animal studies (Markaverich et al., 2005).

It is known that some dihydroxy-derivatives could give signals at around 3.42-3.45 ppm (see Table S1, signals "j1+j2"), and indeed the appearance of a signal near this chemical shift is observed during the AS process of sample RSO1 and of those enriched with γ-T (see Figure 1 for RSO1+2γT, signal "j"). In addition, the AS process of all these samples gives rise to the emergence of another signal at 3.62 ppm (see Figure 1 for RSO1+2γT, signal "k"), which could also be due to compounds supporting hydroxy groups (see Table S1).

The evolution of the concentrations of this type of compounds are shown in Figure 5A. This reveals that in all the samples the compounds giving signal "j" are detected earlier than the ones originating signal "k", their respective progressions being similar in RSO1 and in RSO1+0.02γT. At this point, it is worth mentioning that significant differences were not found between the concentrations of the various kinds of oxidation products in these two samples.

In the oils with γ-T proportions higher than 0.02% a delay in the formation of compounds giving signals "j" and "k" is observed, more as higher is the γ-T amount, while the opposite effect is observed in the case of α-T (see Figure 5B). However, in both kinds of samples, the addition of tocopherol at the highest level provokes a reduction in the growing rate of the concentration of these compounds, more marked for γ-T.

Finally, it must be noticed that, in the complementary study mentioned in section 2.1, carried out with γ-T of higher purity at the highest enrichment level (2%) (data not shown), the same effect on RSO evolution was observed, so any influence of the purity of the γ-T used on the results obtained can be discarded.

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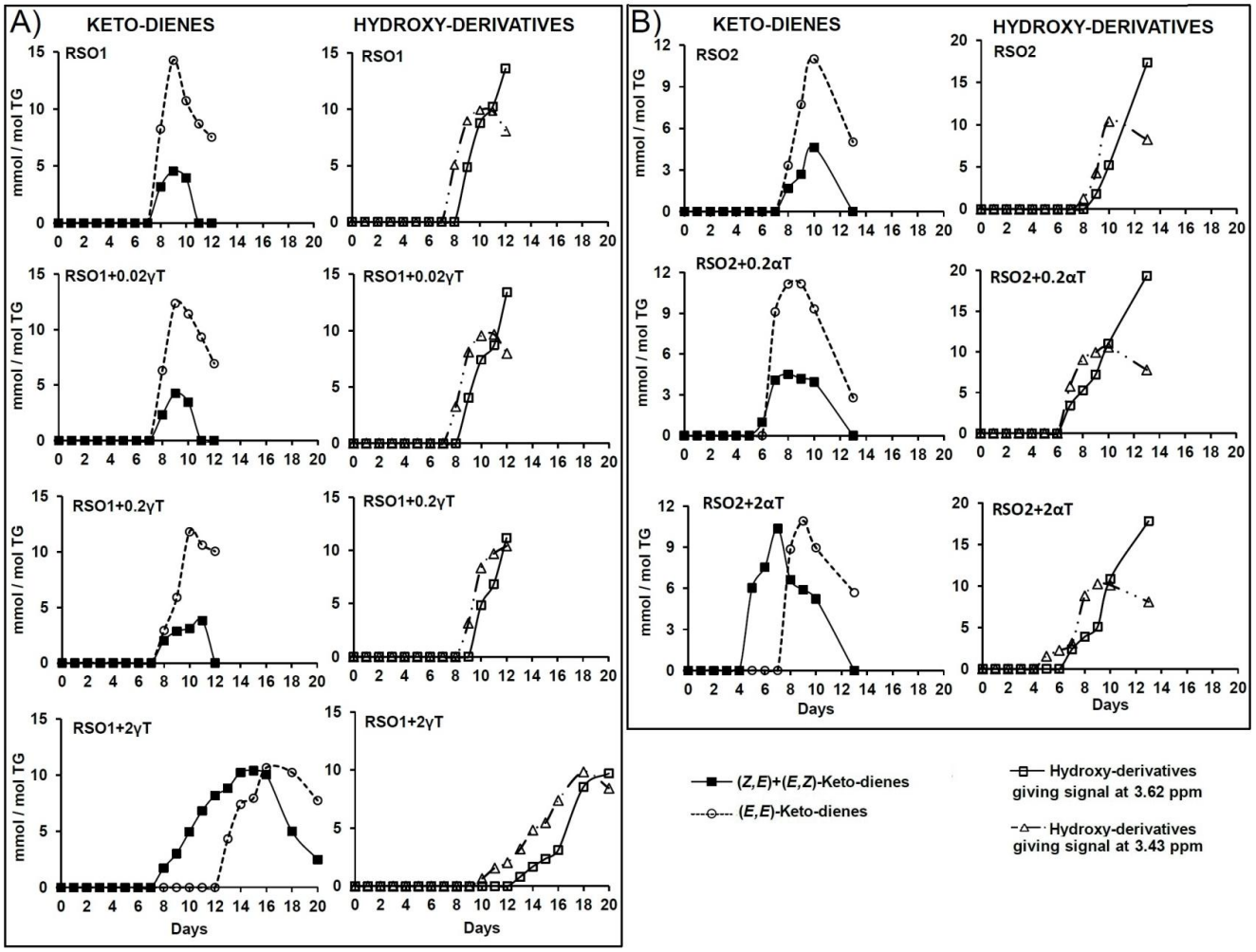

Figure 5. Evolution throughout the accelerated storage process of the concentrations, in mmol/mol TG, of the different types of conjugated keto-dienes and of the different types of hydroxy-derivatives in: A) the samples with varying proportions of γ-T and their reference oil (RSO1); B) the samples with varying proportions of α-T and their reference oil (RSO2). All the figures reported are mean values.

4. CONCLUSIONS

As far as we know, this is the first time that the action of γ-T on the oxidation process of a real edible oil has been studied in a global way by ^{1}H NMR and compared with that of α-T.

Although under the conditions of this study the enrichment of refined soybean oil with 0.2% and 2% of either γ-T or α-T accelerates the formation of (*Z,E*)-hydroperoxides, the rate of generation is considerably higher in the case of α-T. By contrast, a high tocopherol enrichment level (2%) slows down and reduces the formation of (*E,E*)-hydroperoxides. In addition, both hydroperoxides decomposition and polymerization proceed more slowly in the sample with 2% γ-T added than in the one with 2% α-T.

The differences between the effect of γ-T and α-T on hydroperoxides evolution cause, in turn, noticeable divergences in the generation of secondary oxidation products, which is delayed in most cases in the samples enriched with more than 0.02% γ-T, and accelerated in those with α-T added.

Irrespective of the type of tocopherol, the 2% enrichment level modifies the oil oxidation pathway, promoting the generation of certain types of compounds such as (*Z,E*)-hydroperoxides, (*Z,E*)-hydroxy-dienes, (*Z,E*)-keto-dienes, and (*Z,E*)-2,4-alkadienals, these latter not usually observed by ^{1}H NMR under these same conditions in the absence of such high levels of tocopherols. To this should be added that not all the various classes of oxidation products are affected in the same way by the tocopherol enrichment. Therefore, this work strengthens and confirms the importance of monitoring the whole oxidation process and considering different types of oxidation compounds, in order to obtain sound results and gain further insight into the effect and action mechanisms of potentially antioxidant compounds.

Due to the complex effect of γ-T on the generation of oxidation products, it seems difficult to define their action on the oil oxidation process as antioxidant or prooxidant. Notwithstanding, it can be stated that, under the conditions of this study, α-T is more prooxidant than γ-T. The acceleration in the generation of hydroperoxides and of some epoxides when γ-T is in high concentration should be considered when regulating the addition of this compound to edible oils.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Alberdi-Cedeño, J., Ibargoitia, M. L., Cristillo, G., Sopelana, P., & Guillén, M. D. (2017). A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components. *Food Chemistry, 221*, 1135-1144.

Azizkhani M., Kamkar A., & Nejad, A. S. M. (2011). Effects of tocopherols on oxidative stability of margarine. *Journal of the Chemical Society of Pakistan, 33,* 134-137.

Belloque, J., & Ramos, M. (1999). Application of NMR spectroscopy to milk and dairy products. *Trends in Food Science & Technology, 10*, 313-320.

Byrdwell, W. C., & Neff., W. E. (2001). Autoxidation products of normal and genetically modified canola oil varieties determined using liquid chromatography with mass spectrometric detection. *Journal of Chromatography A*, 905, 85-102.

Cillard, J., Cillard, P., Cormier, M., & Girre, L. (1980). α-Tocopherol prooxidant effect in aqueous media: increased autoxidation rate of linoleic acid. *Journal of the American Oil Chemists' Society, 57*, 252-255.

Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union*, 12.11.2011, L 295/1-177.

Dolde, D., & Wang, T. (2011). Oxidation of corn oils with spiked tocols. *Journal of the American Oil Chemists' Society, 88,* 1759-1765.

Fuster, M. D., Lampi, A. M., Hopia, A., & Kamal-Eldin, A. (1998). Effects of α-and γ-tocopherols on the autooxidation of purified sunflower triacylglycerols. *Lipids, 33*, 715-722.

Goicoechea, E., & Guillén, M. D. (2010). Analysis of hydroperoxides, aldehydes and epoxides by ^{1}H nuclear magnetic resonance in sunflower oil oxidized at 70 and 100 ºC. *Journal of Agricultural and Food Chemistry, 58*, 6234-6245.

Gottstein, T., & Grosch, W. (1990). Model study of different antioxidant properties of α- and γ-tocopherol in fats. *European Journal of Lipid Science and Technology, 92*, 139-144.

Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau, C., & Hammoc, B. D. (2000). Metabolism of monoepoxides of methyl linoleate: bioactivation and detoxification. *Archives of Biochemistry and Biophysics, 376*, 420-432.

Guillén, M. D., & Ruiz, A. (2003). Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups. *European Journal of Lipid Science and Technology, 105*, 688–696.

Guillén, M. D., & Ruiz, A. (2005). Oxidation process of oils with high content of linoleic acyl groups and formation of toxic hydroperoxy- and hydroxyalkenals. A study by ^{1}H nuclear magnetic resonance. *Journal of the Science of Food and Agriculture, 85*, 2413–2420.

Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α, β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition, 48*, 119-136.

Guillén, M. D., & Uriarte, P. S. (2009). Contribution to further understanding of the evolution of sunflower oil submitted to frying temperature in a domestic fryer: study by ^{1}H Nuclear Magnetic Resonance. *Journal of Agricultural and Food Chemistry, 57*, 7790–7799.

Guillén, M. D., & Uriarte, P. S. (2012). Study by ^{1}H NMR spectroscopy of the evolution of extra virgin olive oil composition submitted to frying temperature in an industrial fryer for a prolonged period of time. *Food Chemistry, 134*, 162-172.

Heinonen, M., Haila, K., Lampi, A. M., & Piironen, V. (1997). Inhibition of oxidation in 10% oil-in-water emulsions by β-carotene with α- and γ-tocopherols. *Journal of the American Oil Chemists' Society, 74*, 1047-1052.

Hidalgo, F. J., & Zamora, R. (2003). Edible oil analysis by high-resolution nuclear magnetic resonance spectroscopy: recent advances and future perspectives. *Trends in Food Science & Technology*, *14*, 499-506.

Hopia, A., Huang, S. W., & Frankel, E. N. (1996). Effect of α-tocopherol and trolox on the decomposition of methyl linoleate hydroperoxides. *Lipids*, *31*, 357-365.

Huang, S. W., Frankel, E. N., & German, J. B. (1994). Antioxidant activity of α- and γ-tocopherols in bulk oils and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *42*, 2108-2114.

Huang, S. W., Frankel, E. N., & German, J. B. (1995). Effects of individual tocopherols and tocopherol mixtures on the oxidative stability of corn oil triglycerides. *Journal of Agricultural and Food Chemistry, 43*, 2345-2350.

Isnardy, B., Wagner K.-H., & Elmadfa, I. (2003). Effects of α-, γ-, and δ- tocopherols on the autoxidation of purified rapeseed oil triacylglycerols in a system containing low oxygen. *Journal of Agricultural and Food Chemistry, 51*, 7775–7780.

Jung, M. Y., & Min, D. B. (1990). Effects of α-, γ-, and δ-tocopherols on oxidative stability of soybean oil. *Journal of Food Science, 55*, 1464-1465.

Kamal-Eldin, A., Lampi, A. M., & Hopia, A. (2001). α-, γ- and δ-Tocopherols as inhibitors of isomerization and decomposition of *cis,trans* methyl linoleate hydroperoxides. *European Journal of Lipid Science and Technology, 103*, 286-291.

King, R. E., Min, D. B., & Min, S. C. (2011). Study of α-, γ-, and δ-tocopherols in the oxidative stability of lard. *Food Science and Biotechnology*, *20*, 817-822.

Koskas, J. P., Cillard, J., & Cillard, P. (1984). Autoxidation of linoleic acid and behavior of its hydroperoxides with and without tocopherols. *Journal of the American Oil Chemists' Society, 61*, 1466-1469.

Lampi, A.-M., Kataja, L., Kamal-Eldin, A., & Vieno, P. (1999). Antioxidant activities of α- and γ-tocopherols in the oxidation of rapeseed oil triacylglycerols. *Journal of the American Oil Chemists' Society, 76*, 749-755.

Let, M. B., Jacobsen, C., & Meyer, A. S. (2007). Ascorbyl palmitate, γ-tocopherol, and EDTA affect lipid oxidation in fish oil enriched salad dressing differently. *Journal of Agricultural and Food Chemistry, 55*, 2369-2375.

Markaverich, B. M., Crowley, J. R., Alejandro, M. A., Shoulars, K., Casajuna, N., Mani, S., & Sharp, J. (2005). Leukotoxin diols from ground corncob bedding disrupt

estrous cyclicity in rats and stimulate MCF-7 breast cancer cell proliferation. *Environmental Health Perspectives, 113,* 1698-1704.

Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L., & Guillén, M. D. (2018). Prooxidant effect of α-tocopherol on soybean oil. Global monitoring of its oxidation process under accelerated storage conditions by ^{1}H Nuclear Magnetic Resonance. *Food Chemistry, 245*, 312-323.

Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). A review of thermo-oxidative degradation of food lipids studied by ^{1}H NMR spectroscopy: Influence of degradative conditions and food lipid nature. *Comprehensive Reviews in Food Science and Food Safety, 13*, 838-859.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016). The influence of frying technique, cooking oil and fish species on the changes occurring in fish lipids and oil during shallow-frying, studied by ^{1}H NMR. *Food Research International, 84,* 150-159.

Ruiz-Aracama, A., Goicoechea, E., & Guillén, M. D. (2017). Direct study of minor extra-virgin olive oil components without any sample modification. ^{1}H NMR multisupression experiment: A powerful tool. *Food Chemistry, 228,* 301-314.

Sacchi, R., Mannina, L., Fiordiponti, P., Barone, P., Paolillo, L., Patumi, M., & Segre, A. (1998). Characterization of Italian extra virgin olive oils using ^{1}H-NMR spectroscopy. *Journal of Agricultural and Food Chemistry, 46*, 3947-3951.

Schneider, C. (2005). Chemistry and biology of vitamin E. *Molecular Nutrition and Food Research, 49,* 7-30.

Schripsema, J. (2008). Comprehensive analysis of polar and apolar constituents of butter and margarine by nuclear magnetic resonance, reflecting quality and production processes. *Journal of Agricultural and Food Chemistry, 56,* 2547-2552.

Schwartz, H., Ollilainen, V., Piironen, V., & Lampi, A. M. (2008). Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. *Journal of Food Composition and Analysis, 21,* 152-161.

Sopelana, P., Arizabaleta, I., Ibargoitia, M. L., & Guillén, M. D. (2013). Characterisation of the lipidic components of margarines by ^{1}H Nuclear Magnetic Resonance. *Food Chemistry, 141,* 3357-3364.

Tyl, C. E., Brecker, L., & Wagner, K. H. (2008). ^{1}H NMR spectroscopy as tool to follow changes in the fatty acids of fish oils. *European Journal of Lipid Science and Technology, 110,* 141-148.

Vidal, N. P., Manzanos, M. J., Goicoechea, E., & Guillén, M. D. (2012). Quality of farmed and wild sea bass lipids studied by ^{1}H NMR: Usefulness of this technique for differentiation on a qualitative and a quantitative basis. *Food Chemistry, 135,* 1583-1591.

Wagner, K. H., & Elmadfa, I. (2000). Effects of tocopherols and their mixtures on the oxidative stability of olive oil and linseed oil under heating. *European Journal of Lipid Science and Technology, 102,* 624-629.

Wagner, K. H., Isnardy, B., & Elmadfa, I. (2004). γ- and δ-tocopherols are more effective than α-tocopherol on the autoxidation of a 10% rapeseed oil triacylglycerol-in-water emulsion with and without a radical initiator. *European Journal of Lipid Science and Technology, 106,* 44-51.

SUPPLEMENTARY MATERIAL OF MANUSCRIPT 3

A THOROUGH INSIGHT INTO THE COMPLEX EFFECT OF GAMMA-TOCOPHEROL ON THE OXIDATION PROCESS OF SOYBEAN OIL BY MEANS OF ^{1}H NUCLEAR MAGNETIC RESONANCE. COMPARISON WITH ALPHA-TOCOPHEROL

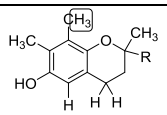
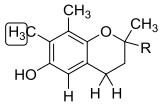
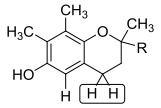
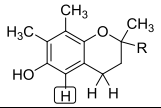
A.S. Martin-Rubio; P. Sopelana; María D. Guillén

Table S1. Chemical shifts, multiplicities and assignments of the ^{1}H NMR signals in $CDCl_3$ of the main types of triglyceride (TG) protons and of some oxidation compounds present in the different soybean oil samples, before and throughout the accelerated storage process. The signal letters agree with those given in Figure 1.

Signal	Chemical shift (ppm)	Multi-plicity	Functional group	
			Type of protons	Compound
Main acyl groups[a]				
A	0.88	t	-C**H**$_3$	saturated and monounsaturated ω-9 acyl groups
	0.89	t	-C**H**$_3$	linoleic acyl groups
B	0.97	t	-C**H**$_3$	linolenic acyl groups
C	1.19-1.42	m*	-(C**H**$_2$)$_n$-	acyl groups
D	1.61	m	-OCO-CH$_2$-C**H**$_2$-	acyl groups in TG
E	1.94-2.14	m**	-C**H**$_2$-CH=CH-	unsaturated acyl groups
F	2.26-2.36	dt	-OCO-C**H**$_2$-	acyl groups in TG
G	2.77	t	=HC-C**H**$_2$-CH=	linoleic acyl groups
H	2.80	t	=HC-C**H**$_2$-CH=	linolenic acyl groups
I	4.22	dd,dd	ROC**H**$_2$-CH(OR')-C**H**$_2$OR''	glyceryl groups
J	5.27	m	ROCH$_2$-C**H**(OR')-CH$_2$OR''	glyceryl groups
K	5.28-5.46	m	-C**H**=C**H**-	acyl groups
Oxidation compounds				
Hydroperoxides[b]				
a	8.3-9.0	bs	-OO**H**	monohydroperoxide group
Conjugated dienic systems[b,c]				
-	5.44	ddd	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroxy group in octadecadienoic acyl groups[d]
-	5.66	dd		
-	5.97	t		
b	6.49	dd		
-	5.47	ddm	-C**H**=C**H**-C**H**=C**H**-	(*E*,*E*)-conjugated double bonds
-	5.76	dtm		

-	6.06	ddtd		associated with
c	6.27	ddm		hydroperoxy group in octadecadienoic acyl groups
-	5.51	dtm	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated
-	5.56	ddm		double bonds
-	6.00	ddtd		associated with
d	6.58	dddd		hydroperoxy group in octadecadienoic acyl groups[e]
Epoxides				
Epoxy-derivatives				
e	2.63[f]	m	-C**H**O**H**C-	(*E*)-9,10-epoxystearate
f1	2.88[f]	m	-C**H**O**H**C-	(*Z*)-9,10-epoxystearate
f2	2.9[g,h]	m	-C**H**O**H**C-	monoepoxy-octadecenoate/-octadecadienoate groups
			-C**H**OHC-CH_2-CHO**H**C-	diepoxides
g1	3.10[g]	m	-CHO**H**C-CH_2-C**H**OHC-	diepoxides
Epoxy-keto-derivatives				
f3	2.89[i]/2.90[j]	td[i]/m[j]	-CO-CH=CH-CHO**H**C-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
f4	2.91[i]	td	-C**H**OHC-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
h	3.20[i,j]	dd	-CO-CH=CH-C**H**OHC-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
			-CHO**H**C-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-C**H**OHC-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-CO-CH=CH-CHO**H**C-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
i1	3.52[i]	dd	-CHO**H**C-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
i2	3.53[i]	dd	-CO-CH=CH-C**H**OHC-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-

				octadecenoate
Epoxy-hydroxy-derivatives				
f5	2.93[k]	dt	-C**H**OHC-CHOH-CH=CH-	*threo*-11-hydroxy-(*E*)-12,13-epoxy-(*Z*)-9-octadecenoate
g2	3.09[l]/3.097[m]	dd	-CHO**H**C-CH=CH-CHOH-	9-hydroxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate
Epoxy-hydroperoxy-derivatives				
g3	3.11[l]	dd	-CHO**H**C-CH=CH-CHOOH-	9-hydroperoxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate[n]
Alcohols				
j1	3.45[o,p]/3.48-3.41[q]	m[o,q]/bs[p]	-C**H**OH-C**H**OH-	9,10-dihydroxy-12-octadecenoate (leukotoxin diol, methyl ester)
j2	3.45[o]/3.42[p]	m[o]/bs[p]	-C**H**OH-C**H**OH-	12,13-dihydroxy-9-octadecenoate (isoleukotoxin diol, methyl ester)
k	3.62***	m	-C**H**OH-	12R-hydroxy-9(*Z*)-octadecenoate
Keto-dienes				
l	7.13***	dm	-CO−CH=C**H**−CH=CH-	(*E*,*E*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups[r]
m	7.50[r]/7.43[s]	dd[r]/ddd[s]	-CO−CH=C**H**−CH=CH-	(*Z*,*E*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
	7.49[r]/7.47[s]	ddd	-CO−CH=C**H**−CH=CH-	(*E*,*Z*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
Aldehydes				
n	9.49[t]	d	−C**H**O	(*E*)-2-alkenals
o	9.52[t]	d	−C**H**O	(*E*,*E*)-2,4-alkadienals

p	9.55[t]	d	−C**H**O	4,5-epoxy-2-alkenals
q	9.57[t]	d	−C**H**O	4-hydroxy-(*E*)-2-alkenals
r	9.58[t]	d	−C**H**O	4-hydroperoxy-(*E*)-2-alkenals
s	9.60[u]	d	-C**H**O	(*Z,E*)-2,4-alkadienals
t	9.75[t]	t	-C**H**O	n-alkanals
			γ-Tocopherol	
u1	2.095***	s		γ-tocopherol[v]
u2	2.123***	s		γ-tocopherol[v]
v	2.652***	m		γ-tocopherol[v]
w	6.362***	s		γ-tocopherol[v]

t: triplet; m: mutiplet; d: doublet; bs: broad signal; s: singlet; *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***Assignment made with the aid of standard compounds

[a]Assignments taken from Guillén, M. D., & Ruiz, A. (2003). *Journal of the Science of Food and Agriculture, 83*, 338–346.

[b]Data taken from Goicoechea, E., & Guillén, M. D. (2010). *Journal of Agricultural and Food Chemistry, 58*, 6234-6245 (hydroperoxides and conjugated (*Z,E*)- and (*E,E*)-hydroperoxy-dienes).

[c]Data taken from Dong, M., Oda, Y., & Hirota, M. (2000). *Bioscience, Biotechnology and Biochemistry, 64*, 882-886 (conjugated (*Z,E*)-hydroxy-dienes).

[d]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Kuklev, D. V., Christie, W. W., Durand, T., Rossi, J. C., Vidal, J. P., Kasyanov, S. P., Akulin, V. N., & Bezuglov, V. V. (1997). *Chemistry and Physics of Lipids, 85*, 125-134.

[e]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Chan, H. W. S., & Levett, G. (1977). *Lipids, 12*, 99-104.

[f]Data taken from Du, G., Tekin, A., Hammond, E. G., & Woo, L. K. (2004). *Journal of the American Oil Chemists' Society, 81*, 477–80.

[g]Data taken from Aerts, H. A. J., & Jacobs, P. A. (2004). *Journal of the American Oil Chemists' Society, 81*, 841-846 (monoepoxy-octadecenoates and diepoxides).

[h]Data taken from Cui, P. H., Duke, R. K., & Duke, C. C. (2008). *Chemistry and Physics of Lipids, 152*, 122-130 (monoepoxy-octadecadienoates).

[i]Data taken from Lin, D., Zhang, J., & Sayre, L. M. (2007). *The Journal of Organic Chemistry, 72*, 9471-9480.

[j]Data taken from Gardner, H. W., Kleiman, R., & Weisleder, D. (1974). *Lipids, 9*, 696-706.

[k]Data taken from Garssen, G. J., Veldink, G. A., Vliegenthart, J. F., & Boldingh, J. (1976). *The FEBS Journal, 62*, 33-36.

[l]Data taken from Gardner, H. W., Weisleder, D., & Kleiman, R. (1978). *Lipids, 13*, 246-252.

[m]Data taken from Van Os Cornelis, P. A., Vliegenthart, J. F. G., Crawford, C. G., & Gardner, H. W. (1982). *Biochimica et Biophysica Acta, 713*, 173-176.

[n]δ-Ketols (hydroxy-keto-derivatives) could also contribute to this signal, according to data from Gardner et al. (1974).

[o]Data taken from Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau C., & Hammoc B. D. (2000). *Archives of Biochemistry and Biophysics, 376*, 420-43.

[p]Data taken from Yang, J., Morton, M. D., Hill, D. W., & Grant, D. F. (2006). *Chemistry and Physics of Lipids, 140*, 75-87.

[q]Data taken from Nilewski, C., Chapelain, C. L., Wolfrum, S., & Carreira, E. M. (2015). *Organic Letters, 17*, 5602-5605.

[r]Assignment ("l") and data ("m") taken from Dufour, C., & Loonis, M. (2005). *Chemistry and Physics of Lipids, 138*, 60-68.

[s]Data taken from Kuklev et al. (1997).

[t]Data taken from Guillén, M. D., & Ruiz, A. (2004). *European Journal of Lipid Science and Technology, 106*, 680–687.

[u]Data taken from Guillén, M. D., & Uriarte, P. S. (2012). *Food Chemistry, 134*, 162-172.

[v]Assignments taken from Baker, J. K., & Myers, C. W. (1991). *Pharmaceutical Research, 8*, 763-770.

Manuscript 4

THE POTENTIAL OF LYSINE TO EXTEND THE SHELF LIFE OF SOYBEAN OIL EVIDENCED BY ^{1}H NUCLEAR MAGNETIC RESONANCE

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ABSTRACT

The effect of 1% and 2% proportions of L-lysine on the oxidation process of soybean oil, performed at 70 ºC under stirring conditions, has been studied by ^{1}H Nuclear Magnetic Resonance. This technique allows one to monitor the generation and further evolution of a wide variety of oxidation products (hydroperoxides, epoxides, aldehydes, keto-dienes and hydroxy-derivatives), providing a comprehensive view of the oxidation process. The addition of lysine considerably delays the degradation of the oil acyl groups and the generation of both primary and secondary oxidation compounds, while preserving the oil content of γ-tocopherol for longer, especially at the highest enrichment level. A diminution in the maximum concentration reached by some types of secondary oxidation products is also observed in the lysine-enriched samples. This is particularly noticeable for aldehydes, whose concentration is markedly reduced, above all that of the toxic oxygenated α,β-unsaturated ones, probably due to their reaction with lysine. Therefore, the ability of lysine to extend the shelf life of soybean oil is proved in this work, as well as its feasibility as a potential antioxidant for use in lipidic foods. These findings could also contribute to a better understanding of the antioxidant effect of proteins.

KEYWORDS: soybean oil, L-lysine, antioxidant, ^{1}H nuclear magnetic resonance, oxidation products

1. INTRODUCTION

In recent times, dietary components with antioxidant ability have raised great interest in the field of food science and nutrition, not only among researchers but also in the food industry, due to the potential of this type of compounds to reduce food lipid oxidation and to their generally accepted benefits for human health. This is the case of polyphenols and tocopherols, as well as of other vegetable components that have contributed to forge the conviction that a high consumption of fruits, vegetables or plant-derived foods contributes to a healthy diet (Khan, Afaq, & Mukhtar, 2008; Shahidi & de Camargo, 2016). However, some of these compounds like for example α-tocopherol, carotenoids and even polyphenols are surrounded by certain controversy due to their prooxidant effects in various systems (Carocho & Ferreira, 2013; Halliwell, 2007; Martin-Rubio, Sopelana, Ibargoitia, & Guillén, 2018). In this context, another class of dietary components with antioxidant potential are amino acids, some of which are essential nutrients; despite this, there are not many studies dealing with this issue, and comparatively more interest has been focused on proteins and peptides (Samaranayaka & Li-Chan, 2011).

Regarding the antioxidant activity of amino acids, this has been demonstrated in various methyl linoleate systems (Gopala Krishna & Prabhakar, 1994; Karel, Tannenbaum, Wallace, & Maloney, 1966; Marcuse, 1962), oil-in-water emulsions (Filippenko & Gribova, 2011; Riisom, Sims, & Fioriti, 1980), soybean oil (Hwang & Winkler-Moser, 2017), refined olive oil (Hidalgo, León, & Zamora, 2006), safflower and bint oils (Ahmad, Al-Hakim, Adel, & Shehata, 1983) or milk fat (Chen & Nawar, 1991). Notwithstanding, it must be noticed that prooxidative effects for some amino acids under certain conditions have also been reported (Ahmad et al., 1983; Farag, Osman, Hallabo, & Nasr., 1978; Marcuse, 1962; Park, Murakami, & Matsumura, 2005; Riisom et al., 1980). In short, although the antioxidant ability of proteins seems to be generally accepted (Elias & Decker, 2010), few is known about the specific action of amino acids on lipid oxidation under varying conditions and with regard to different types of oxidation products.

In the above mentioned studies the antioxidant effect of amino acids were assessed, in general terms, by determining oxygen consumption, hydroperoxides, conjugated dienes and/or secondary oxidation products; these latter have been determined mainly by means of the thiobarbituric acid assay (TBARs), but also by analyzing specific

aldehydes like propanal, pentanal and hexanal in the headspace of the samples. Hwang and Winkler-Moser (2017) also made use of a more advanced technique, ^{1}H Nuclear Magnetic Resonance, when studying the effect of several amino acids on the behaviour of soybean oil at frying temperature, to determine the loss of olefinic and bis-allylic protons after some hours of heating at 180 ºC. However, it could generally be said that the methodologies employed to assess the antioxidant action of amino acids offer only a partial view of the oxidation process.

It must also be noticed that despite several mechanisms have been described to explain the antioxidant action of amino acids and proteins (Elias & Decker, 2010), the specific effect of the different types of amino acids on the course of lipid oxidation processes needs further investigation. Actually, in line with the observations of Park et al. (2005), this specific knowledge could be very useful not only to design antioxidant peptides, but also to develop particular applications of free amino acids for lipid preservation. Moreover, the potential of free amino acids as antioxidants in foods and edible oils might tackle some inconveniences stemming from the allergenic potential of some proteins and peptides.

Taking into account all the aforementioned, this work focuses on the effect of L-lysine, an essential amino acid, on the oxidation process of soybean oil; lysine contains a reactive amino group at the end of its side chain that enables its interaction with other components present in the system, and its antioxidant potential has been shown in some studies (Ahmad et al., 1983; Karel et al., 1966; Riisom et al., 1980). To this end, both refined soybean oil and this same oil containing either 1% or 2% by weight of lysine were heated at 70 ºC under continuous stirring in order to achieve a homogeneous distribution of the amino acid in the oil. The evolution of the oil was monitored by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR), paying special attention to both acyl groups degradation and the generation of a wide range of oxidation products; this technique allows one to obtain a global picture of the oxidation process, thus overcoming some of the disadvantages of the methodologies most frequently used to monitor lipid oxidation processes, which only provide a limited, and in some instances misleading, view of the oxidation course.

The results of this work will provide information about the antioxidant potential of lysine in lipid food systems, thus opening up new possibilities to extend the shelf life of oils or lipid food systems. In addition, nutritional benefits might also derived from the

use of lysine in case of its final ingestion took place. It must be noticed that not only lysine has multiple health benefits (Singh, 2016) but also adverse effects due to the consumption of lysine doses up to 6 g/day and even more have not been observed in humans (VKM, 2016).

2. MATERIALS AND METHODS

2.1. Samples

The oil employed was a refined soybean oil (RSO). Each one of the samples enriched with L-lysine (RSO+LYS) was prepared by adding either 1% or 2% by weight of lysine to 10 g of this oil in separate beakers; the oils with lysine added were designated as RSO+LYS1 (1% of lysine) and RSO+LYS2 (2% of lysine). L-lysine with a purity ≥ 98% was purchased from Cymit Quimica (Barcelona, Spain).

The molar percentages of the different types of oil acyl groups were determined by ^{1}H NMR, as in previous works (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012). These were 5.2±0.1 for linolenic, 47.2±0.2 for linoleic, 29.9±0.2 for oleic and 17.7±0.0 for saturated groups.

2.2. Oxidation process

10 g samples of RSO and of the RSO+LYS oils were prepared in several beakers of 6.5 cm diameter (250 ml), one per day of sampling. These were placed in a multiple magnetic stirrer with calefaction and heated at approximately 70º C. Aliquots were taken periodically from each respective beaker throughout the oil oxidation process for their study by ^{1}H NMR. The evolution of the samples was monitored until the stirring magnet stopped rotating due to the polymerization of the oil. The oxidation process was carried out in duplicate in order to obtain average values for all the studied compounds.

2.3. Monitoring by ^{1}H NMR of the evolution of RSO and of the lysine-enriched oils throughout the oxidation process

2.3.1. Operating conditions

The ^{1}H NMR spectra of the starting oils and of the corresponding aliquots taken throughout the oxidation process were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz. The weight of each aliquot was approximately 0.16 g. These were mixed in a 5 mm diameter tube with 400 μl of deuterated chloroform that

contained 0.2% of non deuterated chloroform and a small amount (0.03%) of tetramethylsilane as internal references. The acquisition parameters used were: spectral width 5000 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.744 s and pulse width 90º, with a total acquisition time of 8 min 55 s. The relaxation delay and acquisition time selected allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making possible their use for quantitative purposes. The experiments were carried out at 25 ºC, as in previous works (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012).

2.3.2. Identification of some compounds

The identification of the oil acyl groups, of γ-tocopherol and of the oxidation products formed throughout the oxidation process was carried out on the basis of the signal assignment shown in Table S1 (see supplementary material), made from bibliographic data and with the aid of several standard compounds. In this latter case, the spectra of the commercial standards mentioned in the supplementary material were acquired, both alone and after being mixed with oil samples in order to know their exact chemical shifts and multiplicities in the system subject of study.

2.3.3. Quantitative data estimated from the ^{1}H NMR spectra

The molar percentages of the several kinds of oil acyl groups throughout the oxidation process were estimated as in previous studies (Guillén & Uriarte, 2012). For this purpose, trilinolein and trilinolenin, acquired from Sigma-Aldrich, were used as references.

The concentrations of γ-tocopherol and of the different types of oxidation products generated were estimated as millimoles per mole of triglyceride (mmol/mol TG), in the way described in the supplementary material.

3. RESULTS AND DISCUSSION

As mentioned above, the evolution of the oxidation process of the several oils was monitored by ^{1}H NMR, paying attention to acyl groups and γ-tocoperol degradation, as well as to new compounds formation. The appearance and further evolution of signals due to oxidation products in the ^{1}H NMR spectrum of the oil with the highest lysine level (RSO+LYS2) can be observed in Figure 1.

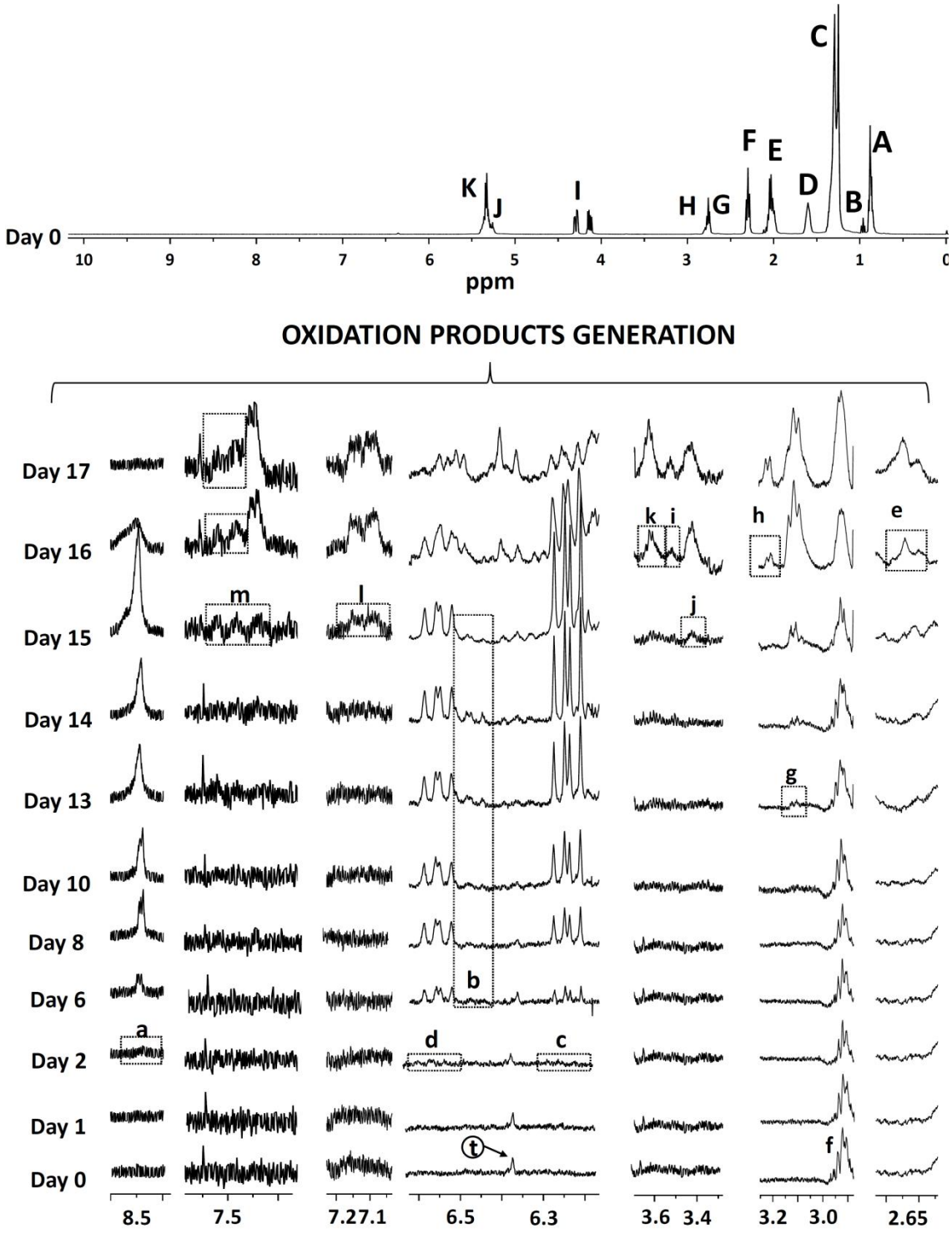

Figure 1. ^{1}H NMR spectrum of sample RSO+LYS2 before being subjected to the oxidation process, together with the enlargements of some spectral regions where changes occur throughout time. Letters agree with those in Table S1, considering that "f" includes signals "f1-f6", "g" signals "g1-g3" and "j" signals "j1+j2". The plots corresponding to the same ^{1}H NMR spectral region are presented at a fixed value of absolute intensity, for them to be valid for comparative purposes.

3.1. Effect of lysine on the evolution of oil acyl groups

The evolutions of the different kinds of oil acyl groups, expressed in molar percentages, are represented *versus* time in days in Figure 2A. This graph shows that the molar percentages of unsaturated groups decrease with time in RSO, especially those of the polyunsaturated ones (linolenic and linoleic); this diminution is slow during a first stage, but very quick afterwards. In consequence, the molar percentage of saturated+modified (S+M) groups increases accordingly. The addition of lysine to the oil delays this process, in line with increasing lysine concentration, by enlarging the first phase of acyl groups degradation from approximately 4 days in RSO to 11 in RSO+LYS1 and 14 in RSO+LYS2. Moreover, the enrichment of RSO with 1% of lysine considerably extends the oil total polymerization process from 7 to 15 days, while the addition of 2% lysine lengthens this process by only two more days.

3.2. Effect of lysine on the evolution of γ-tocopherol

γ-Tocopherol (γ-T) is the most abundant tocol in soybean oil (Alberdi-Cedeño, Ibargoitia, Cristillo, Sopelana, & Guillén, 2017) and, as Figure 1 shows, it can be detected by ^{1}H NMR (see signal "t") during part of the oxidation process. Given that tocopherols are considered important components with antioxidant ability in soybean oil, the evolution of γ-T throughout the oxidation course was monitored in order to assess if the lysine enrichment could affect its consumption rate. Figure 2B, which shows the progress of the γ-T concentration with time reveals that increased lysine levels reduce the rate of γ-T degradation. This suggests that, when lysine is added to RSO, it prevents γ-T from being consumed in antioxidant reactions. Therefore, the addition of lysine not only exerts an antioxidant action regarding acyl groups degradation, but also preserves the γ-T oil content. A slower degradation of γ-T in the presence of lysine was also observed by Hwang and Winkler-Moser (2017) in soybean oil at frying temperature.

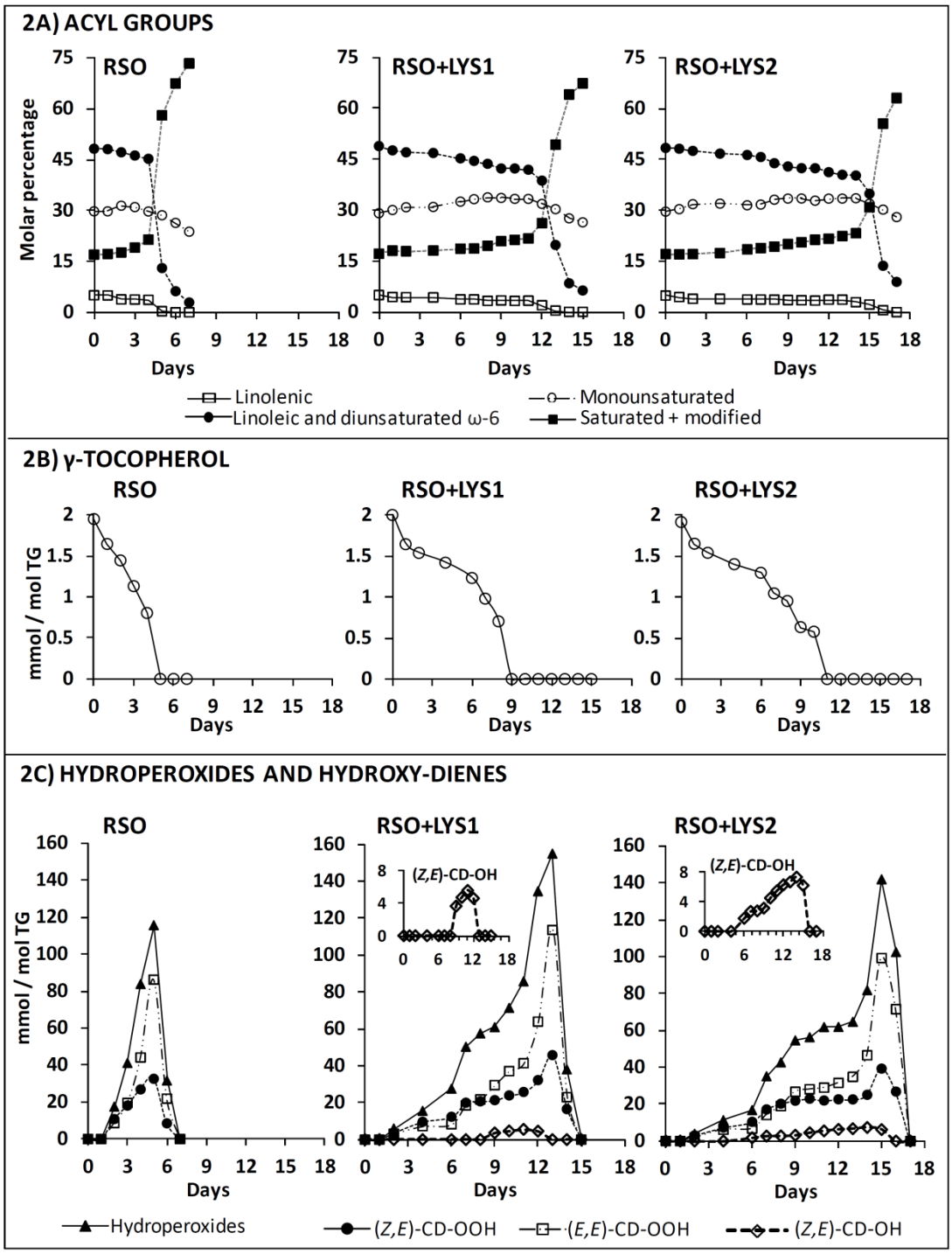

Figure 2. Evolution throughout the oxidation process, in the reference oil (RSO) and in the samples enriched with lysine (RSO+LYS), of: A) the molar percentages of linolenic, linoleic and diunsaturated ω-6, monounsaturated and saturated+modified acyl groups; B) the concentration, in mmol/mol TG, of γ-tocopherol; and C) the concentrations, in mmol/mol TG, of hydroperoxides and their associated conjugated (*Z*,*E*)- and (*E*,*E*)-dienes ((*Z*,*E*)- and (*E*,*E*)-CD-OOH), and of conjugated (*Z*,*E*)-hydroxy-dienes ((*Z*,*E*)-CD-OH). All the figures reported are mean values.

3.3. Influence of the lysine enrichment on the formation and evolution of oxidation products

3.3.1. Evolution of the concentration of hydroperoxides

Hydroperoxides generation takes place when thermodegradation of unsaturated acyl groups occurs. The evolutions of the concentrations of hydroperoxide groups and of their associated (*Z,E*)- and (*E,E*)-conjugated dienes can be observed in Figure 2C, while the progress of their respective signals in sample RSO+LYS2 is shown in Figure 1 (see letter "a" for hydroperoxide groups, and letters "d" and "c" for (*Z,E*)- and (*E,E*)-conjugated dienes, respectively).

3.3.1.1. Hydroperoxides giving signals between 8.3 and 9.0 ppm

Figure 2C shows that in RSO hydroperoxides concentration increases rapidly and at a practically constant rate from days 1 to 5, when acyl groups degradation is already considerable (see Figure 2A); then, it exhibits a sharp drop. In the lysine-enriched samples hydroperoxides are detected at the same time as in the reference oil, namely after 2 days under oxidative conditions, although in concentrations that decrease in line with lysine enrichment. In addition, and in agreement with acyl groups degradation, hydroperoxides concentration rises much more slowly in the oils with lysine added than in the non-enriched oil, particularly in RSO+LYS2, where a certain stabilization in the level of hydroperoxides is observed between days 9 and 13.

These findings, which evidence the ability of lysine to retard the oxidation of RSO, are in agreement with the antioxidant effect of lysine reported by other authors in soybean oil at frying temperature (Hwang & Winkler-Moser, 2017) and in other types of vegetable oils (Ahmad et al., 1983). Notwithstanding, the maximum concentrations of hydroperoxides are higher in the oils with lysine added than in the original oil. Given that when these maximum levels are reached, the extent of acyl groups degradation, especially of the linoleic ones, is lower in the oils containing lysine than in RSO (see Figure 2A), it might be thought that the slower progress of the oxidation process in the former could result in a greater accumulation of hydroperoxides before their decomposition occurs.

3.3.1.2. Conjugated (*Z,E*)- and (*E,E*)-hydroperoxy-dienes

As mentioned above, the progress of (*Z,E*)- and (*E,E*)-hydroperoxy-dienes ((*Z,E*)- and (*E,E*)-CD-OOH) can be followed separately from the evolutions of signals "d" and "c", respectively (see Figure 1).

As can be observed in Figure 2C, the addition of lysine slows down the concentration increase of both (*Z,E*)- and (*E,E*)-CD-OOH, as in the case of hydroperoxide groups. However, it does not affect either their respective evolutions with time or their relative proportions. However, in line with hydroperoxide groups, the maximum concentrations reached by both types of conjugated dienes are somewhat higher in the lysine-enriched oils.

3.3.2. Conjugated (*Z,E*)-hydroxy-dienes

The presence of small signals that could be tentatively assigned to (*Z,E*)-hydroxy-dienes ((*Z,E*)-CD-OH) was detected in some of the spectra corresponding to the oils enriched with lysine, but not in those of the original oil; this is shown for sample RSO+LYS2 in Figure 1 (signal "b"). As can be observed in Figure 2C, this type of compounds could be monitored over more days in RSO+LYS2 than in RSO+LYS1, and their maximum concentration is also higher in the oil with the highest lysine concentration (RSO+LYS2). It is also worthwhile noticing that, especially in this latter sample, the period when (*Z,E*)-CD-OH exhibit their main concentration increase coincides approximately with the time interval when hydroperoxides concentration is stabilized to a certain extent. This might be partly explained by the findings of Ishino et al. (2008), according to whom hydroperoxides can be reduced to alkyl-hydroxides due to their involvement in the formation of adducts between lysine and saturated aldehydes generated during the oil oxidation process, even before this type of aldehydes can be detected by ^{1}H NMR.

3.3.3. Epoxides

As can be observed in Figure 1, the unoxidized refined soybean oil exhibits a signal (letter "f") that could be tentatively assigned in part to compounds supporting epoxy groups, which overlap with the side-band of the bis-allylic protons signal (see Table S1). However, not only does the intensity of this signal increase throughout the oxidation course, but also new signals that could also be tentatively attributed to epoxides are generated over time. Table S1, which compiles some of these compounds, shows that only (*E*)-epoxystearates (letter "e") and (*E*)-epoxy-keto-enes (letters "h" and

"i"), coming probably from oleic and polyunsaturated groups respectively, generate signals isolated from those of the rest of epoxides. The evolution of the signals given by this kind of oxidation products can be observed in Figure 1 (letters "e", "f", "g", "h" and "i"), while the progress of their concentrations is shown in Figure 3.

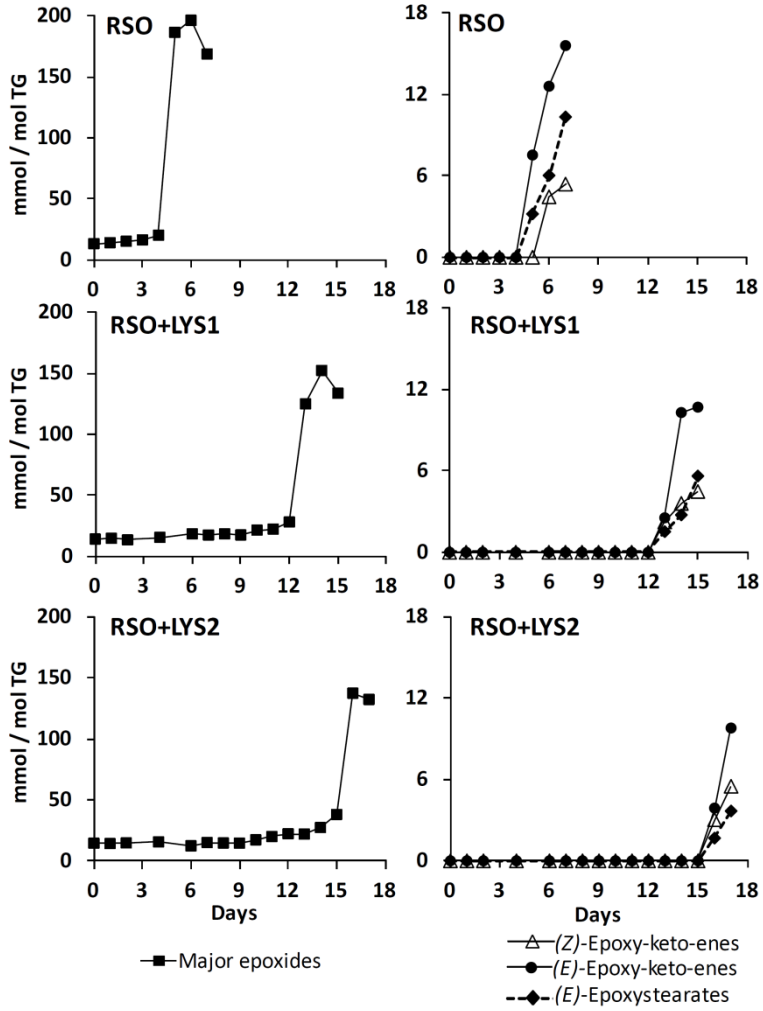

Figure 3. Evolution throughout the oxidation process, in the reference oil (RSO) and in the samples enriched with lysine (RSO+LYS), of the concentration, in mmol/mol TG, of different types of epoxides. All the figures reported are mean values.

3.3.3.1. Major epoxides

As mentioned in the supplementary maerial, the epoxides considered under this designation give their signals between 2.87 and 3.17 ppm approximately (see Table S1), and they have been estimated as a whole. As was the case of hydroperoxides evolution (see Figure 2C), the addition of lysine to RSO caused a remarkable delay in the generation of these major epoxides (see Figure 3), above all in RSO+LYS2. In addition,

the maximum concentration attained by this kind of compounds diminishes with lysine concentration. This latter finding can be considered of interest since some epoxides derived from linoleic groups that might be present among these major epoxides can exhibit toxic effects (Greene, Williamson, Newman, Morisseau, & Hammoc, 2000).

3.3.3.2. Other minor epoxides: (*E*)-epoxystearates and epoxy-keto-enes

As in the case of the so-called major epoxides, the generation of (*E*)-epoxystearates from oleic groups also takes place later in the samples containing lysine than in the original oil, their onset being concomitant with the degradation of oleic groups in all cases (see Figure 2A). The maximum concentration of (*E*)-epoxystearates also decreases with the lysine enrichment level, in agreement with the lower degradation extent of oleic groups.

In general terms, the same can be said of the tentatively identified epoxy-keto-enes, derived from polyunsaturated groups.

3.3.4. Aldehydes

The generation and evolution of the ^{1}H NMR signals of this type of secondary oxidation products, very relevant due to the toxicity of some of them (Guillén & Goicoechea, 2008), is shown in Figure 4A, where all the plots have been drawn at a fixed value of absolute intensity to be valid for comparative purposes. The progress of their concentrations, in turn, can be observed in the graphs displayed in Figure 4B. As Figure 4A reveals, the addition of lysine noticeably delays the time when the first aldehydic signals are spotted in the ^{1}H NMR spectra from day 5 in RSO to days 12 and 14 in RSO+LYS1 and RSO+LYS2, respectively. Moreover, it must be highlighted that, unlike that observed for other oxidation compounds commented above, differences in the appearance rate and in the relative concentrations of the various types of aldehydes are observed in the lysine-enriched samples in comparison with the original oil. Actually, as Figure 4A shows, in RSO all the different types of aldehydes appear suddenly after 5 days under oxidative conditions. By contrast, in RSO+LYS samples the process of aldehydes generation is more progressive, since n-alkanals (letter “s”) and (*E*)-2-alkenals (letter “n”) in RSO+LYS1, and n-alkanals in RSO+LYS2 are the first groups of aldehydes detected, while the remaining ones appear later.

Regarding the evolution of the concentration of the different types of aldehydes, it can be observed in Figures 4A and 4B that in RSO 4-hydroperoxy+4-hydroxy-(*E*)-2-alkenals (quantified together, as mentioned in the supplementary material), (*E*)-2-

alkenals and n-alkanals are the most abundantly generated aldehydes. In addition, (*E*,*E*)-2,4-alkadienals and 4,5-epoxy-2-alkenals are also formed, although in lower concentrations than those previously mentioned.

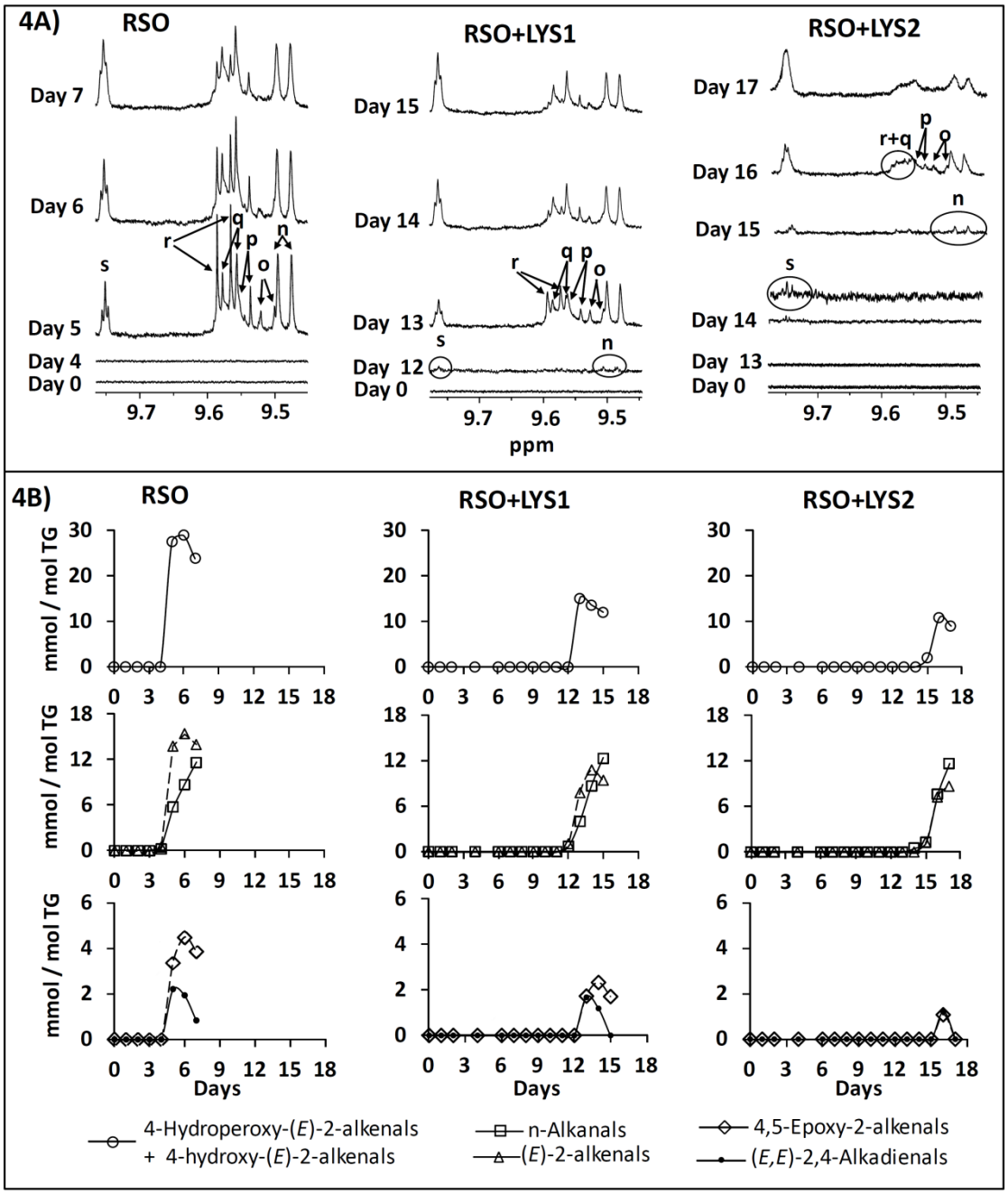

Figure 4. Evolution throughout the oxidation process, in the reference oil (RSO) and in the samples enriched with lysine (RSO+LYS), of: A) the ^{1}H NMR spectral signals of aldehydes: n-alkanals (signal "s", see Table S1), 4-hydroperoxy-(*E*)-2-alkenals (signal "r"), (*E*)-2-alkenals (signal "n"), 4-hydroxy-(*E*)-2-alkenals (signal "q"), 4,5-epoxy-2-alkenals (signal "p") and (*E*,*E*)-2,4-alkadienals (signal "o"); and B) the concentrations, in mmol/mol TG, of the different types of aldehydes. All the figures reported are mean values.

As far as the lysine-enriched samples are concerned, it is noticeable that the concentrations of all the different kinds of oxygenated α,β-unsaturated aldehydes (4-hydroperoxy-+4-hydroxy-(*E*)-2-alkenals and 4,5-epoxy-2-alkenals), considered the most toxic ones (Guillén & Goicoechea, 2008), are notably lower than in the original oil, especially in RSO+LYS2. A decrease in the level of the rest of α,β-unsaturated aldehydes, above all of (*E*)-2-alkenals, is also observed in the oils with lysine added, but to a lower extent than in the case of the oxygenated ones. By contrast, the concentration of n-alkanals does not seem to be affected by the addition of lysine.

Taking into account that the ability of lysine to react with aldehydes is well-known (Uchida, 2015), and that the oxygenated α,β-unsaturated ones (4-hydroperoxy-, 4-hydroxy- and 4,5-epoxy-2-alkenals) are considered among the most reactive (Guillén & Goicoechea, 2008), the markedly decreased concentration of these latter could be due to their reaction with lysine. Actually, the ability of this type of aldehydes to form adducts with lysine has been proved in several works (Shimozu, Hirano, Shibata, Shibata, & Uchida, 2011; Uchida, 2015; Zamora & Hidalgo, 1994).

3.3.5. Conjugated keto-dienes

As in observations made in a previous work (Martin-Rubio et al., 2018), conjugated keto-dienes with (*Z*,*E*)- and (*E*,*E*)-isomerism are also formed throughout the oxidation process of the studied oils. These secondary oxidation compounds have been identified according to the data given in Table S1. Figure 5A shows the evolution of their concentrations in all the oils studied, whereas the progress of their signals in RSO+LYS2 sample can be observed in Figure 1 (see letters “m” and “l”). As Figure 5A reveals, in RSO the generation of (*Z*,*E*)- and (*E*,*E*)-keto-dienes is detected after 5 days under oxidative conditions, their maximum concentrations being observed this same day. Similarly to that commented on epoxides and aldehydes, the addition of lysine to RSO delays the appearance of keto-dienes, more so the higher the lysine concentration; however, it does not practically affect either their maximum concentrations or their relative proportions. Notwithstanding, in the lysine-enriched samples the keto-dienes concentration increase and its subsequent decrease happens more slowly than in RSO, especially in the oil with the highest lysine level; this results in higher concentrations of this type of compounds at the end of the oxidation process in the samples with lysine added. This finding exemplifies the importance of monitoring the entire oxidation

process, as the determination of the concentration of certain compounds at only specific points or at the end of the oxidation process could lead to erroneous conclusions.

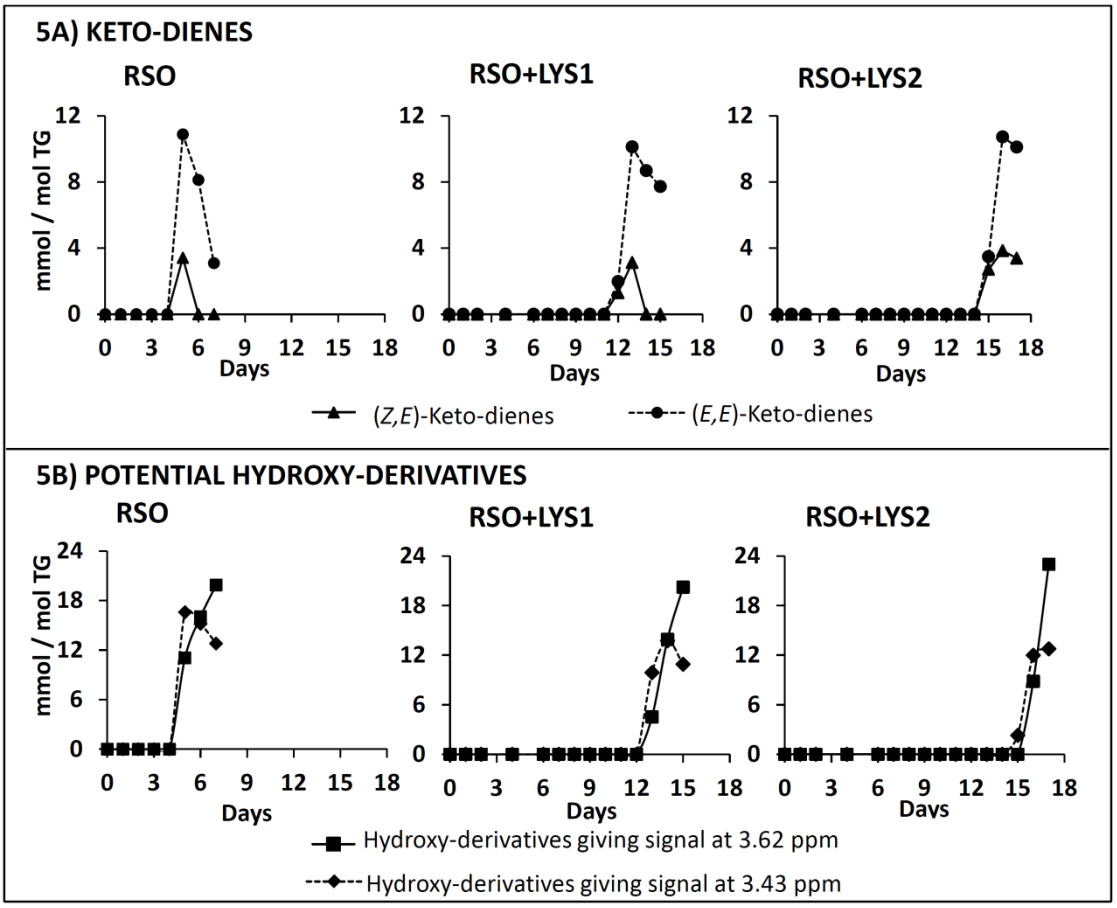

Figure 5. Evolution throughout the oxidation process, in the reference oil (RSO) and in the samples enriched with lysine (RSO+LYS), of the concentrations, in mmol/mol TG, of: A) keto-dienes; and B) alcohols. All the figures reported are mean values.

3.3.6. Compounds with alcohol groups

This type of compounds have been considered separately from the hydroxy-derivatives supporting conjugated dienes discussed in section 3.3.2, and signals that could be tentatively associated to their occurrence have been detected in the oils studied. Considering data in Table S1, it has been assumed that both monohydroxy- and dihydroxy-derivatives could have been generated throughout the RSO oxidation process, since signals coinciding with those designed with letters “j” (3.43 ppm) and “k” (3.62 ppm) have been detected. The evolution of these signals in the RSO+LYS2 oil can be observed in Figure 1 and the progress of their concentrations in all the studied oils in the graphs of Figure 5B; this latter shows that in RSO both types of compounds are

detected simultaneously after 5 days under oxidative conditions. The presence of lysine not only postpones considerably the emergence of this type of secondary oxidation products, particularly in RSO+LYS2, but similarly to that observed for keto-dienes (see Figure 5A), in the case of the tentatively identified dihydroxy-derivatives (signal at 3.43 ppm) it also slows down their concentration increase with time. It is worth pointing out that in the lysine-enriched oils the concentration of these latter ones are lower than in the reference oil. This might be related to the reduction observed in major epoxides in these same samples (see Figure 3), since these may include linoleic monoepoxides precursors of some dihydroxy-derivatives like leukotoxin- and isoleukotoxin-diols.

4. CONCLUSIONS

As far as we know, this is the first time that such a detailed study has been conducted on the effect of an amino acid on the oxidation process of an edible oil, determining a wide range of oxidation products by ^{1}H NMR.

The results obtained evidence that the enrichment of refined soybean oil with lysine in proportions of 1 and 2% in weight has a clear antioxidant effect under the conditions of this study, even though the rise in lysine proportion from 1 to 2% does not greatly affect either the oil oxidative stability or the time needed to reach total polymerization. Thus, although the addition of lysine does not postpone hydroperoxides emergence, it noticeably delays the degradation of oil acyl groups and the rate of hydroperoxides concentration increase, in such a way that the generation of secondary oxidation compounds is detected considerably later than in the non-enriched oil. All this extends considerably the oil shelf life, while preserving the oil content of γ-T, considered a bioactive and beneficial compound.

It is also noticeable that as the lysine level gets higher, the concentration of major epoxides, epoxy-keto-enes, (*E*)-epoxystearates, dihydroxy-derivatives and, above all, of α,β-unsaturated aldehydes, is reduced. While the diminution in the level of toxic oxygenated α,β-unsaturated aldehydes might be due to their reaction with lysine, to the best of our knowledge, the influence of lysine on the concentration of other secondary oxidation products had not been reported before in a food system.

The outcomes of this work not only prove the feasibility of lysine as a potential antioxidant for use in lipidic food systems but also they could help in adjusting the addition of exogenous antioxidants to foodstuffs containing this amino acid. Thus, lysine could constitute an alternative to other antioxidants, either synthetic or natural,

whose use is surrounded by controversies regarding either their impact on human health or their prooxidant potential under certain conditions. Moreover, the knowledge derived from this work might also contribute to go deeper into the antioxidant effect of proteins and peptides.

CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

ACKNOWLEDGEMENTS

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REFERENCES

Ahmad, M. M., Al-Hakim, S., Adel, A., & Shehata, Y. (1983). The antioxidant activity of amino acids in two vegetable oils. *Journal of the American Oil Chemists' Society, 60*, 837-840.

Alberdi-Cedeño, J., Ibargoitia, M. L., Cristillo, G., Sopelana, P., & Guillén, M. D. (2017). A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components. *Food Chemistry, 221*, 1135-1144. https://doi.org/10.1016/j.foodchem.2016.11.046

Carocho, M., & Ferreira, I. C. (2013). A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology, 51*, 15-25. https://doi.org/10.1016/j.fct.2012.09.021

Chen, Z. Y., & Nawar, W. W. (1991). The role of amino acids in the autoxidation of milk fat. *Journal of the American Oil Chemists' Society, 68*, 47-50. https://doi.org/10.1007/BF02660309

Elias, R. J., & Decker, E. A. (2010). Protein antioxidants for the stabilization of lipid foods: current and potential applications. In E. A. Decker, R. J. Elias, & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications,*

Volume 1: Understanding mechanisms of oxidation and antioxidant activity (pp. 249-271). Cambridge, UK: Woodhead Publishing Limited. https://doi.org/10.1533/9780857090447.2.249

Farag, R. S., Osman, S. A., Hallabo, S. A. S., & Nasr, A. A. (1978). Cottonseed oil oxidation catalyzed by amino acids and albumin in aqueous and nonaqueous media. *Journal of the American Oil Chemists' Society, 55*, 613-615. https://doi.org/10.1007/BF02678956

Filippenko, T. A., & Gribova, N. Y. (2011). Antioxidant activity of amino acids during oxidation of sunflower oil in an emulsion. *Pharmaceutical Chemistry Journal*, *45*, 296-298.

Gopala Krishna, A. G., & Prabhakar, J. V. (1994). Antioxidant efficacy of amino acids in methyl linoleate at different relative humidities. *Journal of the American Oil Chemists' Society, 71*, 645-647. https://doi.org/10.1007/BF02540594

Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau, C., & Hammoc, B. D. (2000). Metabolism of monoepoxides of methyl linoleate: bioactivation and detoxification. *Archives of Biochemistry and Biophysics, 376*, 420-432. https://doi.org/10.1006/abbi.2000.1753

Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α,β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition*, *48*, 119-136. https://doi.org/10.1080/10408390601177613

Guillén, M. D., & Ruiz, A. (2003). Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups. *European Journal of Lipid Science and Technology, 105*, 688-696. https://doi.org/10.1002/ejlt.200300866

Guillén, M. D., & Uriarte, P. S. (2012). Study by ^{1}H NMR spectroscopy of the evolution of extra virgin olive oil composition submitted to frying temperature in an industrial fryer for a prolonged period of time. *Food Chemistry, 134*, 162-172. https://doi.org/10.1016/j.foodchem.2012.02.083

Halliwell, B. (2007). Dietary polyphenols: good, bad, or indifferent for your health?. *Cardiovascular Research, 73*, 341-347. https://doi.org/10.1016/j.cardiores.2006.10.004

Hidalgo, F. J., León, M. M., & Zamora, R. (2006). Antioxidative activity of amino phospholipids and phospholipid/amino acid mixtures in edible oils as determined by

the Rancimat method. *Journal of Agricultural and Food Chemistry*, *54*, 5461-5467. DOI: 10.1021/jf060848s

Hwang, H. S., & Winkler-Moser, J. K. (2017). Antioxidant activity of amino acids in soybean oil at frying temperature: Structural effects and synergism with tocopherols. *Food Chemistry*, *221*, 1168-1177. https://doi.org/10.1016/j.foodchem.2016.11.042

Ishino, K., Shibata, T., Ishii, T., Liu, Y. T., Toyokuni, S., Zhu, X., Sayre. L. M., Uchida, K. (2008). Protein N-acylation: H_2O_2-mediated covalent modification of protein by lipid peroxidation-derived saturated aldehydes. *Chemical Research in Toxicology*, *21*, 1261-1270. DOI: 10.1021/tx800080x

Karel, M., Tannenbaum, S. R., Wallace, D. H., & Maloney, H. (1966). Autoxidation of methyl linoleate in freeze-dried model systems. III. Effects of added amino acids. *Journal of Food Science, 31*, 892-896. https://doi.org/10.1111/j.1365-2621.1966.tb03266.x

Khan, N., Afaq, F., & Mukhtar, H. (2008). Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxidants & Redox Signaling*, *10*, 475-510. https://doi.org/10.1089/ars.2007.1740

Marcuse, R. (1962). The effect of some amino acids on the oxidation of linoleic acid and its methyl ester. *Journal of the American Oil Chemists' Society, 39*, 97-103.

Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L., & Guillén, M. D. (2018). Prooxidant effect of α-tocopherol on soybean oil. Global monitoring of its oxidation process under accelerated storage conditions by ^{1}H nuclear magnetic resonance. *Food Chemistry*, *245*, 312-323. http://dx.doi.org/10.1016/j.foodchem.2017.10.098

Park, E. Y., Murakami, H., & Matsumura, Y. (2005). Effects of the addition of amino acids and peptides on lipid oxidation in a powdery model system. *Journal of Agricultural and Food Chemistry*, *53*, 8334-8341. DOI: 10.1021/jf058063u

Riisom, T., Sims, R. J., & Fioriti, J. A. (1980). Effect of amino acids on the autoxidation of safflower oil in emulsions. *Journal of the American Oil Chemists' Society, 57*, 354-359. https://doi.org/10.1007/BF02662057

Samaranayaka, A. G., & Li-Chan, E. C. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of Functional Foods*, *3*, 229-254. https://doi.org/10.1016/j.jff.2011.05.006

Shahidi, F., & de Camargo, A. C. (2016). Tocopherols and tocotrienols in common and emerging dietary sources: occurrence, applications, and health benefits. *International Journal of Molecular Sciences, 17*, 1745. https://doi.org/10.3390/ijms17101745

Shimozu, Y., Hirano, K., Shibata, T., Shibata, N., & Uchida, K. (2011). 4-Hydroperoxy-2-nonenal is not just an intermediate but a reactive molecule that covalently modifies proteins to generate unique intramolecular oxidation products. *Journal of Biological Chemistry, 286*, 29313-29324. doi: 10.1074/jbc.M111.255737

Singh, M. (2016). Medicinal uses of L-Lysine: Past and future. *International Journal of Research in Pharmaceutical Sciences, 2*, 637-642.

Uchida, K. (2015). Aldehyde adducts generated during lipid peroxidation modification of proteins. *Free Radical Research, 49*, 896-904. https://doi.org/10.3109/10715762.2015.1036052

VKM (Norwegian Scientific Committee for Food Safety). (2016). Risk assessment of "other substances" - L-lysine opinion of the Panel on Nutrition, Dietetic Products, Novel Food and Allergy of the Norwegian Scientific Committee for Food Safety, ISBN: 987-82-8259-252-9, Oslo, Norway.

Zamora, R., & Hidalgo, F. J. (1994). Modification of lysine amino groups by the lipid peroxidation product 4,5(*E*)-Epoxy-2(*E*)-heptenal. *Lipids, 29*, 243-249

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SUPPLEMENTARY MATERIAL OF MANUSCRIPT 4

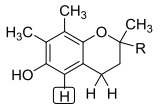
THE POTENTIAL OF LYSINE TO EXTEND THE SHELF LIFE OF SOYBEAN OIL EVIDENCED BY ^{1}H NUCLEAR MAGNETIC RESONANCE

A.S. Martin-Rubio; P. Sopelana; María D. Guillén

Table S1. Chemical shifts, multiplicities and assignments of the ^{1}H NMR signals in $CDCl_3$ of the main types of triglyceride (TG) protons, of some oxidation compounds and of γ-tocopherol, present in the different soybean oil samples, before and throughout the oxidation process.

Signal	Chemical shift (ppm)	Multi-plicity	Functional group	
			Type of protons	Compound
Main acyl groups[a]				
A	0.88	t	-C**H**$_3$	saturated and monounsaturated ω-9 acyl groups
	0.89	t	-C**H**$_3$	linoleic acyl groups
B	0.97	t	-C**H**$_3$	linolenic acyl groups
C	1.19-1.42	m*	-(C**H**$_2$)$_n$-	acyl groups
D	1.61	m	-OCO-CH$_2$-C**H**$_2$-	acyl groups in TG
E	1.94-2.14	m**	-C**H**$_2$-CH=CH-	unsaturated acyl groups
F	2.26-2.36	dt	-OCO-C**H**$_2$-	acyl groups in TG
G	2.77	t	=HC-C**H**$_2$-CH=	linoleic acyl groups
H	2.80	t	=HC-C**H**$_2$-CH=	linolenic acyl groups
I	4.22	dd,dd	ROC**H**$_2$-CH(OR')-C**H**$_2$OR''	glyceryl groups
J	5.27	m	ROCH$_2$-C**H**(OR')-CH$_2$OR''	glyceryl groups
K	5.28-5.46	m	-C**H**=C**H**-	acyl groups
Oxidation compounds				
Hydroperoxides[b]				
a	8.3-9.0	bs	-OO**H**	monohydroperoxide group
Conjugated dienic systems[b,c]				
-	5.44	ddd	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroxy group in octadecadienoic acyl groups[d]
-	5.66	dd		
-	5.97	t		
b	6.49	dd		
-	5.47	ddm	-C**H**=C**H**-C**H**=C**H**-	(*E*,*E*)-conjugated double bonds associated with hydroperoxy group in octadecadienoic acyl groups
-	5.76	dtm		
-	6.06	ddtd		
c	6.27	ddm		

-	5.51	dtm	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroperoxy group in octadecadienoic acyl groups[e]
-	5.56	ddm		
-	6.00	ddtd		
d	6.58	dddd		
Epoxides				
Epoxy-derivatives				
e	2.63[f]	m	-C**H**O**H**C-	(*E*)-9,10-epoxystearate
f1	2.88[f]	m	-C**H**O**H**C-	(*Z*)-9,10-epoxystearate
f2	2.9[g]	m	-C**H**O**H**C-	monoepoxy-octadecenoate groups
			-C**H**OHC-CH_2-CHO**H**C-	diepoxides
f3	2.94***	m	-C**H**O**H**C-	(*Z*)-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid
g1	3.10[g]	m	-CHO**H**C-CH_2-C**H**OHC-	diepoxides
Epoxy-keto-derivatives				
f4	2.89[h]/2.90[i]	td[h]/m[i]	-CO-CH=CH-CHO**H**C-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
f5	2.91[h]	td	-C**H**OHC-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
h	3.20[h,i]	dd	-CO-CH=CH-C**H**OHC-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
			-CHO**H**C-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-C**H**OHC-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-CO-CH=CH-CHO**H**C-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
i1	3.52[h]	dd	-CHO**H**C-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
i2	3.53[h]	dd	-CO-CH=CH-C**H**OHC-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
Epoxy-hydroxy-derivatives				
f6	2.93[j]	dt	-C**H**OHC-CHOH-CH=CH-	*threo*-11-hydroxy-(*E*)-12,13-epoxy-(*Z*)-9-octadecenoate
g2	3.09[k]/3.097[l]	dd	-CHO**H**C-CH=CH-CHOH-	9-hydroxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate
Epoxy-hydroperoxy-derivatives				
g3	3.11[k]	dd	-CHO**H**C-CH=CH-CHOOH-	9-hydroperoxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate[m]
Alcohols				

j1	3.45[n,o]/3.48-3.41[p]	m[n,p]/bs[o]	-C**H**OH-C**H**OH-	9,10-dihydroxy-12-octadecenoate (leukotoxin diol, methyl ester)
j2	3.45[n]/3.42[o]	m[n]/bs[o]	-C**H**OH-C**H**OH-	12,13-dihydroxy-9-octadecenoate (isoleukotoxin diol, methyl ester)
k	3.62***	m	-C**H**OH-	12R-hydroxy-9(*Z*)-octadecenoate
Keto-dienes				
l	7.13***	dm	-CO−CH=C**H**−CH=CH-	(*E*,*E*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups[q]
m	7.50[q]/7.43[r]	dd[q]/ddd[r]	-CO−CH=C**H**−CH=CH-	(*Z*,*E*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
	7.49[q]/7.47[r]	ddd	-CO−CH=C**H**−CH=CH-	(*E*,*Z*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
Aldehydes				
n	9.49[s]	d	−C**H**O	(*E*)-2-alkenals
o	9.52[s]	d	−C**H**O	(*E*,*E*)-2,4-alkadienals
p	9.55[s]	d	−C**H**O	4,5-epoxy-2-alkenals
q	9.57[s]	d	−C**H**O	4-hydroxy-(*E*)-2-alkenals
r	9.58[s]	d	−C**H**O	4-hydroperoxy-(*E*)-2-alkenals
s	9.75[s]	t	-C**H**O	n-alkanals
			γ-Tocopherol[t]	
t	6.362***	s		

t: triplet; m: multiplet; d: doublet; bs: broad signal; *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***Assignment made with the aid of standard compounds

[a]Assignments taken from Guillén, M. D., & Ruiz, A. (2003). *Journal of the Science of Food and Agriculture*, *83*, 338-346.

[b]Data taken from Goicoechea, E., & Guillén, M. D. (2010). *Journal of Agricultural and Food Chemistry*, *58*, 6234-6245 (hydroperoxides and conjugated (*Z*,*E*)- and (*E*,*E*)-hydroperoxy-dienes).

[c]Data taken from Dong, M., Oda, Y., & Hirota, M. (2000). *Bioscience, Biotechnology and Biochemistry, 64*, 882-886 (conjugated (*Z,E*)-hydroxy-dienes).

[d]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Kuklev, D. V., Christie, W. W., Durand, T., Rossi, J. C., Vidal, J. P., Kasyanov, S. P., Akulin, V. N., & Bezuglov, V. V. (1997). *Chemistry and Physics of Lipids, 85*, 125-134.

[e]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Chan, H. W. S., & Levett, G. (1977). *Lipids, 12*, 99-104.

[f]Data taken from Du, G., Tekin, A., Hammond, E. G., & Woo, L. K. (2004). *Journal of the American Oil Chemists' Society, 81*, 477-80.

[g]Data taken from Aerts, H. A. J., & Jacobs, P. A. (2004). *Journal of the American Oil Chemists' Society, 81*, 841-846 (monoepoxy-octadecenoates and diepoxides).

[h]Data taken from Lin, D., Zhang, J., & Sayre, L. M. (2007). *The Journal of Organic Chemistry, 72*, 9471-9480.

[i]Data taken from Gardner, H. W., Kleiman, R., & Weisleder, D. (1974). *Lipids, 9*, 696-706.

[j]Data taken from Garssen, G. J., Veldink, G. A., Vliegenthart, J. F., & Boldingh, J. (1976). *The FEBS Journal*, *62*, 33-36.

[k]Data taken from Gardner, H. W., Weisleder, D., & Kleiman, R. (1978). *Lipids, 13*, 246-252.

[l]Data taken from Van Os Cornelis, P. A., Vliegenthart, J. F. G., Crawford, C. G., & Gardner, H. W. (1982). *Biochimica et Biophysica Acta, 713*, 173-176.

[m]δ-Ketols (hydroxy-keto-derivatives) could also contribute to this signal (Gardner et al., 1974).

[n]Data taken from Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau C., & Hammoc B. D. (2000). *Archives of Biochemistry and Biophysics, 376*, 420-43.

[o]Data taken from Yang, J., Morton, M. D., Hill, D. W., & Grant, D. F. (2006). *Chemistry and Physics of Lipids, 140*, 75-87.

[p]Data taken from Nilewski, C., Chapelain, C. L., Wolfrum, S., & Carreira, E. M. (2015). *Organic Letters, 17*, 5602-5605.

[q]Data taken from Dufour, C., & Loonis, M. (2005). *Chemistry and Physics of Lipids, 138*, 60-68.

[r]Data taken from Kuklev et al. (1997).

[s]Data taken from Guillén, M. D., & Ruiz, A. (2004). *European Journal of Lipid Science and Technology, 106*, 680-687.

[t]Assignment taken from Baker, J. K., & Myers, C. W. (1991). *Pharmaceutical Research, 8*, 763-770.

Standard compounds used for the identification of various oil components by ^{1}H NMR

γ-Tocopherol, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E,E*)-2,4-hexadienal, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, 4,5-epoxy-(*E*)-2-decenal and 12,13-epoxy-9(*Z*)-octadecenoic acid methyl ester (isoleukotoxin methyl ester), acquired from Sigma-Aldrich; 4-hydroxy-(*E*)-2-nonenal, 4-hydroperoxy-(*E*)-2-nonenal, 9,10-dihydroxy-12(*Z*)-octadecenoic acid (leukotoxin diol), 12,13-dihydroxy-9(*Z*)-octadecenoic acid (isoleukotoxin diol), *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, 9-keto-10(*E*),12(*E*)-octadecadienoic acid and 12R-hydroxy-9(*Z*)-octadecenoic acid methyl ester (ricinoleic acid methyl ester), purchased from Cayman Chemical (Ann Arbor, MI, USA); and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica, were used for the identification of some signals present in the spectra of the samples subject of study.

Quantification of γ-tocopherol and of the different types of oxidation products from ^{1}H NMR spectral data

The concentrations of γ-tocopherol and of the different types of oxidation products generated throughout the oil oxidation process were estimated as millimoles per mole of triglyceride (mmol/mol TG) by using the following equations:

$[\gamma\text{-T}] = [A_{\gamma T}/(A_{TG}/4)]*1000$

$[OP] = [(A_{OP}/n)/(A_{TG}/4)]*1000$,

where $A_{\gamma T}$ and A_{OP} are the areas of the signals selected for the quantification of γ-T and of each oxidation product (OP), respectively (see Table S1), n the number of protons that generate each signal and A_{TG} the area of the protons at *sn*-1 and *sn*-3 positions in the glycerol backbone of TG (signal "I" in Table S1).

To estimate the concentration of the so-called major epoxides (see section 3.3.3.1), signals between 2.87 and 3.17 ppm approximately were taken together, assuming that the signal at approximately 2.9 ppm corresponds mainly to epoxides contributing with two protons and the one at 3.1 ppm to epoxy-compounds contributing with only one (see Table S1). In the case of the signal at approximately 2.9 ppm, the overlapped area due to the side band of *bis*-allylic protons signals (G and H) must be subtracted.

4-Hydroperoxy- and 4-hydroxy-(*E*)-2-alkenals have been quantified together in order to accurately compare their concentrations in all the samples, because in sample RSO+LYS2 it is very difficult to determine each one of these kinds of aldehydes separately, due to the almost total overlap of their signals (see Figure 4A, letters "r" and "q", respectively).

Manuscript 5

STUDY OF THE EFFECT OF THE COOXIDATION OF SOYBEAN OIL AND LYSINE ON THEIR RESPECTIVE EVOLUTIONS: A COMBINED ASSESSMENT BY ^{1}H NMR AND LC/MS

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Submitted for publication

ABSTRACT

The objective of this work was to analyze the evolution of a system consisting of soybean oil with a 2% of L-lysine under heating at 70 ºC and stirring conditions, analyzing how the cooxidation of the oil and of the amino acid affects their respective evolutions, trying to obtain information about the action mechanism of lysine on soybean oil oxidation. The study of the oil progress by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR), shows that lysine delays oil degradation and oxidation products generation in comparison with a reference oil without lysine. Regarding lysine evolution, the analysis by ^{1}H NMR and Liquid Chromatography/Mass Spectrometry of a series of aqueous extracts obtained from the studied system over time reveals the generation of lysine adducts, most of them at the ε-position, with n-alkanals, malondialdehyde, (*E*)-2-alkenals and oxygenated α,β-unsaturated aldehydes. However, this does not seem enough to explain the antioxidant action of lysine.

Chemical compounds studied in this article: L-Lysine (PubChem CID: 5962); (*E*,*E*)-2,4-Decadienal (PubChem CID: 5283349); 4-Hydroxy-(*E*)-2-nonenal (PubChem CID: 1693); 4-Hydroperoxy-(*E*)-2-nonenal (PubChem CID: 6435432); (*E*)-9,10-epoxystearate (PubChem CID: 12235226); Nε-Formyl-lysine (PubChem CID: 70923); Nε-Acetyl-lysine (PubChem CID: 92832); Nε-Hexanoyl-lysine (PubChem CID: 76128638); Nε-(2-Propenal)-lysine (PubChem CID: 6439214); Nα-Acetyl-lysine (PubChem CID: 92907)

KEYWORDS: soybean oil, L-lysine, antioxidant, ^{1}H Nuclear Magnetic Resonance, Liquid Chromatography/Mass Spectrometry, hydroperoxides, aldehydes, epoxides, lysine adducts

1. INTRODUCTION

The antioxidant ability of proteins and peptides (Elias, Kellerby & Decker, 2008) has long generated great interest among food scientists, especially in the last two decades when numerous studies have been conducted to investigate the potential of protein hydrolysates of diverse origin as antioxidants in food systems (Elias, Bridgewater, Vachet, Waraho, McClements & Decker, 2006; Elias et al., 2008; Jónsdóttir, Geirsdóttir, Hamaguchi, Jamnik, Kristinsson & Undeland, 2016). In this context, the monitoring of lipid oxidation is usually carried out by means of classical methodologies such as peroxide value, conjugated dienes and/or TBARs, in some cases combined with the determination of volatile aldehydes like propanal or hexanal. However, in most of the studies a global view of the oxidation process is not achieved and this hinders the intricate task of delving into antioxidant mechanisms.

It is also noteworthy that most of these studies mainly focus on the evolution of the lipid matrix, and little attention is given to the changes provoked in amino acids. In fact, only a reduced number of the multiple works dealing with the antioxidant effect of proteins or amino acids on food systems combine the simultaneous monitoring of both the lipid oxidation process and the modifications suffered by amino acids (Elias et al., 2006; Jónsdóttir et al., 2016). Among these latter, the formation of carbonyl groups from basic amino acids, the oxidation of free thiols to give disulfide bridges or the hydroxylation of aromatic amino acids should be mentioned. Protein oxidation can also lead to cleavage of the polypeptide chain and to formation of cross-linked protein aggregates (Stadtman & Levine, 2003). Furthermore, functional groups of proteins can react with lipid oxidation products like hydroperoxides or aldehydes (Gardner, 1979; Uchida, 2003).

Various mechanisms have been proposed to explain the antioxidant action of proteins, such as binding of metal ions (Ahmad, Al-Hakim, Adel & Shehata, 1983; Xu, Zheng, Zhu, Li & Zhou, 2018) or free radical scavenging ability (Xu et al., 2018), among others. However, the specific pathways through which amino acid residues or free amino acids are able to affect the course of lipid oxidation require further investigation for a better understanding of their antioxidant effect.

Another issue worth mentioning is related to the potential of amino acids as antioxidants in food systems. Unlike what can happen in proteins, where certain antioxidant amino acids can be buried into the protein structure, free amino acids are

directly available to take part in different types of reactions and this might enhance their antioxidant action. Despite this, studies aimed at investigating the effect of free amino acids on the oxidative stability of food lipids are comparatively less abundant than those concerning proteins and their hydrolysates (Ahmad et al., 1983; Filippenko & Gribova, 2011; Hwang & Winkler-Moser, 2017; Martin-Rubio, Sopelana & Guillén, 2019).

Taking into account all the above, this work addresses the effect of L-lysine on the oxidation process of soybean oil, analyzing how the cooxidation of the oil and of the amino acid affects their respective evolutions. Given that the antioxidant effect of lysine on soybean oil has already been proved in a previous work (Martin-Rubio et al., 2019) interest has been focused on the evolution of the amino acid; notwithstanding, the progress of the oil during oxidation has also been monitored in order to establish relationships between both processes. It must be noticed that oxidation of amino acids and proteins is awakening increasing awareness due to its biological and health implications.

To achieve the aims of this study, refined soybean oil and the same oil containing a 2% in weight of lysine were heated under continuous stirring in a magnetic stirrer at 70 ºC. The oil progress was monitored by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR), while the changes in lysine were studied by Liquid Chromatography followed by Mass Spectrometry (LC/MS) and also by ^{1}H NMR, in order to explore the potential of this latter technique to follow the evolution of lysine under oxidative conditions. Thus, the outcomes of this work might contribute to gaining further insight into the cooxidation of lipids and amino acids, and to some extent of lipids and proteins; for this purpose a food system which would be feasible in the context of human nutrition has been studied from a dual perspective: oil and amino acid, since it is expected that there is a relationship between both evolutions, which might provide information about the action mechanism of lysine on soybean oil.

2. MATERIALS AND METHODS

2.1. Samples

The original samples were refined soybean oil (RSO) purchased from a multinational company and the same oil enriched with 2% by weight of L-lysine (RSO+2LYS). The molar percentages of the different types of oil acyl groups were determined by ^{1}H NMR, as in previous works (Guillén & Uriarte, 2012). These were 6.3±0.1 for linolenic,

49.5±0.1 for linoleic, 25.5±0.3 for oleic and 18.7±0.3 for saturated groups. The L-lysine used had a purity ≥ 98% and was purchased from Sigma-Aldrich (St. Louis, MO, USA). Due to the difficulty of uniformly distributing lysine into the oil, RSO+2LYS samples were prepared by directly weighing 8 g of RSO in a beaker and then adding 2% by weight of lysine.

2.2. Oxidation process

Samples of RSO and of RSO+2LYS (8 g of oil in all cases) were prepared in beakers of 5 cm diameter, one per day of sampling, placed on a multiple magnetic stirrer heated at around 70 ºC and stirred at 180 rpm with magnets of 4.5 cm long. Samples submitted to oxidative conditions for different periods were taken throughout the oxidation process for their study. Their evolution was monitored until the stirring magnet stopped rotating due to the polymerization of the oil. The oxidation process was carried out in duplicate in order to obtain average values for all the compounds studied.

2.2.1. Monitoring by ^{1}H NMR of the evolution of RSO and of RSO+2LYS samples throughout the oxidation process

The ^{1}H NMR spectra both of the original RSO and RSO+2LYS samples and of these samples after their being submitted to oxidative conditions over different periods of time were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz, following the same procedure as in previous works (Guillén & Uriarte, 2012). Experimental details and information about acquisition parameters are given in the supplementary material.

The identification of the oil acyl groups and of some of their derived products formed throughout the oxidation process was carried out from the ^{1}H NMR spectra signal assignments shown in Table S1 (see supplementary material). This contains bibliographic data and data coming from several standard compounds, also indicated in the supplementary material.

Furthermore, the molar percentage of the several kinds of oil acyl groups throughout the oxidation process were estimated from these ^{1}H NMR spectra, as in previous studies (Guillén & Uriarte, 2012). For this purpose, trilinolein and trilinolenin, acquired from Sigma-Aldrich, were used. The concentrations of the oxidation products generated were estimated as described in the supplementary material.

2.2.2. Extraction of lysine and some of its derivatives from the RSO+2LYS sample after being submitted for different periods of time to oxidative conditions

Lysine and some of its derivatives formed during the oxidation process were extracted from the corresponding RSO+2LYS sample as follows; 0.16 g of each sample were poured into a 1.5 ml Eppendorf microtube and mixed with 800 μl of the extraction solvent. Several deuterated solvents were tested to extract the amino acid and its derivatives from the system and to study their recovery by means of ^{1}H NMR: water, water with different methanol percentages, methanol and acid water (0.5 M of HCl, pH close to 1.5). The best extraction efficiency was achieved using acid water, so this was the solvent chosen. After adding the solvent, each Eppendorf microtube was shaken for 10 min with an automatic tube stirrer and then centrifuged for another 10 min. The aqueous phase was taken out with a pipette, filtered through a 0.45 μm filter (GL Science Inc., Tokyo, Japan) using a 1 ml syringe (Terumo corporation, Tokyo, Japan) and poured into another Eppendorf microtube. The extracts were analyzed both by ^{1}H NMR and by LC/MS. It must be pointed out that milliQ water was used to obtain the extracts intended for the LC/MS analysis, while deuterated water was used for ^{1}H NMR.

2.2.2.1. Study of the extracts by LC/MS

The aqueous extracts obtained from the RSO+2LYS sample throughout the oxidation process were studied by LC/MS. However, due to analytical requirements, in this case it was necessary to add milliQ water to these extracts before their analysis in order to make the pH less acid (near 2.0). The LC/MS analysis conditions, together with the procedures followed for the identification and quantification of lysine and its derivatives are given in the supplementary material.

The evolutions of lysine and their derivatives were monitored only up to the 22nd day of the oxidation process; afterwards, the oil polymerization degree impaired the extraction of lysine and lysine derivatives in the aqueous phase, so it was not possible to obtain extracts directly comparable to the previous ones.

2.2.2.2. Study of the extracts by ^{1}H NMR

The aqueous extracts obtained from the RSO+2LYS sample throughout the oxidation process were also studied by ^{1}H NMR. The procedure followed was the same as for the

lipid samples but, in this case, 600 μl of the aqueous extract were taken directly from the Eppendorf microtube (see section 2.2.2) and placed in a NMR tube for analysis.

Identification of the compounds in these extracts was made from the assignment of the ^{1}H NMR spectral signals obtained by means of standard compounds. To this aim L-lysine, Nε-formyl-lysine, Nα-acetyl-lysine and Nε-acetyl-lysine were purchased from Cymit Quimica. The chemical shifts, multiplicities and assignments of their signals are given in Table S2 (supplementary material).

The quantification of lysine and its derivatives by ^{1}H NMR constitutes one of the goals of this work, so the procedure developed for this purpose will be described below, in the Results and Discussion section.

3. RESULTS AND DISCUSSION

3.1. Effect of the presence of lysine on soybean oil evolution. Monitoring by ^{1}H NMR.

Given that a more exhaustive analysis of the effect of different lysine proportions on the evolution of refined soybean oil under very similar conditions to those in this study was performed before (Martin-Rubio et al., 2019). Therefore, in the present work a less detailed discussion will be carried out; however, it is considered necessary to include these data in order to relate oil evolution to that of lysine.

3.1.1. Effect on the degradation rate of polyunsaturated acyl groups

The degradation of soybean oil can be estimated from the evolution of the molar percentages of the two types of polyunsaturated acyl groups present in this oil (linolenic and linoleic), determined from ^{1}H NMR spectral data, which is represented *versus* time in days in Figure 1A. As this graph shows, the evolution of polyunsaturated groups splits into two very distinct stages, characterized by markedly different degradation rates. Regardless of the stage considered, the molar percentages of both kinds of acyl groups diminish at a higher rate in RSO than in RSO+2LYS sample. This indicates that lysine provokes an important slowdown in oil degradation, in such a way that when 2% of lysine is added to the oil it is not until day 22 that a sudden decrease of the molar percentages of polyunsaturated groups occurs, whereas in RSO this takes place after 10 days. It is also noticeable that the degradation extent of linoleic groups at the end of the oxidation process in the lysine-enriched sample is somewhat lower than in the original

oil. These results indicate that lysine exerts a clear antioxidant effect and it is to be expected that this will also affect the formation of soybean oil oxidation derived compounds such as hydroperoxides, epoxides and aldehydes.

3.1.2. Effect on hydroperoxide formation

As a consequence of acyl group degradation, hydroperoxides giving ^{1}H NMR signals between 8.3 and 9.0 ppm (see Table S1) are generated. The evolution of the concentration of these hydroperoxides and of their associated (*Z*,*E*)- and (*E*,*E*)-conjugated dienes, expressed in mmol/mol TG, is shown in Figure 1B. This shows that, in agreement with observations made in the degradation of linolenic and linoleic acyl groups (see Figure 1A), hydroperoxide concentration rises at a much higher rate in RSO than in RSO+2LYS, reaching in the first case its maximum concentration on day 12 (247 mmol/mol TG) and on day 23 in the second (210 mmol/mol TG).

A comparison of the evolution of hydroperoxide concentration in the sample containing lysine and in the reference oil suggests that lysine could be acting as a free radical scavenger, in line with the findings of Xu, Zheng, Zhu, Li and Zhou (2018). According to this hypothesis, it might interact with radicals coming from polyunsaturated acyl groups and/or from the earliest formed hydroperoxides, thus limiting the propagation stage of the radicalary reaction to the point when it no longer seems possible to slow down the oxidation course and hydroperoxide accumulation occurs. Indeed, in the extensive review of the reactions between proteins and oxidized lipids carried out by Schaich in 2008, it is stated that radical transfer from lipids to proteins occurs early in lipid oxidation and this provokes an antioxidant effect on lipids.

If hydroperoxides with (*Z*,*E*)- and (*E*,*E*)-hydroperoxy-dienes ((*Z*,*E*)- and (*E*,*E*)-CD-OOH) are monitored separately from their respective signals “d” and “c”(see Table S1), it can be observed in Figure 1B that, in agreement with findings for total hydroperoxides, the addition of lysine delays the concentration increase of both types of CD-OOH. However, it does not affect either their respective evolutions with time, maximum concentrations or relative proportions when compared to the reference oil.

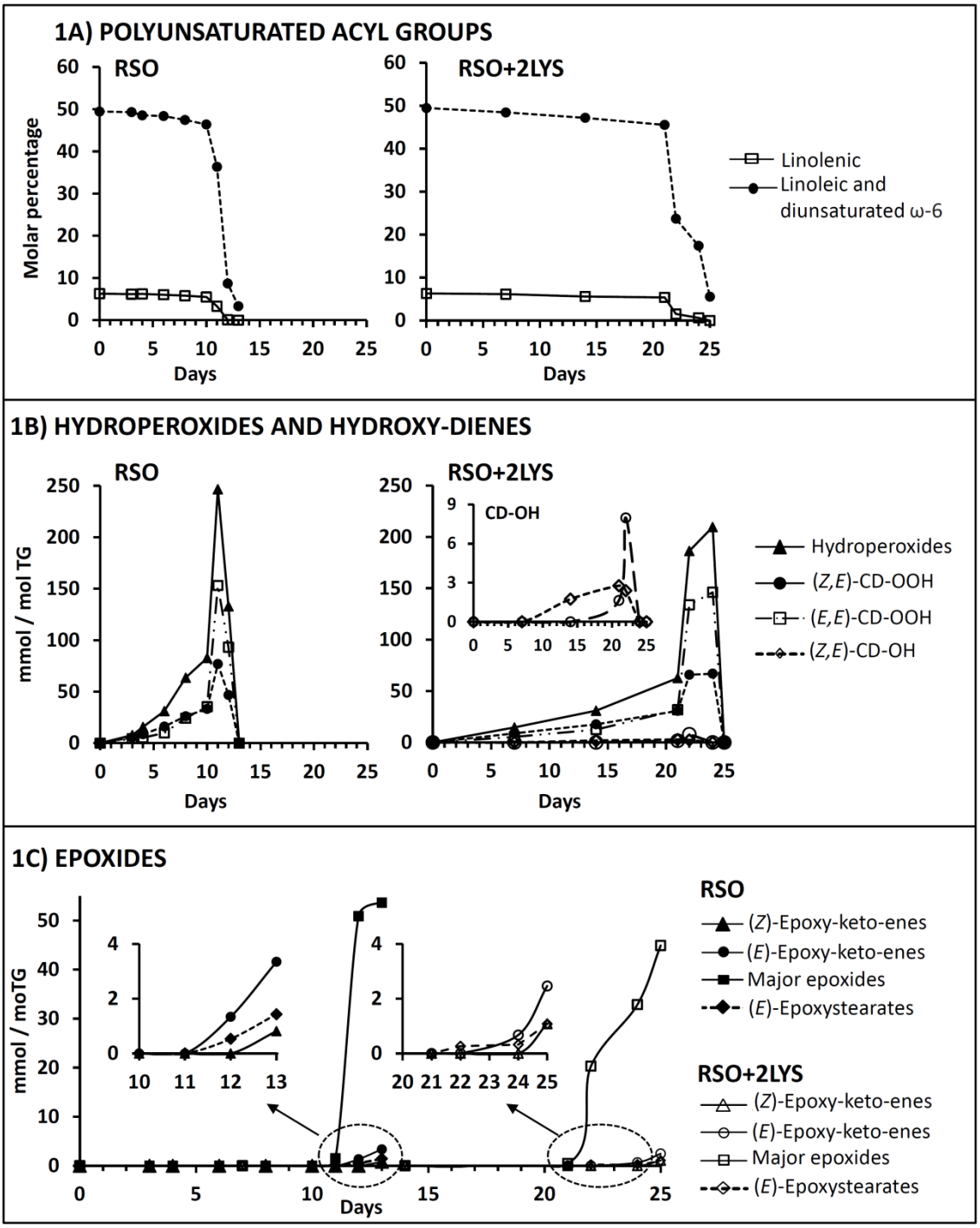


Figure 1. Evolution throughout the oxidation process, in the reference oil (RSO) and in the sample with lysine added (RSO+2LYS), of: A) the molar percentages of linolenic and linoleic acyl groups; B) the concentrations, in mmol/mol TG, of hydroperoxides and their associated conjugated (*Z*,*E*)- and (*E*,*E*)-dienes ((*Z*,*E*)- and (*E*,*E*)-CD-OOH), and of conjugated (*Z*,*E*)- and (*E*,*E*)-hydroxy-dienes ((*Z*,*E*)- and (*E*,*E*)-CD-OH); and C) the concentrations, in mmol/mol TG, of epoxides. All the figures reported are mean values.

3.1.3. Effect on epoxide formation

The addition of 2% of lysine to RSO delays the appearance of the different types of epoxides monitored to a remarkable extent. This can be observed in Figure 1C, which displays the respective evolutions of the concentrations of: i) the so-called major epoxides, which include various classes of epoxides giving signals at 2.9 and 3.1 ppm approximately (see Table S1); ii) (*Z*)- and (*E*)-epoxy-keto-enes, presumably derived from polyunsaturated groups (see signals "i", "j1" and "j2" in Table S1), and; iii) (*E*)-epoxystearates, generated from oleic groups (see signal "f" in Table S1). Moreover, the concentration increase of major epoxides over time is also somewhat slower in RSO+2LYS sample, in such a way that the maximum reached in this system (44.92 mmol/mol TG) is slightly lower than in the original oil (53.66 mmol/mol TG).

3.1.4. Effect on aldehyde formation and on evolution of their concentration

The appearance and evolution of aldehyde signals in the ^{1}H NMR oil spectra is shown in Figure 2A and the evolution of their concentrations, expressed in mmol/mol TG, in the graphs displayed in Figure 2B.

The addition of lysine to soybean oil affects aldehyde evolution somewhat differently than the rest of oxidation products above mentioned. Although, as can be observed in Figure 2A, the addition of lysine to RSO notably delays the time at which the first aldehydic signals are spotted in the ^{1}H NMR spectra (from day 8 in RSO to day 21 in RSO+2LYS), it also causes a pronounced reduction in the concentration of the different types of oxygenated α,β-unsaturated aldehydes (letters "m", "n" and "o" in Figure 2A). A diminution in the level of (*E*)-2-alkenals (see Figure 2B) is also observed in the oil containing lysine, although not so striking as in the case of the aldehydes previously mentioned. Therefore, given that the ability of lysine to react with aldehydes is well-known (Uchida, 2003), the marked decrease observed in the concentration of aldehydes, especially in the case of the most reactive ones like 4-hydroperoxy- and 4-hydroxy-(*E*)-2-alkenals (Guillén & Goicoechea, 2008), could be due to their reaction with lysine.

It is worth noticing that, unlike unsaturated aldehydes, the concentration of n-alkanals does not decline but rather slightly increases in the sample containing lysine in comparison with the reference oil (see Figure 2B). In this sense, saturated aldehydes could proceed from pyrrolization reactions of lysine with epoxy-alkenals like 4,5-epoxy-decenal (Zamora & Hidalgo, 1995). Notwithstanding, the occurrence of other

types of reactions leading to the generation of saturated aldehydes should not be discarded either.

Finally, it only remains to add, in relation to aldehyde formation and the evolution of their concentration, that in the oxidation of RSO a singlet at near 8 ppm appears in the ^{1}H NMR spectrum, simultaneous to the appearance of aldehyde proton signals, whose intensity increases parallel to that of aldehydes. This singlet has been tentatively attributed to the proton of the hemiacetal group formed from aldehydes (Sacchi et al., 1996). It is noteworthy that this signal does not appear in the RSO+2LYS spectra, maybe because the presence of lysine could provoke competitive reactions with aldehydes that hinder the formation of hemiacetals.

3.1.5. Effect on conjugated (*Z*,*E*)-and (*E*,*E*)-hydroxy-dienes formation

In RSO+2LYS submitted to oxidative conditions, between days 14 and 22, very small signals that could be tentatively assigned to (*Z*,*E*)- and (*E*,*E*)-hydroxy-dienes ((*Z*,*E*)- and (*E*,*E*)-CD-OH, signals "b" and "e" in Table S1) appear. However, these signals are not perceived in any of the spectra of RSO. As can be observed in the enlargement of Figure 1B, the maximum concentrations of (*Z*,*E*)- and (*E*,*E*)-CD-OH are reached after 21 and 22 days under oxidative conditions, respectively. Therefore, the formation of hydroxy-dienes could be due to the ability of lysine to reduce hydroperoxides to more stable hydroxides, and this would contribute to its global antioxidant effect, since this reaction avoids to a certain extent the decomposition of hydroperoxides to give other types of reactive oxidation products.

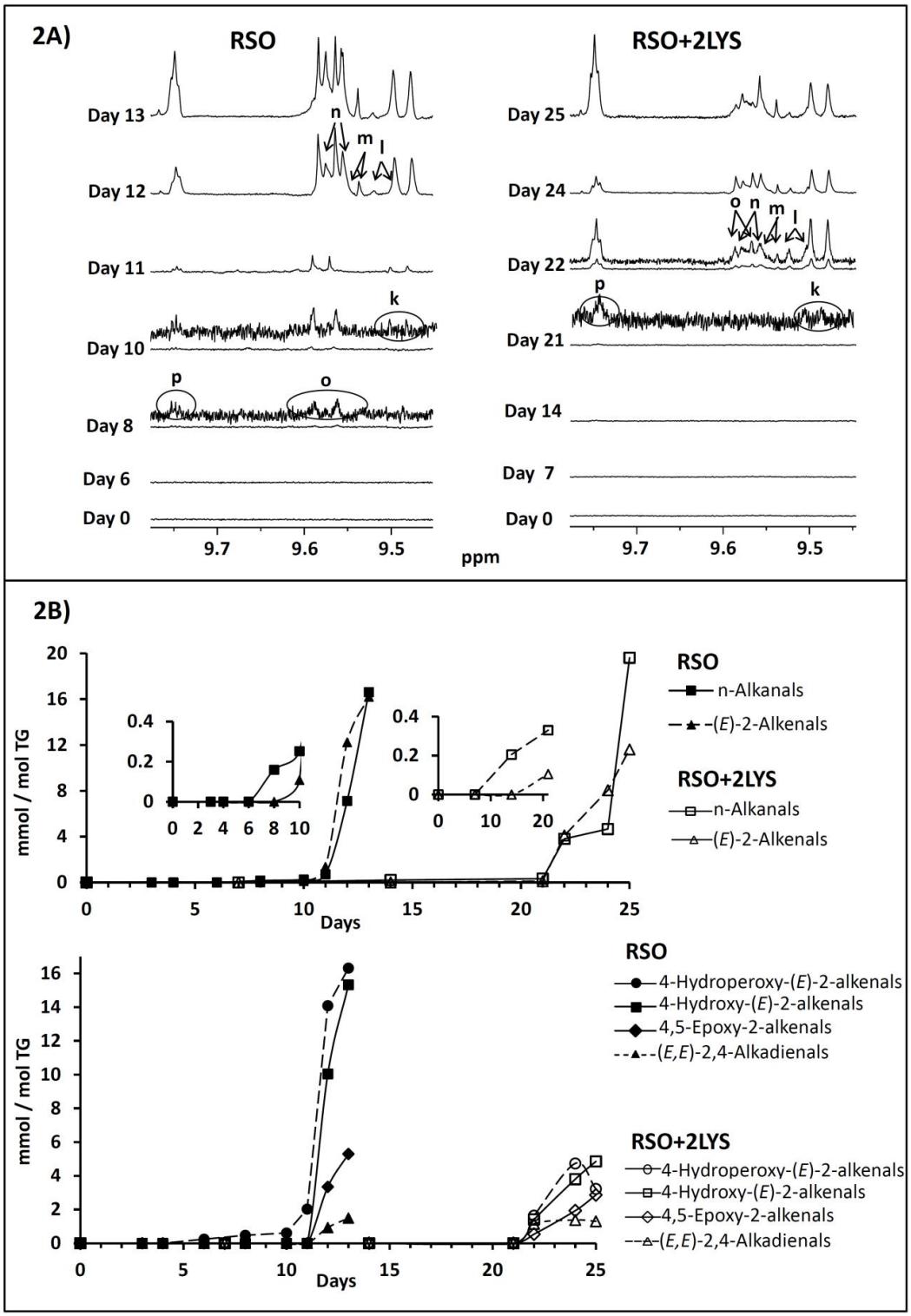


Figure 2. Evolution throughout the oxidation process, in the reference oil (RSO) and in the sample with lysine added (RSO+2LYS), of: A) the ^{1}H NMR spectral signals of aldehydes: n-alkanals (signal "p"), 4-hydroperoxy-(*E*)-2-alkenals (signal "o"), (*E*)-2-alkenals (signal "k"), 4-hydroxy-(*E*)-2-alkenals (signal "n"), 4,5-epoxy-2-alkenals (signal "m") and (*E,E*)-2,4-alkadienals (signal "l"); and B) their respective concentrations, in mmol/mol TG, given as mean values. Letters in Figure A) agree with those in Table S1, and all the plots have been drawn at a fixed value of absolute intensity to be valid for comparative purposes. 4-OOH: 4-hydroperoxy.

3.2. Analysis of the changes that lysine might undergo or the reactions in which it could be involved during the oxidation process of RSO+2LYS sample

The evolution of lysine in RSO+2LYS sample throughout the oxidation process, as above mentioned, has been tackled through the study by LC/MS and ^{1}H NMR of the aqueous extracts of RSO+2LYS sample obtained throughout the oxidation process.

3.2.1. Decrease in lysine concentration

This was monitored by LC/MS through the measurement of the abundance of its mass spectrum base peak in the several aqueous extracts until day 22, since beyond this sampling time the state of the sample did not allow one to obtain extracts under comparable conditions (see section 2.2.2.2). As Figure 3A shows, the abundance of lysine decreases only slightly during most of the oil oxidation process until day 22, when it exhibits a sharp decrease. This coincides with the moment when oil degradation becomes very fast, in such a way that hydroperoxides are close to their maximum concentration and the levels of all the secondary oxidation products monitored begin to increase (see Figures 1 and 2).

3.2.2. Metal chelation reactions

Lysine has been shown to exhibit ability to bind metals (Sadler, Tucker & Viles, 1994; Xu et al., 2018). Regarding this issue, changes in the ^{1}H NMR spectrum of lysine might occur due to its hypothetical interaction with metals present in the system, since according to some authors (Sadler et al., 1994; Selvakannan, Mandal, Phadtare, Pasricha & Sastry, 2003) this can provoke the broadening of some ^{1}H NMR lysine signals. However, as can be observed from Figure 4A, which shows the ^{1}H NMR spectrum of the aqueous extract of RSO+2LYS sample before heating (day 0) and its evolution throughout the oxidation process, this is not observed in our work. Hence, a metal-binding effect of lysine cannot be inferred from this study.

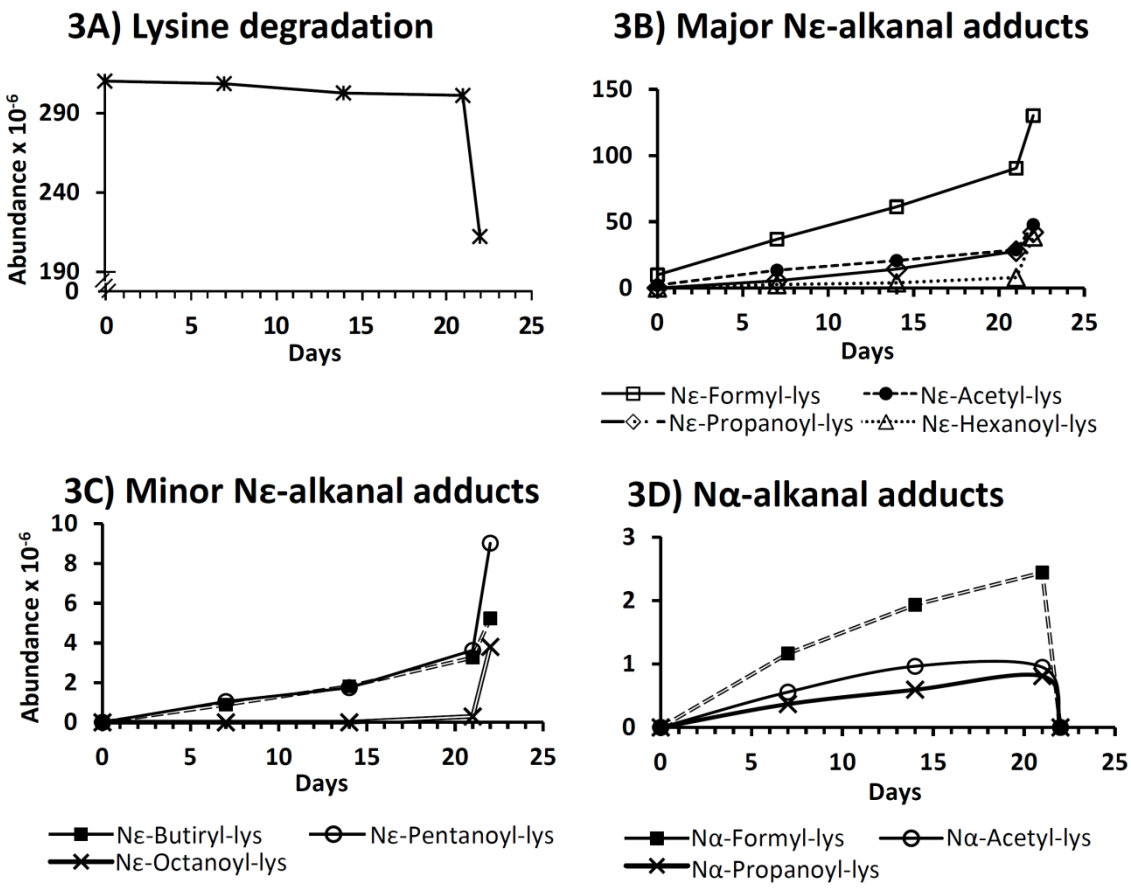


Figure 3. Evolution of the abundances of the base peaks of lysine and of each of the lysine adducts with n-alkanals in the aqueous extracts of the RSO+2LYS oil sample obtained throughout the oxidation process, determined by LC/MS. All the figures reported are mean values.

3.2.3. Oxidation reactions yielding α-aminoadipic semialdehyde

In the presence of reactive oxygen species and transition metals such as iron and copper, an oxidative deamination of lysine can occur, leading to the formation of α-aminoadipic semialdehyde (AAS) (Akagawa et al., 2006). Although this compound has been regarded as one of the most abundant carbonyl products of metal-catalyzed oxidation of proteins in food and biological systems (Requena, Chao, Levine & Stadtman, 2001), it has not been detected either by LC/MS or by ^{1}H NMR in this study. This could be due, among other factors, to the oxidative conditions used in our investigation.

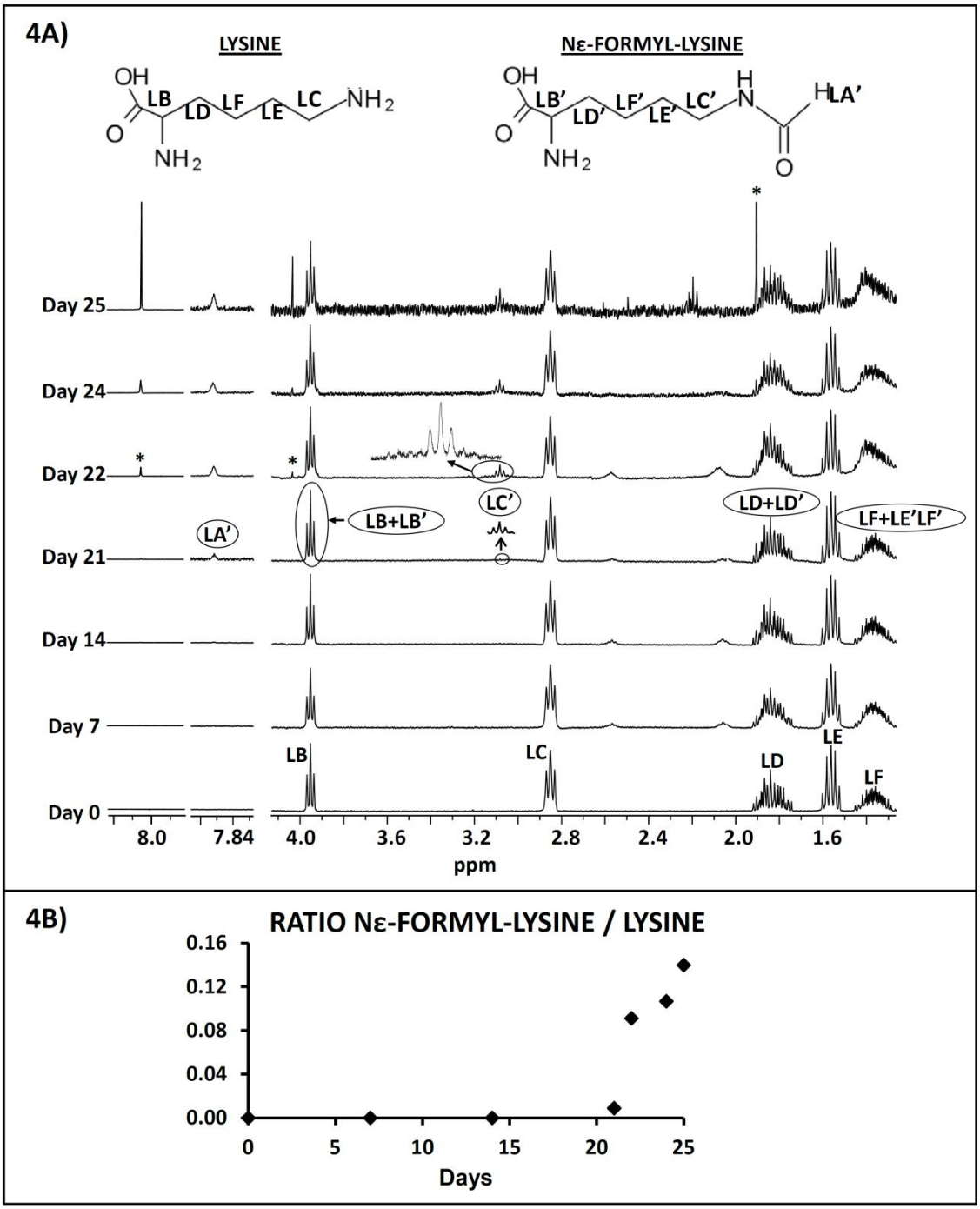


Figure 4. A) ^{1}H NMR spectrum of the aqueous extract of RSO+2LYS sample before being heated (day 0) and its evolution throughout the oxidation process. Letters agree with those in Table S2. All the plots have been drawn at a fixed value of absolute intensity to be valid for comparative purposes. B) Evolution of the ratio between the molar concentrations of formyl-lysine and lysine, determined by ^{1}H NMR, in the aqueous extracts of the RSO+2LYS sample obtained throughout the oxidation process. All the figures reported are mean values.

3.2.4. Reactions with lipid oxidation products

In addition to other oxidative changes, the simultaneous oxidation of lipids and amino acids can induce the reaction of the latter with lipid oxidation products. Among these the available scientific literature mainly concerns on reactions with aldehydes of varying natures and, to a much lesser extent, with hydroperoxides, whereas only very few studies can be found concerning other types of oxidation products, as will be commented below.

3.2.4.1. Reaction with hydroperoxides

Although there are several studies dealing with the reaction of hydroperoxides with proteins (Hidalgo & Kinsella, 1989; Wu, Hou, Zhang, Kong & Hua, 2009), to the best of our knowledge, little is known about the structure of the products that can be generated as a consequence of such reactions. In this regard, the ability of lysine to react with linoleic group hydroperoxides, giving rise to an amide-type adduct designated as Nε-hexanoyl-lysine, has been suggested by some researchers (Kato et al., 1999). This compound, was detected by LC/MS in the aqueous extract of the RSO+2LYS sample after 7 days under oxidative conditions, and its abundance exhibits an increase with time (see Figure 3B), especially at the end of the monitoring period. However, as far as we know, the exact mechanism leading to the generation of this compound from hydroperoxides has not been described.

Despite the lack of information related to compounds resulting from the reaction of hydroperoxides and lysine, it has been reported that this type of oxidation products can mediate covalent modifications of lysine by saturated aldehydes, giving rise to N-alkanoyl (amide type) lysine adducts while hydroperoxides are reduced to alkyl-hydroxides (Ishino et al., 2008). Indeed, hydroxy-dienes have been detected throughout the oxidation process of the sample containing lysine (see section 3.1.6) though not in the oxidation of RSO.

3.2.4.2. Reaction with epoxides

Even when epoxides are considered very reactive and toxic compounds (Schaich, 2008), little is known about their reactions with proteins or amino acids. In this regard, Pokorny, Klein and Koren (1966) observed the binding of 9,10-epoxystearic acid to albumin, but the structure of the compound generated was not identified. However, in later studies the formation of aminols derived from this type of reactions was reported

by Lederer (1996). With respect to lysine, its reaction with long chain epoxy-keto-ene fatty acids exhibiting a 4,5-epoxy-1-keto-2-pentene system to give long chain pyrrole fatty esters has also been observed (Hidalgo & Zamora, 1995).

Although neither the just mentioned types of pyrrole derivatives nor aminols have been identified by any of the analytical techniques used in this work, as Figure 1C shows, a slightly lower level of epoxides was noticed at the end of the oxidation process in RSO+2LYS sample than in RSO. Therefore, while this could be in part attributable to the later detection of epoxides in the oil enriched with lysine and to their slower increase with time before total oil polymerization is reached, the possible reaction of epoxides with lysine should not be discarded.

3.2.4.3. Reaction with saturated aldehydes

Aldehydes are by far the most extensively studied of all the different types of lipid oxidation compounds in terms of their reactivity towards proteins, peptides and amino acids, and indeed the ability of lysine to take part in such reactions, has already been described (Ishino et al., 2008; Schaich, 2008; Uchida, 2003).

Some lysine adducts with saturated aldehydes were identified by means of LC/MS in the aqueous extracts of RSO+2LYS sample from day 7 onwards. This finding would support the involvement of hydroperoxides in the generation of this type of adducts, mentioned in section 3.2.4.1, and their role in the generation of hydroxy-dienes (see section 3.1.6). Actually, the maximum concentrations of both hydroxy-dienes and lysine-alkanal adducts are observed at the same time (see Figures 1B and 3).

The identification of this type of adducts, all of them of the amide type, was carried out, as described in the supplementary material, by comparison of their mass spectra with those of the n-alkanals adducts synthesized in the laboratory. Their respective retention times, base peaks and rupture ions are shown in Table S3, together with the corresponding mass spectra (see supplementary material). It can be observed that, although both adducts at the Nε and Nα positions were detected, the former were more numerous than the latter, what evidences the higher reactivity of the Nε position.

Regarding the Nε-adducts, the mass spectra obtained with cone potential 35 V (see Table S3) exhibit three rupture fragments characteristic of lysine: 84 (loss of the $-NH_2$ group and of the -COOH group at the α-position), 130 (loss of $-NH_2$ at ε-position) and 147 (molecular weight of lysine+1) (Shibata et al., 2011). In addition, another

distinctive fragment is also observed (underlined in Table S3), which coincides with the molecular weight of the corresponding adduct subtracting the amino and the carboxylic groups supported on the α-carbon; this is not observed in the Nα-adducts, where the only fragment found in the spectra obtained with cone potential 35 V, apart from that of the base peak, is that with m/z 84.

Evolutions of the abundances of the mass spectra base peaks of each of the above mentioned derivatives during RSO+2LYS sample oxidation are shown in Figure 3 (Figures 3B, 3C and 3D). As can be observed from these graphs, the abundances of the several lysine adducts increase with time; this growth is particularly noticeable at the end of the oxidation process (between days 21 and 22), when lysine concentration shows a steep decline (see Figure 3A), and it takes place alongside generation of most aldehydes in RSO+2LYS sample (see Figure 2A). Among the former, the one due to the reaction of lysine with formaldehyde (*Nε-formyl-lysin*e) exhibits the highest abundance (see Figure 3B), followed by lysine adducts with acetaldehyde (*Nε-acetyl-lysine*), propanal (*Nε-propanoyl-lysine*) and hexanal (*Nε-hexanoyl-lysine*). These findings reveal either that the adducts with the lowest molecular weight aldehydes are the most profusely generated, in agreement with Benedetti and Comporti (1987), or that these are more water-soluble, and so better extracted, than those having a higher number of carbon atoms; notwithstanding, both factors might even simultaneously influence the results obtained.

Regarding Nε-hexanoyl-lysine, it is worth noticing that, as commented in section 3.2.4.1, this compound might also derive from the direct reaction of lysine with hydroperoxides (Kato et al., 1999). However, considering that the abundance of this adduct only increases considerably at the end of the monitoring period (see Figure 3B), it seems more plausible to think of hexanal as the compound mainly involved in the generation of this type of adducts (Ishino et al., 2008).

Furthermore, Nε-lysine adducts with butanal, pentanal and octanal were also detected, though in lower abundances than those of the previously mentioned compounds (see Figure 3C). It is worth noticing that of the volatile aldehydes generated throughout the oxidation process of soybean oil (data not shown) nonanal is in higher abundance than octanal, though adducts with the former have not been detected; this reinforces all the aforementioned regarding the influence of both aldehyde reactivity and water solubility of the adducts on the results obtained.

Finally, as mentioned above, Nα-adducts were also detected from day 7 to day 21, but in very small abundances (see Figure 3D).

All the previously mentioned adducts except that with octanal (see Figure 3C) were detected from day 7 onwards although saturated aldehydes were not noticed by ^{1}H NMR in RSO+2LYS sample until day 21 (see Figure 2A). In this sense, it must be taken into account that, despite not being detected by ^{1}H NMR, aldehydes in small concentrations can be present in RSO+2LYS sample, either coming from oxidation reactions in the earliest stage of hydroperoxide decomposition or as components of the starting soybean oil.

As described before, the aqueous extracts of RSO+2LYS sample were also studied by ^{1}H NMR and the corresponding spectra are shown in Figure 4A, where the presence of various signals can be observed, most of them due to lysine (see Table S2). It is noteworthy that while LC/MS analysis of the aqueous extracts revealed the presence of various lysine adducts with n-alkanals (see Figures 3B-3D), most of them seem to be in too low concentrations to be detected by ^{1}H NMR, which is a less sensitive technique. Thus, according to the signal assignments displayed in Table S2, only *Nε-formyl-lysine*, the most abundant adduct detected by LC/MS, could be identified from day 21 onwards (see signals LA' and LC').

With the aim of monitoring the evolution of *Nε-formyl-lysine* relative to lysine, the ratio between the moles of *Nε-formyl-lysine* and lysine at each sampling point was calculated from the areas of signals LA' and LC, respectively (see Figure 4A and Table S2). The equations employed to make this calculation were the following:

$N_{Lys} = kA_{LC}/2$; $N_{N\varepsilon\text{-formyl-lysine}} = kA_{LA'}$,

where N is the number of moles of each compound, k the proportionality constant between the area of the ^{1}H NMR signal and the number of protons that generate it, and A_{LC} and $A_{LA'}$ the respective areas of signals LC and LA'.

The evolution of this ratio throughout the RSO+2LYS sample oxidation process, which is shown in Figure 4B, reveals that, as the process advances, the proportion of *Nε-formyl-lysine* relative to lysine increases, especially between days 22 and 25, the time when oxidation proceeds at the fastest rate (see Figures 1 and 2). Precisely, day 22 is when all the Nε-alkanal adducts including Nε-formyl-lysine exhibit the greatest abundance increase (see Figures 3B and 3C).

If the results obtained from the analysis of the aqueous extracts of RSO+2LYS sample are compared with those coming from the oil phase, it appears striking that, despite the generation of several adducts between lysine and n-alkanals, the final concentration of this type of aldehydes in RSO+2LYS sample is not lower than in the non-enriched oil (see Figure 2B). In this sense, considering that the adducts generated between lysine and n-alkanals of low molecular weight like formaldehyde, acetaldehyde or propanal are the most abundant, it could be thought that lysine mainly forms adducts with very volatile aldehydes which may tend to escape from the liquid oil matrix in sample RSO. Therefore, it might be thought that this type of aldehydes would make a minor contribution to the total of n-alkanals detected in RSO. However, as mentioned in section 3.1.5, other factors might also be involved in the results observed

3.2.4.4. Reaction with more reactive aldehydes

Unlike saturated, unsaturated aldehydes and dialdehydes have more reactive sites in their molecules, so a greater variety of reactions can take place between lysine and this type of aldehydes. These include the formation of Schiff bases and Michael additions among others (Schaich, 2008). Depending on the extent of the reactions, even more complex products can be generated, like different types of polymers (Zamora, Alaiz & Hidalgo, 2000).

In this regard, LC/MS analysis of the aqueous extracts revealed the presence of new compounds, dissimilar to the aforementioned n-alkanal adducts, above all in the latter stages of oxidation (mainly days 21 and 22). Their appearance coincides with the time when the concentration of aldehydes is noticeable in the ^{1}H NMR spectra of RSO+2LYS sample, and with a marked lower concentration of α,β-unsaturated aldehydes in comparison with the reference RSO oil (see Figure S1). Some of these compounds have been tentatively identified by taking into account bibliographic data concerning the molecular weight and mass spectrum of lysine adducts with different types of unsaturated aldehydes, as indicated in the supplementary material. The potential aldehyde involved in each of the adducts, their respective detection day, structures, mass spectra base peaks and rupture ions, together with the bibliographic references used for their tentative identification, are shown in Table 1; their full mass spectra are also given in the supplementary material. According to the data provided by the TICs obtained with cone potential 20 V, compounds included in Table 1 exhibit as their base peak an ion with a mass matching the molecular weight of certain lysine

adducts described in bibliography plus 1, and in some cases one or more fragments characteristic of lysine (147 and/or 130). In addition, the spectra obtained with cone potential 35 V show further ions that were useful in tentatively identifying some of the adducts. In summary, 7 adducts were identified: 2 coming from malondialdehyde (MDA), 2 from (*E*)-2-alkenals and 3 from oxygenated α,β-unsaturated aldehydes. Among these kinds of aldehydes, (*E*)-2-alkenals and the oxygenated α,β-unsaturated type are detected in considerable concentrations during the oxidation process of RSO oil, but their concentrations are markedly reduced in the presence of lysine, this being especially noticeable in the latter case (see Figures 2B and S1).

Adducts with MDA. When it comes to MDA, a very reactive bifunctional aldehyde (Uchida, 2003), although this compound is not specifically detected under the conditions of this study, it is generated in the peroxidation of polyunsaturated acyl groups or fatty acids. However, detailed studies have evidenced that it is mainly generated from polyunsaturated groups with three or more double bonds, while the linoleic ones, which are the most abundant in soybean oil, are considered weak precursors for MDA (Esterbauer, Schaur & Zollner, 1991). Thus, although MDA would not be expected to be generated in very large amounts from soybean oil due to its relatively low level of triunsaturated acyl groups, it is a very reactive molecule, and in fact this is evidenced in this work. With regard to the MDA adducts here found, the one with base peak m/z 201 (see Table 1) might correspond to the Schiff base-type *Nε-(2-propenal)lysine*, while the compound with m/z 329 has been tentatively identified as an *N,N'-disubstituted 1-amino-3-iminopropene lysine derivative*, resulting from the reaction of MDA with two lysine molecules (Uchida, 2003). Regarding *Nε-(2-propenal)lysine*, it must be noticed that Shimozu, Hirano, Shibata, Shibata and Uchida (2011) also identified this compound as one of the reaction products of lysine with 4-hydroperoxy-(*E*)-2-nonenal (4-HPNE). According to these authors, it is possible that HPNE is first converted to MDA in the presence of lysine, which would then react with lysine.

Adducts with (E)-2-alkenals. Regarding the two adducts between lysine and (*E*)-2-alkenals (see Table 1), the one with base peak 259 would match with a *Michael adduct of lysine with (E)-2-heptenal*. Michael addition is in fact considered by some authors (Meynier, Rampon, Dalgalarrondo & Genot, 2004) to be the most likely pathway for the formation of adducts between lysine and (*E*)-2-alkenals. Lysine adducts with (*E*)-2-

heptenal were also found by Globisch, Schindler, Kreßler and Henle (2014) during the incubation at 37 ºC of lysine with peanut protein; however, the adducts observed by these authors were mainly due to the addition of two molecules of (*E*)-2-heptenal to lysine, which differ from that tentatively reported here. This suggests that the conditions used in the above mentioned study, concerning for example aldehyde proportion relative to amino acid, might have led to different adducts.

As for the compound with base peak 241, only detected at the end of the oxidation process (day 22) and in low abundance, this has been tentatively identified as *Nε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine)*, which is a product derived from the reaction of lysine with two molecules of acrolein (2-propenal) (Uchida, 2003). Although the molecular weight of this compound also matches that of a Schiff base type adduct of lysine with (*E*)-2-heptenal, this reaction can be reversed under acid conditions and indeed these exist in the extracts analyzed.

Adducts with oxygenated α,β-unsaturated aldehydes. With respect to the three adducts presumably coming from the reaction of lysine with oxygenated α,β-unsaturated aldehydes (see Table 1), the ones with base peaks 319 and 301 coincide with the only two specific adducts between lysine and 4-HPNE reported to date (Shimozu et al., 2011). The first, *Nε-4-hydroxynonanoic acid-lysine (HNA-lysine)*, has been suggested as resulting from an initial Michael adduct that undergoes an intramolecular oxidation of the aldehyde group to the carboxyl group catalyzed by the hydroperoxide; the second, *Nε-4-hydroxy-(2Z)-nonenoyl-lysine*, is an amide-type adduct, similar to those formed with n-alkanals. With regard to the adduct with base peak 303, this could arise from 4-hydroxy-(*E*)-2-nonenal (4-HNE) as a result of a Michael addition and further stabilization by cyclization to an hemiacetal. Globisch, Kaden and Henle (2015) also analysed the occurrence of lysine adducts with 4-HNE in protein from toasted peanuts after a previous hydrolysis process, but in that case the compound detected was a 2-pentylpyrrol derivative.

Table 1. Lysine adducts with aldehydes different from n-alkanals tentatively identified by LC/MS in the aqueous extracts of RSO+LYS sample obtained throughout the oxidation process, together with their detection day, structure, mass spectra base peaks (BP) and other fragments of their mass spectra. LYS: lysine. The underlined ions are characteristic of each adduct.

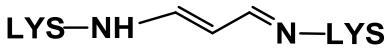
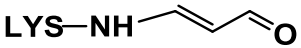
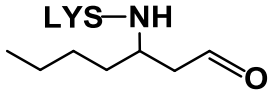
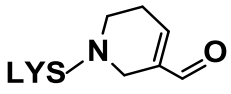
				Cone 20 V		Cone 35 V		
Reaction with	**Detection day**	**Compound**	**Structure**	**BP**	**Other fragments**	**BP**	**Other fragments**	**References**
MDA								
	14	N,N'-disubstituted 1-amino-3-iminopropene LYS derivative		**329**	147	**329**	84	Uchida, 2003
	21	Nε-(2-Propenal)-LYS		**201**		**84**	130, 201, <u>138</u>	Uchida, 2003
(*E*)-2-Alkenals								
Heptenal	21	(*E*)-2-Heptenal-LYS Michael adduct		**259**		**259**	130, <u>196</u>	Meynier et al., 2004
Acrolein								
	22	FDP-LYS		**241**	130	**241**	<u>195</u>, 84, 96, 130	Uchida, 2003

Table 1. Continuation

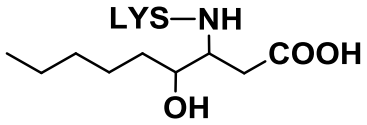
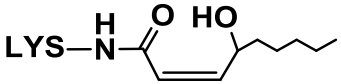
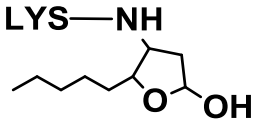
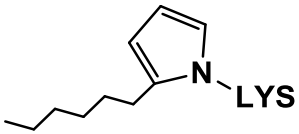
Oxygenated α,β-unsaturated aldehydes								
4-HPNE	14	Nε-4-Hydroxynonanoic acid-LYS (HNA-LYS)		**319**		**319**	130	Shimozu et al, 2011
4-HPNE	21	Nε-4-Hydroxy-(2*Z*)-nonenoyl-LYS		**301**	147, 130	**147**	84, 130, 301	Shimozu et al, 2011
4-HNE	21	4-HNE-LYS Michael adduct stabilized as hemiacetal		**303**	147,130	**303**	84, 130	Uchida, 2003
Adduct with an unidentified aldehyde								
	22	Nε-2-Hexyl-pyrrole-LYS		**281**		**197**	281, 84, 236, 134	Miyashita et al., 2014

Figure 5, which shows the evolution of the abundances of the mass spectra base peaks of each of the aforementioned adducts during the oxidation process, reveal that those detected in the first place were the *N,N'-disubstituted 1-amino-3-iminopropene lysine derivative*, coming from MDA and the most abundant throughout the oxidation process, and *HNA-lysine*, generated from 4-HPNE. As was the case of adducts of lysine with n-alkanals (see section 3.2.4.3), most of the adducts with α,β-unsaturated aldehydes were detected by LC/MS before these aldehydes were noticed in the ^{1}H NMR spectra of RSO+2LYS sample (see Figure 2A). It is also worth noting that although, according to that commented above, MDA would not be the most likely aldehyde to be generated in the greatest quantity in RSO+2LYS oil oxidation, perhaps as a result of its high reactivity the *1-amino-3-iminopropene lysine derivative* is that exhibiting the most increased abundance.

When it comes to the lysine adducts with (*E*)-2-alkenals and oxygenated α,β-unsaturated aldehydes, it is worth noticing that, although these two groups of aldehydes are among the most abundantly detected by ^{1}H NMR during oxidation of RSO oil (see Figure 2B), Figure 5 shows that the adducts with oxygenated α,β-unsaturated aldehydes are more numerous, and in general more abundant, than those with (*E*)-2-alkenals, especially those coming from 4-HPNE; these outcomes seem to reflect the greater reactivity of the oxygenated α,β-unsaturated aldehydes and, in fact, a great decrease in their concentration is noticed in the sample containing lysine in comparison with the reference oil.

Another remarkable feature is the great decrease in oxygenated α,β-unsaturated aldehyde concentration observed by ^{1}H NMR (see Figure 2) when compared to the abundance of lysine adducts with this type of aldehydes determined by LC/MS (see Figure 5). In this regard, it should be noticed that the adducts detected by this latter technique are only those generated between lysine and volatile aldehydes, but lysine adducts with aldehydes supported on acyl groups would not be taken into account.

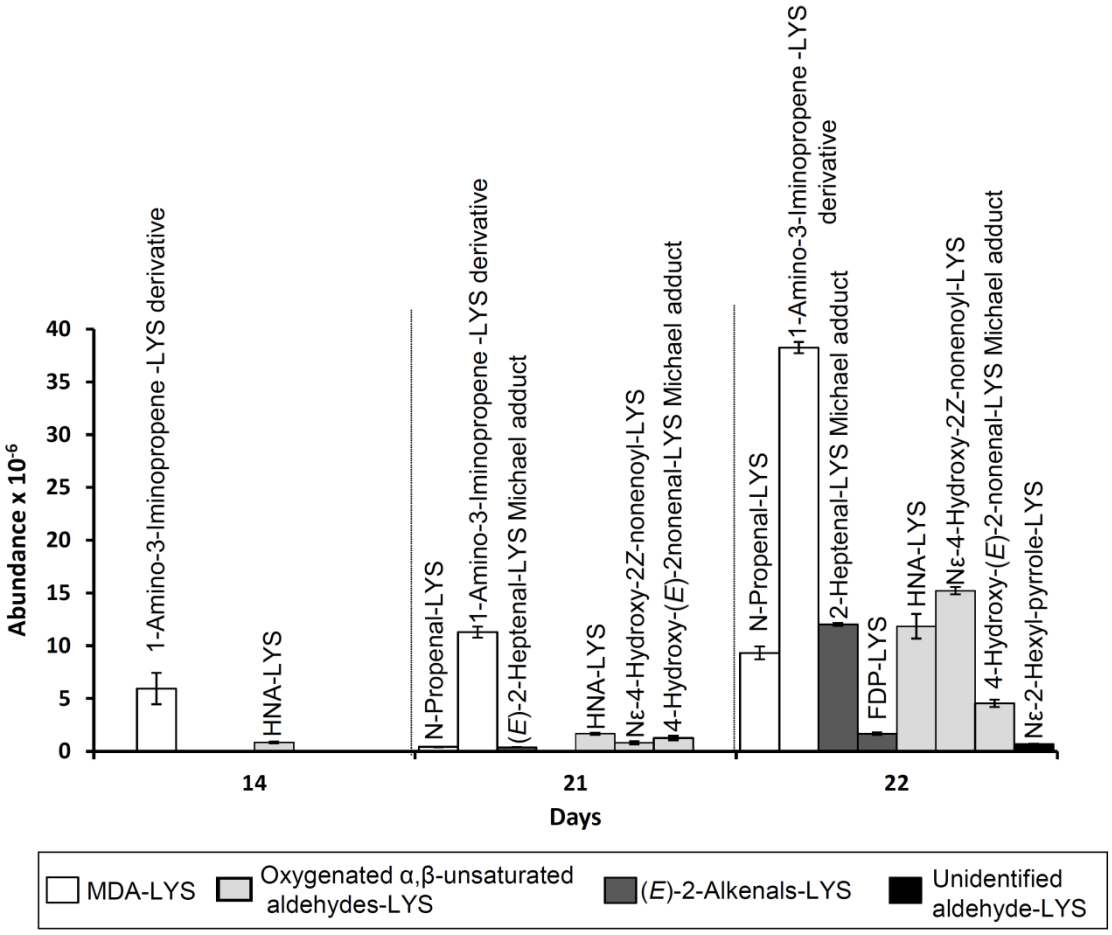


Figure 5. Evolution of the abundance of the base peaks of the different adducts between lysine and other aldehydes different from n-alkanals in the aqueous extracts of the RSO+2LYS sample obtained throughout the oxidation process, determined by LC/MS. All the figures reported are mean values.

3.2.4.5. Pyrrolization reactions

Some oxidation products can lead to the generation of compounds with a pyrrolic structure when reacting with proteins and amino acids. This is the case of some oxygenated α,β-unsaturated aldehydes such as 4,5-epoxy-2-alkenals and 4-hydroxy-(*E*)-2-alkenals (Globisch et al., 2015; Zamora et al., 2000), and also of long chain epoxy-keto-enes with a 4,5-epoxy-1-oxo-2-pentene system, as commented in section 3.2.4.2. Depending on the mechanism involved in the formation of this type of compounds, this process can be accompanied by the concomitant generation of saturated aldehydes (Hidalgo & Zamora, 1995). Among the compounds detected in the LC/MS

chromatograms of the aqueous extracts obtained from RSO+2LYS sample throughout oxidation, a compound with a pyrrolic structure has also been tentatively identified in the light of its mass spectrum, considering data from Miyashita and coworkers (2014): *Nε-2-hexyl-pyrrole* (see Table 1 and Figure 5). As far as we know, this compound has not been previously described either in food or in biological systems, and its precursor remains unknown. Actually, although it has been considered to derive from the reaction of lysine with an aldehyde, another type of oxidation product should not be discarded. The spectrum and the structure of this compound is displayed in the supplementary material.

3.2.4.6. Polymerization reactions

In addition to all the issues above commented, it is remarkable that, although not measured, a change in colour from yellow to dark orange took place during the oxidation process of RSO+2LYS sample. Part of these coloured compounds remained in the lipid phase and another part passed to the acid aqueous extract. Therefore, considering that the reaction between lipid oxidation compounds and amino acids can lead to the formation of coloured polymers (Zamora & Hidalgo, 2005), polymerization reactions might have taken place despite the resulting products not being detected under the conditions of this study.

This hypothetical formation of coloured polymers could help to explain some of the observations made in this work such as: i) the lower degradation extent of linoleic acyl groups in presence of lysine in comparison with the reference oil when total oil polymerization is reached (see Figure 1A), since polymers in which lysine is involved might be contributing to oil polymerization, so the oil would totally polymerize with a lower linoleic degradation extent; ii) the apparently low abundance of lysine adducts with unsaturated aldehydes found by LC/MS in contrast to the high reduction of the concentration of this kind of aldehydes in the oil, since especially α,β-unsaturated aldehydes can be involved in polycondensation reactions leading to colour development to a greater extent than the saturated ones (Burton, McWeeny & Biltcliffe, 1963); and iii) the apparently low decrease in lysine abundance throughout most of the oil oxidation process (see Figure 3A), since lysine might eventually cleave from the polymer, thus restoring the lysine content (Zamora & Hidalgo, 2005).

3.3. Analysis of the potential role of lysine-aldehyde adducts on the antioxidant effect observed

According to Alaiz, Zamora and Hidalgo (1996), some of the products coming from the reaction of lysine with aldehydes such as (*E*)-2-octenal or 4,5-epoxy-alkenals can exhibit antioxidant activity in soybean oil, so it could be thought that the lysine adducts identified might contribute to the lower oxidation rate of the oil containing lysine. However, it must be taken into account, that this type of studies are performed by adding the previously formed compound to the oil. Secondly, the compounds identified in this work do not match with those for which antioxidant ability is claimed, and in fact, N-alkanoyl-lysine adducts, which are practically the only ones detected during the time period when lysine shows its antioxidant effect (the first twenty days), have not been reported to exhibit antioxidant ability. Therefore, a mechanism other than just the reaction of lysine with aldehydes seems to come into play. In this sense, Alaiz and coworkers themselves (Alaiz, Hidalgo & Zamora, 1997) pointed out that proteins modified by oxidized lipids are able to exert an antioxidant action on edible oils at the same time as they are being produced, independently of the reactions occurring with oxidation products.

4. CONCLUSIONS

As far as we know, this is the first time that such a detailed study has been conducted on how the cooxidation of an edible oil and an amino acid affects both the evolution of the oil components and of the amino acid itself by combining ^{1}H NMR and LC/MS.

This study confirms the findings of a previous work, evidencing that the addition of 2% of lysine to refined soybean oil exerts a clear antioxidant effect by noticeably retarding oil acyl group degradation and the generation of primary oxidation compounds. Consequently, all the secondary oxidation products appear later in the oil with added lysine, where a remarkable reduction in the concentration of toxic oxygenated α,β-unsaturated aldehydes is also noticed.

Regarding lysine evolution, the main lysine derivatives identified by means of the techniques used were adducts of lysine and different types of aldehydes generated throughout the oil oxidation process. This does not mean that other lysine derivatives were not generated, but those described here were the only ones that could be identified in the aqueous extracts subject of study.

The most abundant lysine adducts are those with low molecular weight n-alkanals and MDA, followed by the ones with α,β-unsaturated aldehydes like 4-HPNE and (*E*)-2-alkenals, and to a lesser degree, 4-HNE; this latter group of aldehydes, especially the oxygenated ones, are those exhibiting the greatest concentration decrease in the oil with lysine added. To the best of our knowledge, this is the first time that several lysine adducts with aldehydes of varying nature, including the recently identified specific HPNE adducts, have simultaneously been detected in a complex food model system.

Although the ability of lysine to trap very reactive aldehydes could constitute a detoxification mechanism in food systems, it should not be forgotten that this reaction implies a reduction in the biological availability of this essential amino acid.

As it was hypothesized in the Introduction section, a relationship between the evolution of lysine and that of the oil has been observed in this work. However, the formation of adducts between lysine and aldehydes, which is the most outstanding finding, does not seem sufficient to explain the antioxidant effect observed, so further studies would be needed to unravel the specific action mechanism of this amino acid.

Finally, the outcomes of this work may serve to highlight the need to perform the studies about the cooxidation of lipids and proteins in food systems under conditions as similar as possible to those existing during food processing and storage, since this is crucial to select appropriate markers of the reaction between amino acids and lipid oxidation products.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ahmad, M. M., Al-Hakim, S., Adel, A., & Shehata, Y. (1983). The antioxidant activity of amino acids in two vegetable oils. *Journal of the American Oil Chemists' Society, 60*, 837-840. https://doi.org/10.1007/BF02787440

Akagawa, M., Sasaki, D., Ishii, Y., Kurota, Y., Yotsu-Yamashita, M., Uchida, K., & Suyama, K. (2006). New method for the quantitative determination of major protein carbonyls, α-aminoadipic and γ-glutamic semialdehydes: investigation of the formation mechanism and chemical nature *in vitro* and *in vivo*. *Chemical Research in Toxicology, 19*, 1059-1065. https://doi.org/10.1021/tx060026p

Alaiz, M., Hidalgo, F. J., & Zamora, R. (1997). Comparative antioxidant activity of Maillard- and oxidized lipid-damaged bovine serum albumin. *Journal of Agricultural and Food Chemistry, 45*, 3250-3254. https://doi.org/10.1021/jf970217l

Alaiz, M., Zamora, R., & Hidalgo, F. J. (1996). Antioxidative activity of pyrrole, imidazole, dihydropyridine, and pyridinium salt derivatives produced in oxidized lipid/amino acid browning reactions. *Journal of Agricultural and Food Chemistry, 44*, 686-691. https://doi.org/10.1021/jf950606k

Benedetti, A., & Comporti, M. (1987). Formation, reactions and toxicity of aldehydes produced in the course of lipid peroxidation in cellular membranes. *Bioelectrochemistry and Bioenergetics, 18*, 187-202. https://doi.org/10.1016/0302-4598(87)85021-3

Burton, H. S., McWeeny, D. J., & Biltcliffe, D. O. (1963). Non-enzymic browning: The role of unsaturated carbonyl compounds as intermediates and of SO_2 as an inhibitor of browning. *Journal of the Science of Food and Agriculture, 14*, 911-920. https://doi.org/10.1002/jsfa.2740141211

Elias, R. J., Bridgewater, J. D., Vachet, R. W., Waraho, T., McClements, D. J., & Decker, E. A. (2006). Antioxidant mechanisms of enzymatic hydrolysates of β-lactoglobulin in food lipid dispersions. *Journal of Agricultural and Food Chemistry, 54*, 9565-9572. https://doi.org/10.1021/jf062178w

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition,48*, 430-441. https://doi.org/10.1080/10408390701425615

Esterbauer, H., Schaur, R. J., & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine, 11*, 81-128. https://doi.org/10.1016/0891-5849(91)90192-6

Filippenko, T. A., & Gribova, N. Y. (2011). Antioxidant activity of amino acids during oxidation of sunflower oil in an emulsion. *Pharmaceutical Chemistry Journal*, *45*, 296-287. https://doi.org/10.1007/s11094-011-0619-6

Gardner, H. W. (1979). Lipid hydroperoxide reactivity with proteins and amino acids: a review. *Journal of Agricultural and Food Chemistry*, *27*, 220-229. https://doi.org/10.1021/jf60222a034

Globisch, M., Kaden, D., & Henle, T. (2015). 4-Hydroxy-2-nonenal (4-HNE) and its lipation product 2-pentylpyrrole lysine (2-PPL) in peanuts. *Journal of Agricultural and Food Chemistry, 63*, 5273-5281. https://doi.org/10.1021/jf502501f

Globisch, M., Schindler, M., Kreßler, J., & Henle, T. (2014). Studies on the reaction of *trans*-2-heptenal with peanut proteins. *Journal of Agricultural and Food Chemistry, 62*, 8500-8507. https://doi.org/10.1021/acs.jafc.5b01502

Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α,β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition, 48*, 119-136. https://doi.org/10.1080/10408390601177613

Guillén, M. D., & Uriarte, P. S. (2012). Study by ^{1}H NMR spectroscopy of the evolution of extra virgin olive oil composition submitted to frying temperature in an industrial fryer for a prolonged period of time. *Food Chemistry, 134*, 162-172. https://doi.org/10.1016/J.FOODCHEM.2012.02.083

Hidalgo, F. J., & Kinsella, J. E. (1989). Changes induced in beta-lactoglobulin B following interactions with linoleic acid 13-hydroperoxide. *Journal of Agricultural and Food Chemistry, 37*, 860-866. https://doi.org/10.1021/jf00088a006

Hidalgo, F. J., & Zamora, R. (1995). *In vitro* production of long chain pyrrole fatty esters from carbonyl-amine reactions. *Journal of Lipid Research, 36*, 725-735. http://www.ncbi.nlm.nih.gov/pubmed/7616119

Hwang, H. S., & Winkler-Moser, J. K. (2017). Antioxidant activity of amino acids in soybean oil at frying temperature: Structural effects and synergism with tocopherols. *Food Chemistry, 221*, 1168-1177. https://doi.org/10.1016/j.foodchem.2016.11.042

Ishino, K., Shibata, T., Ishii, T., Liu, Y. T., Toyokuni, S., Zhu, X., Sayre. L. M. & Uchida, K. (2008). Protein N-acylation: H_2O_2-mediated covalent modification of

protein by lipid peroxidation-derived saturated aldehydes. *Chemical Research in Toxicology, 21,* 1261-1270. https://doi.org/10.1021/tx800080x

Jónsdóttir, R., Geirsdóttir, M., Hamaguchi, P. Y., Jamnik, P., Kristinsson, H. G., & Undeland, I. (2016). The ability of *in vitro* antioxidant assays to predict the efficiency of a cod protein hydrolysate and brown seaweed extract to prevent oxidation in marine food model systems. *Journal of the Science of Food and Agriculture, 96,* 2125-2135. https://doi.org/10.1002/jsfa.7328

Kato, Y., Mori, Y., Makino, Y., Morimitsu, Y., Hiroi, S., Ishikawa, T., & Osawa, T. (1999). Formation of Nε-(hexanonyl) lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. *Journal of Biological Chemistry, 274,* 20406-20414. https//doi.org/ 10.1074/jbc.274.29.20406

Lederer, M. O. (1996). Reactivity of lysine moieties toward γ-hydroxy-α,β-unsaturated epoxides: A model study on protein-lipid oxidation product interaction. *Journal of Agricultural and Food Chemistry, 44,* 2531-2537. https://doi.org/10.1021/JF950837R

Martin-Rubio, A. S., Sopelana, P., & Guillén, M. D. (2019). The potential of lysine to extend the shelf life of soybean oil evidenced by ^{1}H Nuclear Magnetic Resonance. *LWT, 105,* 169-176. https://doi.org/10.1016/j.lwt.2019.01.070

Meynier, A., Rampon, V., Dalgalarrondo, M., & Genot, C. (2004). Hexanal and t-2-hexenal form covalent bonds with whey proteins and sodium caseinate in aqueous solution. *International Dairy Journal, 14,* 681-690. https://doi.org/10.1016/J.IDAIRYJ.2004.01.003

Miyashita, H., Chikazawa, M., Otaki, N., Hioki, Y., Shimozu, Y., Nakashima, F., Shibata, T., Hagihara, Y., Maruyama, S., Matsumi, N., & Uchida, K. (2014). Lysine pyrrolation is a naturally-occurring covalent modification involved in the production of DNA mimic proteins. *Scientific Reports, 4,* article number: 5343. https://doi.org/10.1038/srep05343

Pokorny, J., Klein, S., & Koren, J. (1966). Reaction of oxidized lipids with protein. II. Reactions of albumin with epoxy derivatives. *Nahrung, 10,* 321-325.

Requena, J. R., Chao, C. C., Levine, R. L., & Stadtman, E. R. (2001). Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed

oxidation of proteins. *Proceedings of the National Academy of Sciences, 98*, 69-74. https://doi.org/10.1073/pnas.98.1.69

Sacchi, R., Patumi, M., Fontanazza, G., Barone, P., Fiordiponti,P., Mannina, L., Rossi, E., & Segre A. L. (1996). A high-field ^{1}H Nuclear Magnetic Resonance study of the minor components in virgin olive oils. *Journal of the American Chemical Society, 73*, 747-758.

Sadler, P. J., Tucker, A., & Viles, J. H. (1994). Involvement of a lysine residue in the N-terminal Ni^{2+} and Cu^{2+} binding site of serum albumins: Comparison with Co^{2+}, Cd^{2+} and Al^{3+}. *European Journal of Biochemistry, 220*, 193-200. https://doi.org/10.1111/j.1432-1033.1994.tb18614.x

Schaich, K. M. (2008). Co-oxidation of proteins by oxidizing lipids. *Lipid Oxidation Pathways, 2*, 181-272.

Selvakannan, P. R., Mandal, S., Phadtare, S., Pasricha, R., & Sastry, M. (2003). Capping of gold nanoparticles by the amino acid lysine renders them water-dispersible. *Langmuir, 19*, 3545-3549. https//doi.org/ 10.1021/la026906v

Shibata, T., Shimozu, Y., Wakita, C., Shibata, N., Kobayashi, M., Machida, S., Kato, R., Itabe, H., Zhu, X., Sayre, L. M., & Uchida, K. (2011). Lipid peroxidation modification of protein generates N^{ε}-(4-oxononanoyl)lysine as a pro-inflammatory ligand. *Journal of Biological Chemistry, 286*, 19943-19957. https://doi.org/10.1074/jbc.M110.187047

Shimozu, Y., Hirano, K., Shibata, T., Shibata, N., & Uchida, K. (2011). 4-Hydroperoxy-2-nonenal is not just an intermediate but a reactive molecule that covalently modifies proteins to generate unique intramolecular oxidation products. *Journal of Biological Chemistry, 286*, 29313-29324. https://doi.org/10.1074/jbc.M111.255737

Stadtman, E. R., & Levine, R. L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids, 25*, 207-218. https://doi.org/10.1007/s00726-003-0011-2

Uchida, K. (2003). Histidine and lysine as targets of oxidative modification. *Amino Acids, 25*, 249-257. https://doi.org/10.1007/s00726-003-0015-y

Wu, W., Hou, L., Zhang, C., Kong, X., & Hua, Y. (2009). Structural modification of soy protein by 13-hydroperoxyoctadecadienoic acid. *European Food Research and Technology, 229*, 771-778. https://doi.org/10.1007/s00217-009-1113-1

Xu, P., Zheng, Y., Zhu, X., Li, S., & Zhou, C. (2018). L-lysine and L-arginine inhibit the oxidation of lipids and proteins of emulsion sausage by chelating iron ion and scavenging radical. *Asian-Australasian Journal of Animal Sciences, 31*, 905-913. https://doi.org/10.5713/ajas.17.0617

Zamora, R., Alaiz, M., & Hidalgo, F. J. (2000). Contribution of pyrrole formation and polymerization to the nonenzymatic browning produced by amino-carbonyl reactions. *Journal of Agricultural and Food Chemistry, 48*, 3152-3158. https//doi.org/ 10.1021/jf991090y

Zamora, R., & Hidalgo, F. J. (1995). Linoleic acid oxidation in the presence of amino compounds produces pyrroles by carbonyl amine reactions. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1258*, 319-327. https://doi.org/10.1016/0005-2760(95)00139-4

Zamora, R., & Hidalgo, F. J. (2005). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition, 45*, 49-59. https://doi.org/10.1080/10408690590900117

SUPPLEMENTARY MATERIAL OF MANUSCRIPT 5

STUDY OF THE EFFECT OF THE COOXIDATION OF SOYBEAN OIL AND LYSINE ON THEIR RESPECTIVE EVOLUTIONS: A COMBINED ASSESSMENT BY ^{1}H NMR AND LC/MS

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Monitoring by ^{1}H NMR of the evolution of RSO and of RSO+2LYS samples throughout the oxidation process: experimental details and information about acquisition parameters

The weight of each sample aliquot was approximately 0.16 g. These were mixed in a 5 mm diameter tube with 400 μl of deuterated chloroform that contained 0.2% of non deuterated chloroform and a small amount (0.03%) of tetramethylsilane as internal references.

The acquisition parameters used were: spectral width 5000 Hz, relaxation delay 3s, number of scans 64, acquisition time 3.744 s and pulse width 90º, with a total acquisition time of 8 min 55 s. The relaxation delay and acquisition time selected allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making it possible to use them for quantitative purposes. The experiments were carried out at 25 ºC, as in previous works (Guillén, M. D., & Ruiz, A. (2003). *European Journal of Lipid Science and Technology, 105*, 688-696).

Standard compounds used for the identification of oxidation products by ^{1}H NMR in the oil samples

The standard compounds used for the identification of some oxidation products from the ^{1}H NMR spectra were the following: (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal and 4,5-epoxy-(*E*)-2-decenal, acquired from Sigma-Aldrich, 4-hydroxy-(*E*)-2-nonenal, 4-hydroperoxy-(*E*)-2-nonenal and *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, purchased from Cayman Chemical (Ann Arbor, MI, USA), and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica (Barcelona, Spain).

Quantification of oxidation products from ^{1}H NMR spectral data in the oil samples

The concentrations of the oxidation products generated from lipid oxidation in sample RSO and in the oil phase of RSO+2LYS sample through the oxidation process were estimated as millimoles per mol of triglyceride (mmol/mol TG). The general equation to carry out this latter determination was the following:

$$OP] = [(A_{OP}/n)/(A_{TG}/4)]*1000,$$

where A_{OP} is the area of the signal selected for the quantification of each oxidation product (OP), n the number of protons that generate the signal and A_{TG} the area of the protons at *sn*-1 and *sn*-3 positions in the glycerol backbone of TG (signal "I" in Table S1).

To estimate the concentration of the so-called major epoxides (see section 3.1.3), signals between 2.87 and 3.17 ppm approximately were taken together, assuming that the signal at approximately 2.9 ppm corresponds mainly to epoxides contributing with two protons and the one at 3.1 ppm to epoxy-compounds contributing with only one (see Table S1). In the case of the signal at approximately 2.9 ppm, the overlapped area due to the side band of *bis*-allylic protons signals (G and H) must be subtracted.

Monitoring by LC/MS of the evolution of lysine in the aqueous extracts obtained from the RSO+2LYS sample throughout the oxidation process: analysis conditions and procedures followed for the identification and quantification of lysine and its derivatives

The LC/MS chromatograms of the aqueous extracts of RSO+2LYS sample were obtained using a Waters Xevo TQD LC/MS equipment. Sample volumes of 10 µl each were injected into an Imtakt, WAA24 Intrada Amino Acid column (100 mm x 2 mm x 3 µm). A discontinuous gradient of solvent A (H_2O containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) was used as follows: 20% B at 0 min, 75% B at 7 min and 99% B at 7.1 min. Mass spectrometric analysis was performed in TIC mode using positive ion chemical ionization (cone potentials 20 V and 35 V).

The identification of lysine derivative**s** was achieved, on the one hand, by comparing the mass spectra of the compounds detected in the TIC chromatograms obtained with cone potentials 20 V and 35 V with those of lysine adducts obtained in the laboratory by making react lysine with n-alkanals (formaldehyde, propanal, butanal, hexanal, heptanal, octanal and nonanal), as in the study conducted by Kawai, Fujii, Okada, Tsuchie, Uchida and Osawa (2006)[1]. For this purpose, lysine (50 mM) was incubated with 50 mM of each alkanal in the presence of H_2O_2 (50 mM) in 50 mM sodium

phosphate buffer (pH 7.2) at 37 °C. Furthermore, bibliographic data were also used to tentatively identify some of the compounds present in the aqueous extracts.

All the identified lysine derivatives were quantified by measuring the area of their corresponding mass spectra base peaks in the chromatograms obtained with cone potential 20 V. The quantifications were made for them to be useful for comparisons between the samples, not to achieve absolute concentrations of each of the compounds formed.

[1]Kawai, Y., Fujii, H., Okada, M., Tsuchie, Y., Uchida, K., & Osawa, T. (2006). Formation of Nε-(succinyl) lysine *in vivo*: a novel marker for docosahexaenoic acid-derived protein modification. *Journal of Lipid Research, 47*, 1386-1398. https//doi.org/ 10.1194/jlr.M600091-JLR200

Table S1. Chemical shifts, multiplicities and assignments of the ^{1}H NMR signals in $CDCl_3$ of the main types of triglyceride (TG) protons, and of some oxidation compounds, present in the different soybean oil samples, before and throughout the oxidation process.

Signal	Chemical shift (ppm)	Multi-plicity	Functional group	
			Type of protons	Compound
			Main acyl groups[a]	
A	0.88	t	-C**H**$_3$	saturated and monounsaturated ω-9 acyl groups
	0.89	t	-C**H**$_3$	linoleic acyl groups
B	0.97	t	-C**H**$_3$	linolenic acyl groups
C	1.19-1.42	m*	-(C**H**$_2$)$_n$-	acyl groups
D	1.61	m	-OCO-CH$_2$-C**H**$_2$-	acyl groups in TG
E	1.94-2.14	m**	-C**H**$_2$-CH=CH-	unsaturated acyl groups
F	2.26-2.36	dt	-OCO-C**H**$_2$-	acyl groups in TG
G	2.77	t	=HC-C**H**$_2$-CH=	linoleic acyl groups
H	2.80	t	=HC-C**H**$_2$-CH=	linolenic acyl groups
I	4.22	dd,dd	ROC**H**$_2$-CH(OR')-C**H**$_2$OR''	glyceryl groups

J	5.27	m	$ROCH_2$-C**H**(OR')-CH_2OR''	glyceryl groups
K	5.28-5.46	m	-C**H**=C**H**-	acyl groups
			Oxidation compounds	
Hydroperoxides[b]				
a	8.3-9.0	bs	-OO**H**	monohydroperoxide group
Conjugated dienic systems[b,c,d]				
-	5.44	ddd	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroxy group in octadecadienoic acyl groups[e]
-	5.66	dd		
-	5.97	t		
b	6.49	dd		
-	5.47	ddm	-C**H**=C**H**-C**H**=C**H**-	(*E*,*E*)-conjugated double bonds associated with hydroperoxy group in octadecadienoic acyl groups
-	5.76	dtm		
-	6.06	ddtd		
c	6.27	ddm		
-	5.51	dtm	-C**H**=C**H**-C**H**=C**H**-	(*Z*,*E*)-conjugated double bonds associated with hydroperoxy group in octadecadienoic acyl groups[f]
-	5.56	ddm		
-	6.00	ddtd		
d	6.58	dddd		
-	5.58	dd	–C**H**=C**H**–C**H**=C**H**–	(*E*,*E*)-conjugated double bonds associated with hydroxy group (OH) in octadecadienoic acyl groups
-	5.71	dd		
-	6.03	dd		
e	6.18	dd		
Epoxides				
Epoxy-derivatives				
f	2.63[g]	m	-C**H**O**H**C-	(*E*)-9,10-epoxystearate
g1	2.88[g]	m	-C**H**O**H**C-	(*Z*)-9,10-epoxystearate
g2	2.9[h]	m	-C**H**O**H**C-	monoepoxy-octadecenoate groups
			-C**H**OHC-CH_2-CHO**H**C-	diepoxides
g3	2.94***	m	-C**H**O**H**C-	(*Z*)-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid
h1	3.10[h]	m	-CHO**H**C-CH_2-C**H**OHC-	diepoxides
Epoxy-keto-derivatives				
g4	2.89[i]/2.90[j]	td[i]/m[j]	-CO-CH=CH-CHO**H**C-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
g5	2.91[i]	td	-C**H**OHC-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate

i	3.20[i,j]	dd	-CO-CH=CH-C**H**OHC-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
			-CHO**H**C-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-C**H**OHC-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
			-CO-CH=CH-CHO**H**C-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
j1	3.52[i]	dd	-CHO**H**C-CH=CH-CO-	(*Z*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
j2	3.53[i]	dd	-CO-CH=CH-C**H**OHC-	(*Z*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
Epoxy-hydroxy-derivatives				
g6	2.93[k]	dt	-C**H**OHC-CHOH-CH=CH-	*threo*-11-hydroxy-(*E*)-12,13-epoxy-(*Z*)-9-octadecenoate
h2	3.09[l]/3.097[m]	dd	-CHO**H**C-CH=CH-CHOH-	9-hydroxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate
Epoxy-hydroperoxy-derivatives				
h3	3.11[l]	dd	-CHO**H**C-CH=CH-CHOOH-	9-hydroperoxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate[n]
Aldehydes				
k	9.49[o]	d	−C**H**O	(*E*)-2-alkenals
l	9.52[o]	d	−C**H**O	(*E,E)*-2,4-alkadienals
m	9.55[o]	d	−C**H**O	4,5-epoxy-2-alkenals
n	9.57[o]	d	−C**H**O	4-hydroxy-(*E*)-2-alkenals
o	9.58[o]	d	−C**H**O	4-hydroperoxy-(*E*)-2-alkenals
p	9.75[o]	t	-C**H**O	n-alkanals

t: triplet; m: mutiplet; d: doublet; bs: broad signal; *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***Assignment made with the aid of standard compounds

[a]Assignments taken from Guillén, M. D., & Ruiz, A. (2003). *Journal of the Science of Food and Agriculture, 83*, 338-346.

[b]Data taken from Goicoechea, E., & Guillén, M. D. (2010). *Journal of Agricultural and Food Chemistry, 58*, 6234-6245 (hydroperoxides and conjugated (*Z,E*)- and (*E,E*)-hydroperoxy-dienes).

[c]Data taken from Dong, M., Oda, Y., & Hirota, M. (2000). *Bioscience, Biotechnology and Biochemistry, 64*, 882-886 (conjugated (*Z,E*)-hydroxy-dienes).

[d]Data taken from Tassignon, P., De Waard, P., De Rijk, T., Tournois, H., de Wit, D., & De Buyck, L. (1994). *Chemistry and Physics of Lipids, 71*, 187-196 (conjugated (*E,E*)-hydroxy-dienes).

[e]The chemical shifts of the (*Z,E*)- and (*E,Z*)-isomers are practically indistinguishable, according to data from Kuklev, D. V., Christie, W. W., Durand, T., Rossi, J. C., Vidal,

J. P., Kasyanov, S. P., Akulin, V. N., & Bezuglov, V. V. (1997). *Chemistry and Physics of Lipids*, *85*, 125-134.

[f]The chemical shifts of the (*Z*,*E*)- and (*E*,*Z*)-isomers are practically indistinguishable, according to data from Chan, H. W. S., & Levett, G. (1977). *Lipids, 12*, 99-104.

[g]Data taken from Du, G., Tekin, A., Hammond, E. G., & Woo, L. K. (2004). *Journal of the American Oil Chemists' Society, 81*, 477-480.

[h]Data taken from Aerts, H. A. J., & Jacobs, P. A. (2004). *Journal of the American Oil Chemists' Society, 81*, 841-846 (monoepoxy-octadecenoates and diepoxides).

[i]Data taken from Lin, D., Zhang, J., & Sayre, L. M. (2007). *The Journal of Organic Chemistry, 72*, 9471-9480.

[j]Data taken from Gardner, H. W., Kleiman, R., & Weisleder, D. (1974). *Lipids, 9*, 696-706.

[k]Data taken from Garssen, G. J., Veldink, G. A., Vliegenthart, J. F., & Boldingh, J. (1976). *The FEBS Journal*, *62*, 33-36.

[l]Data taken from Gardner, H. W., Weisleder, D., & Kleiman, R. (1978). *Lipids, 13*, 246-252.

[m]Data taken from Van Os Cornelis, P. A., Vliegenthart, J. F. G., Crawford, C. G., & Gardner, H. W. (1982). *Biochimica et Biophysica Acta*, *713*, 173-176.

[n]δ-Ketols (hydroxy-keto-derivatives) could also contribute to this signal (Gardner et al., 1974).

[o]Data taken from Guillén, M. D., & Ruiz, A. (2004). *European Journal of Lipid Science and Technology, 106*, 680-687.

Table S2. Chemical shifts of L-lysine, Nε-formyl-lysine, Nα-acetyl-lysine and Nε-acetyl-lysine in deuterated acid water (0.5 M HCl, pH close to 1.5), obtained from reference compounds.

Signal	Chemical shift[1]	Multiplicity[2]	Type of protons	Compound
L-Lysine and Nε-Lysine adducts				
LA'	7.86	s	-NεH-C**H**O	Nε-formyl-lysine
LB	3.95	t	-Cα**H**	lysine
LB'	3.94	t	-Cα**H**	Nε-formyl-lysine
LB''	3.93	t	-Cα**H**	Nε-acetyl-lysine
LC'	3.09	t	-Nε-C**H$_2$**-	Nε-formyl-lysine
LC''	3.04	t	-Nε-C**H$_2$**-	Nε-acetyl-lysine
LC	2.85	t	-Nε-C**H$_2$**-	lysine
LD	1.93 - 1.74	m	-Nε-CH$_2$-CH$_2$-CH$_2$-**CH$_2$**-Cα	lysine
LD'	1.90 -1.71	m	-Nε-CH$_2$-CH$_2$-CH$_2$-**CH$_2$**-Cα	Nε-formyl-lysine
LD''	1.87 - 1.69	m	-Nε-CH$_2$-CH$_2$-CH$_2$-**CH$_2$**-Cα	Nε-acetyl-lysine
LG''	1.84	s	-NεH-CO-C**H$_3$**	Nε-acetyl-lysine
LE	1.61 - 1.52	m	-Nε-CH$_2$-C**H$_2$**-CH$_2$-CH$_2$-Cα	lysine
LE'	1.46 - 1.37	m	-Nε-CH$_2$-C**H$_2$**-CH$_2$-CH$_2$-Cα	Nε-formyl-lysine
LE''	1.45 - 1.36	m	-Nε-CH$_2$-C**H$_2$**-CH$_2$-CH$_2$-Cα	Nε-acetyl-lysine
LF	1.46 - 1.26	m	-Nε-CH$_2$-CH$_2$-C**H$_2$**-CH$_2$-Cα	lysine
LF'	1.38 - 1.19	m	-Nε-CH$_2$-CH$_2$-C**H$_2$**-CH$_2$-Cα	Nε-formyl-lysine
LF''	1.37 - 1.17	m	-Nε-CH$_2$-CH$_2$-C**H$_2$**-CH$_2$-Cα	Nε-acetyl-lysine
Nα-Lysine adducts				
LB'''	4.19 - 4.14	m	-Cα**H**	Nα-acetyl-lysine
LC'''	2.86 - 2.78	t	-Nε-C**H$_2$**-	Nα-acetyl-lysine
LG'''	1.87	s	-NαH-CO-C**H$_3$**	Nα-acetyl-lysine
LD'''	1.79 - 1.56	m	-Nε-CH$_2$-CH$_2$-CH$_2$-**CH$_2$**-Cα	Nα-acetyl-lysine

LE'''	1.57 – 1.47	m	-Nε-CH_2-C**H_2**-CH_2-CH_2-Cα	Nα-acetyl-lysine
LF'''	1.37 – 1.22	m	-Nε-CH_2-CH_2-C**H_2**-CH_2-Cα	Nα-acetyl-lysine

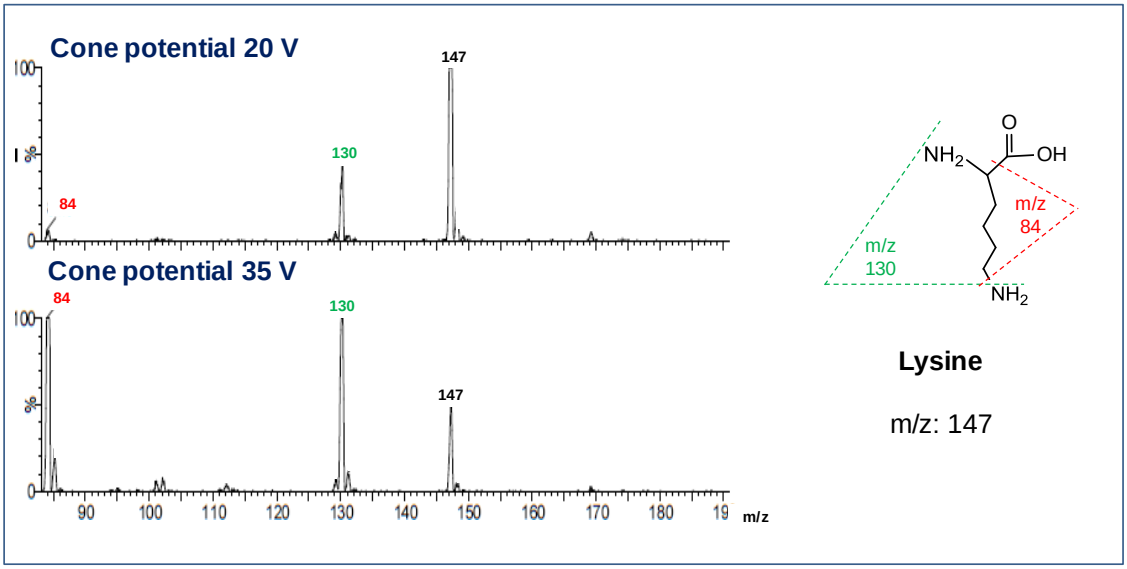
[1] The chemical shifts of the lysine derivatives have been established taking those of lysine as reference
[2] s: singlet; t: triplet; m: mutiplet

Table S3. Lysine adducts with n-alkanals identified by LC/MS in the aqueous extracts of RSO+2LYS sample throughout the oxidation process, together with their retention times (RT), mass spectra base peaks (BP) and other fragments of their mass spectra.

		Cone 20 V		**Cone 35 V**	
RT (min)	**Compound[1]**	**BP[2]**	**Other fragments**	**BP**	**Other fragments[3]**
1:94	Nε-octanoyl-lysine	273	147	273	84, <u>210</u>, 130, 147
2:26	Nε-hexanol-lysine	245	147	245	84, <u>182</u>, 147, 130
2:36	Nε-pentanoyl-lysine	231	147	231	84, <u>168</u>, 147, 130
2:53	Nε-butiryl-lysine	217	147	84	217, 147, <u>154</u>, 130
2:71	Nε-propanoyl-lysine	203		<u>140</u>	203, 84, 130, 147
2:91	Nε-acetyl-lysine	189		<u>126</u>	84, 189, 147, 130
2:99	Nε-formyl-lysine	175	112	<u>112</u>	175, 84, 130, 147
3:45	Nα-propanoyl-lysine	203		84	203
3:71	Nα-acetyl-lysine	189		84	189
3:73	Nα-formyl-lysine	175		175	84

[1] Nε-: adducts formed at the ε position; Nα-: adducts formed at the α position.
[2] The base peak corresponds to the molecular weight of the compound plus 1.
[3] The underlined ion is characteristic of each Nε-adduct.

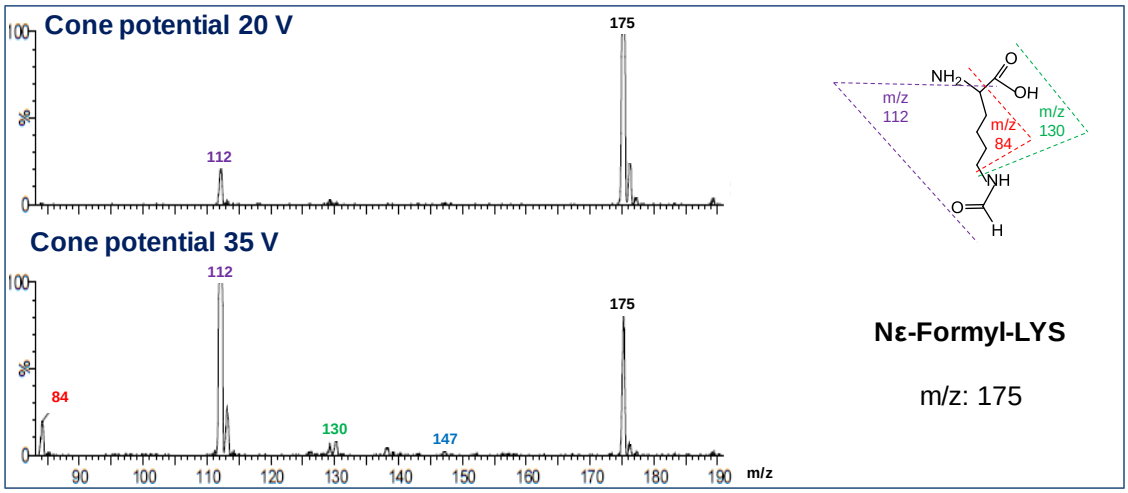
MASS SPECTRA AND STRUCTURES OF LYSINE AND OF THE TENTATIVELY IDENTIFIED LYSINE ADDUCTS



Lysine

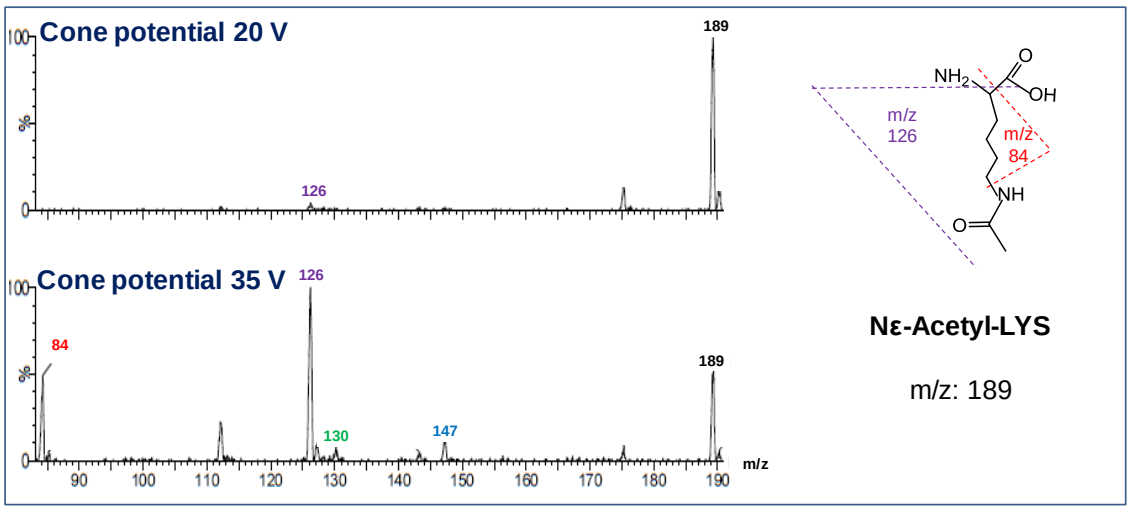
m/z: 147

ALKANAL- Nε LYSINE ADDUCTS



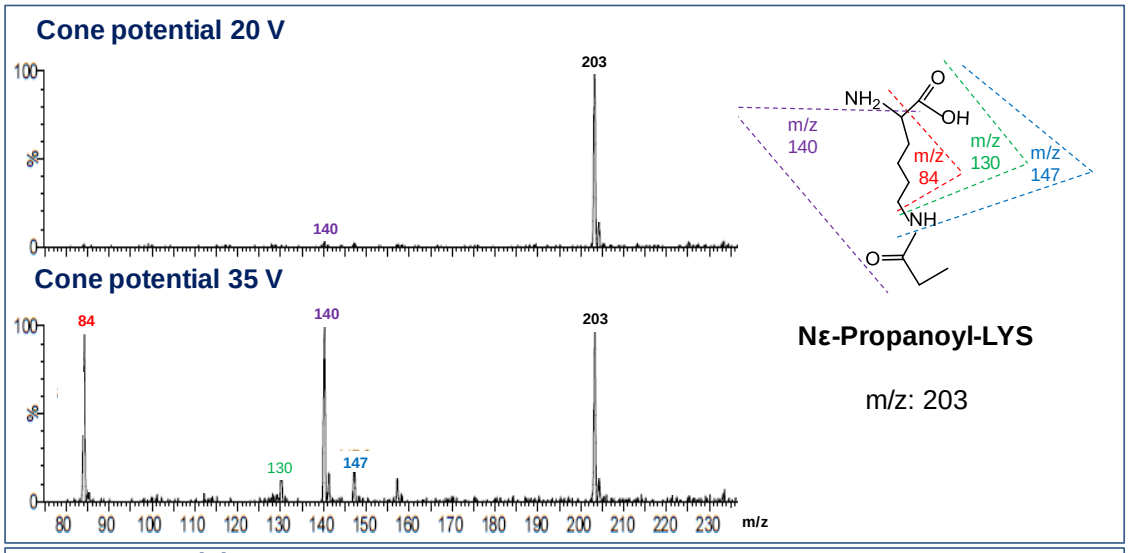
Nε-Formyl-LYS

m/z: 175



Nε-Acetyl-LYS

m/z: 189


Cone potential 20 V
203
140
Cone potential 35 V
84
140
203
130
147
m/z
NH2
O
OH
NH
O
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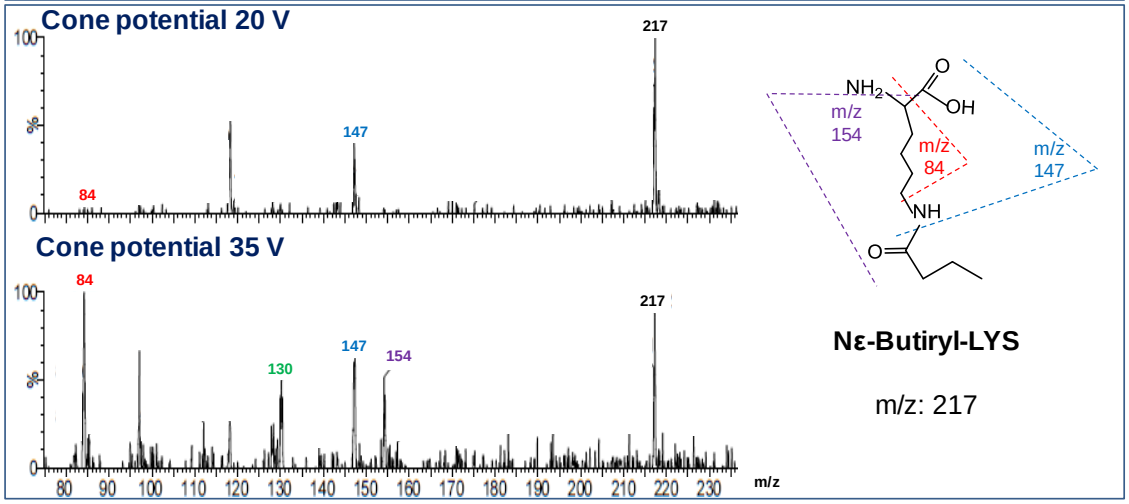
m/z 84
m/z 130
m/z 147
Nε-Propanoyl-LYS
m/z: 203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217
147
84
Cone potential 35 V
84
217
147
154
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m/z
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OH
NH
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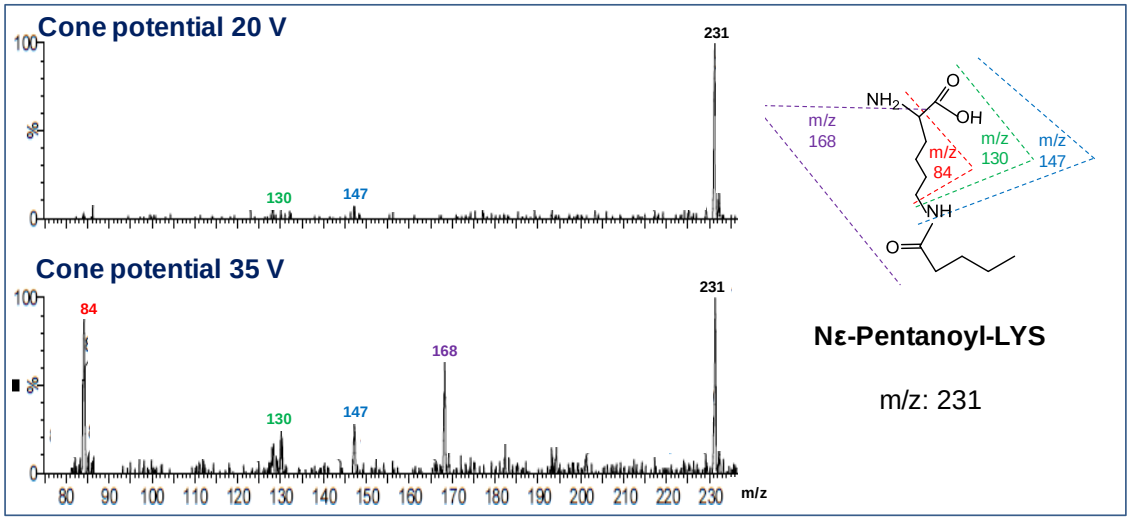
m/z 84
m/z 147
Nε-Butiryl-LYS
m/z: 217


Cone potential 20 V
231
130
147
Cone potential 35 V
84
231
168
130
147
m/z
NH2
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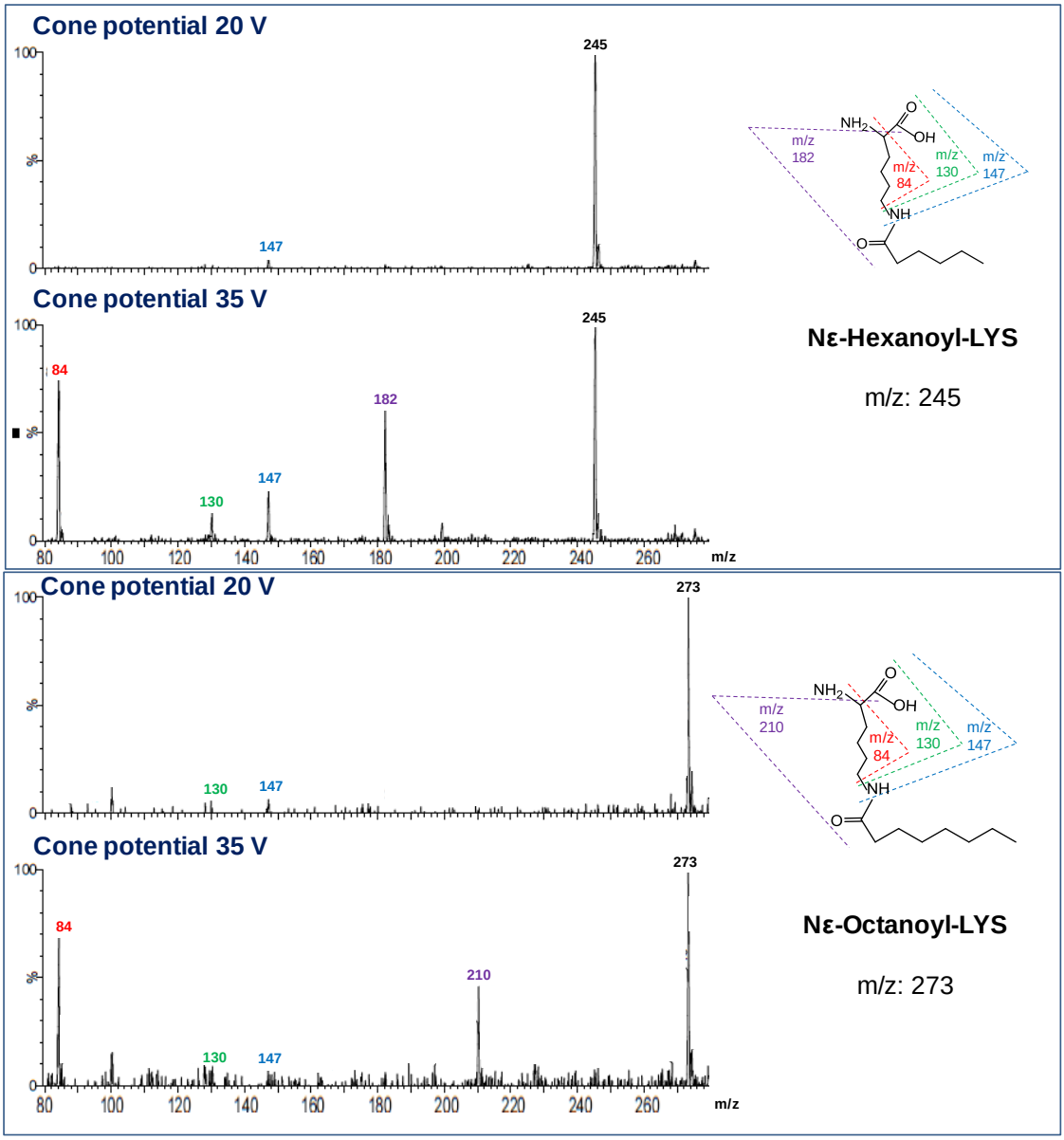
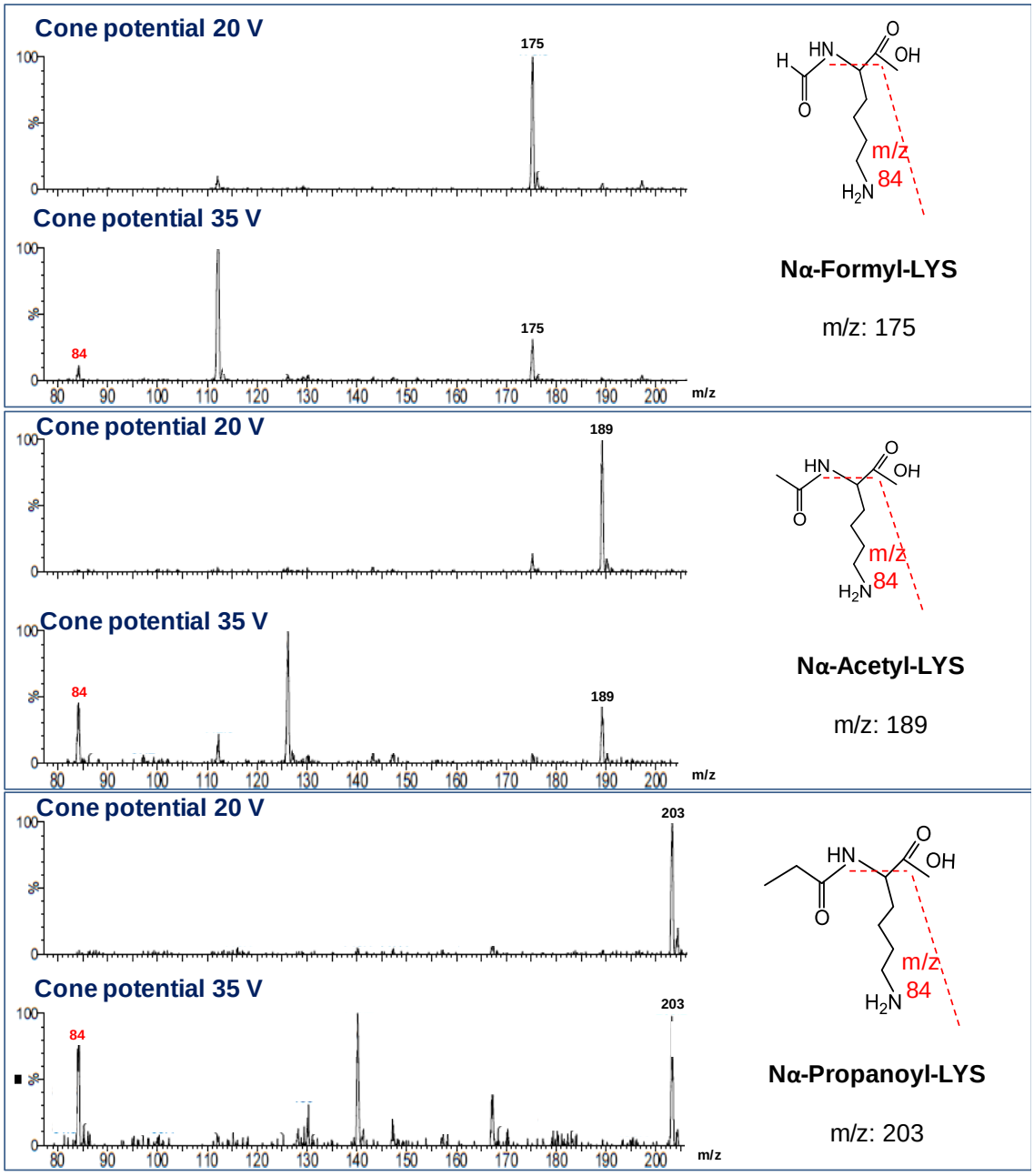
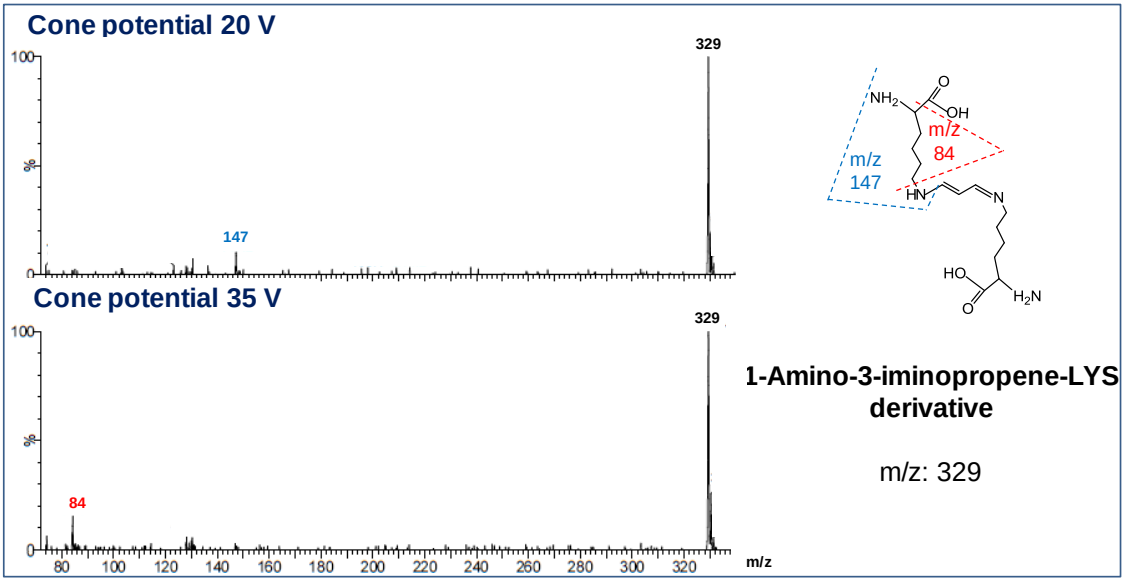
m/z 147
Nε-Pentanoyl-LYS
m/z: 231

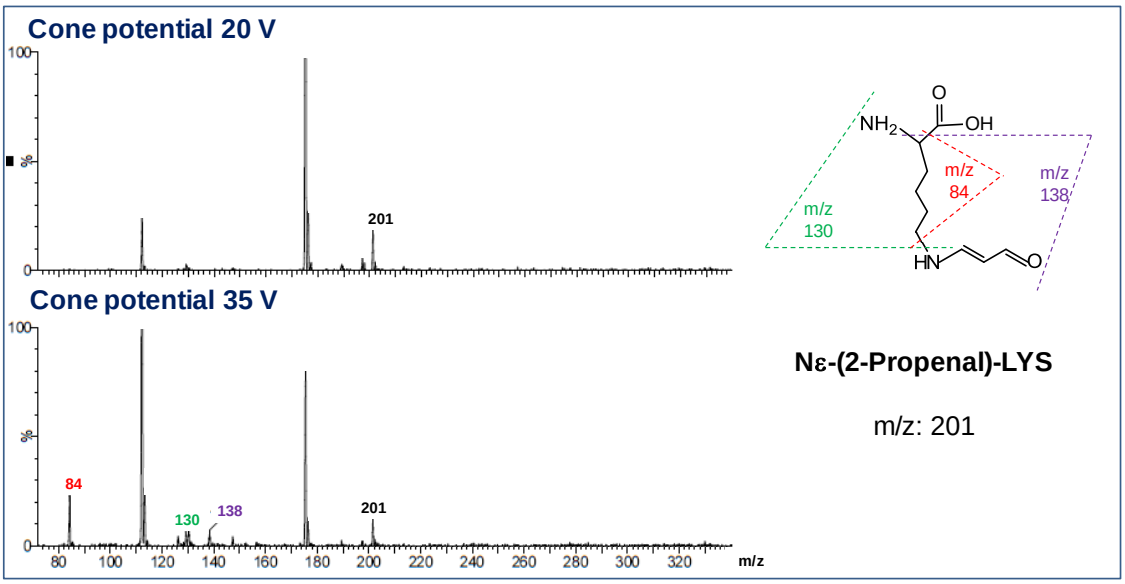

Cone potential 20 V
245
147
Cone potential 35 V
245
84
182
147
130
m/z
Nε-Hexanoyl-LYS
m/z: 245
m/z 182
m/z 84
m/z 130
m/z 147
Cone potential 20 V
273
130
147
Cone potential 35 V
273
84
210
130
147
m/z
Nε-Octanoyl-LYS
m/z: 273
m/z 210
m/z 84
m/z 130
m/z 147

ALKANAL- Nα LYSINE ADDUCTS

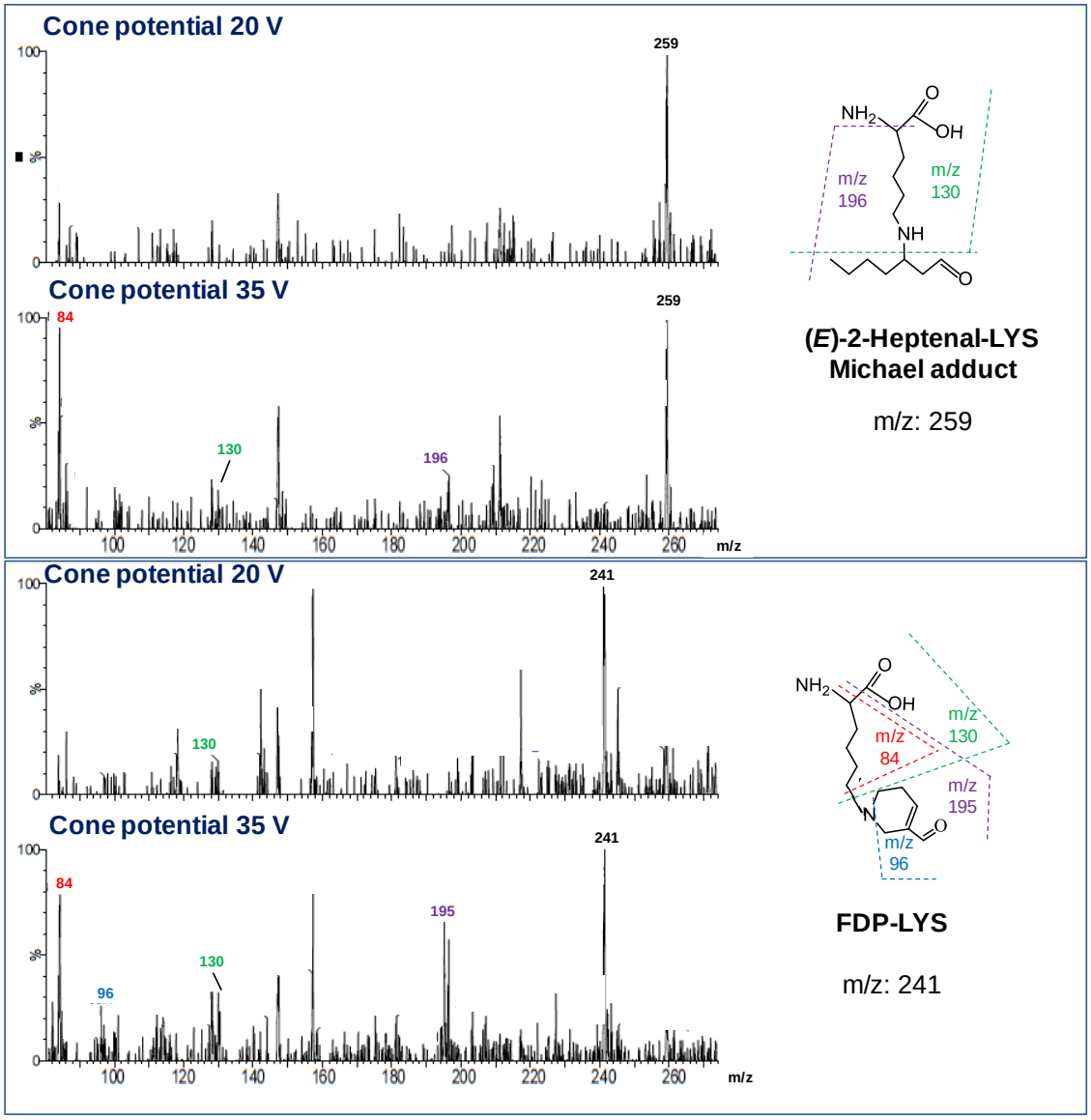


MDA-LYSINE ADDUCTS

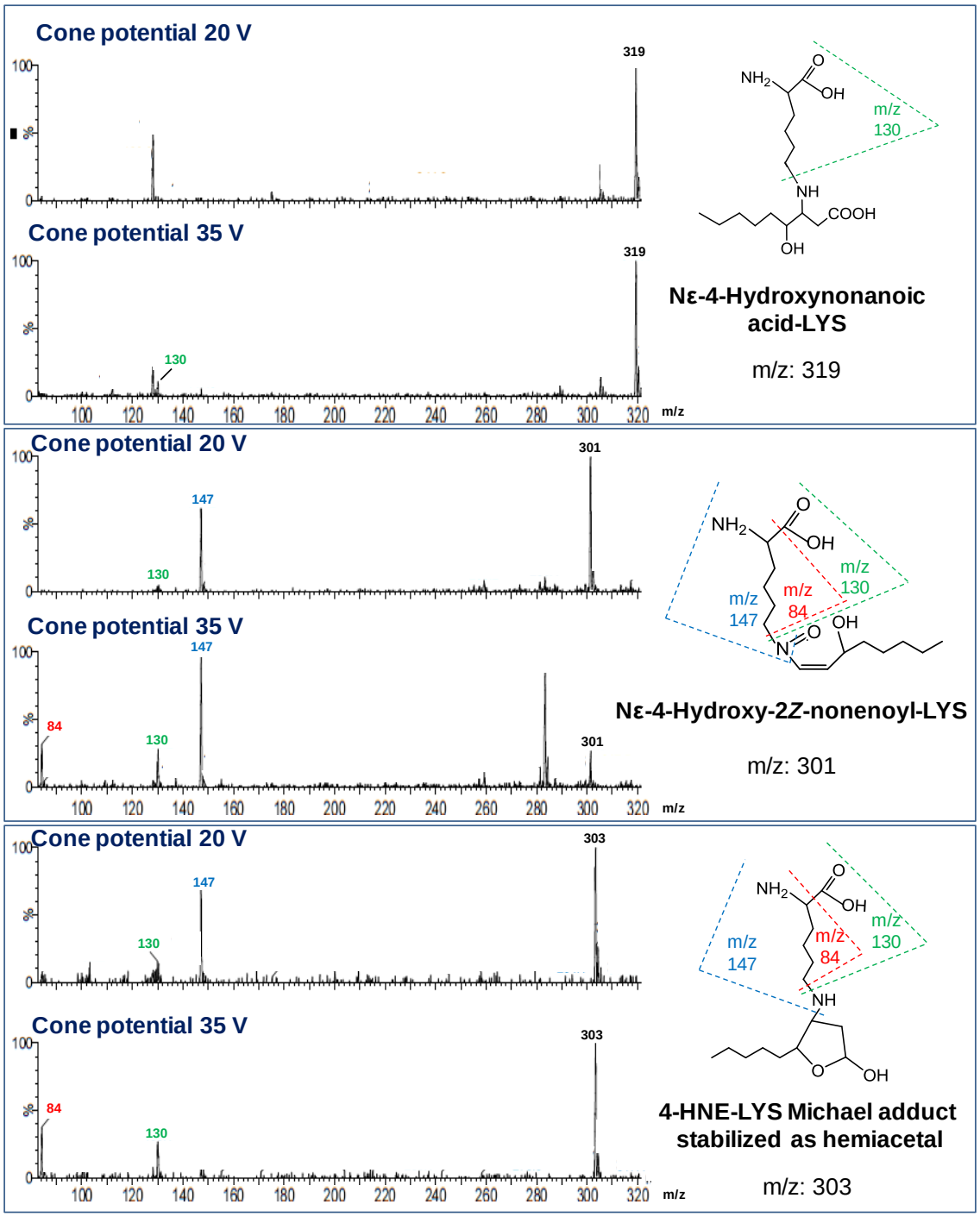




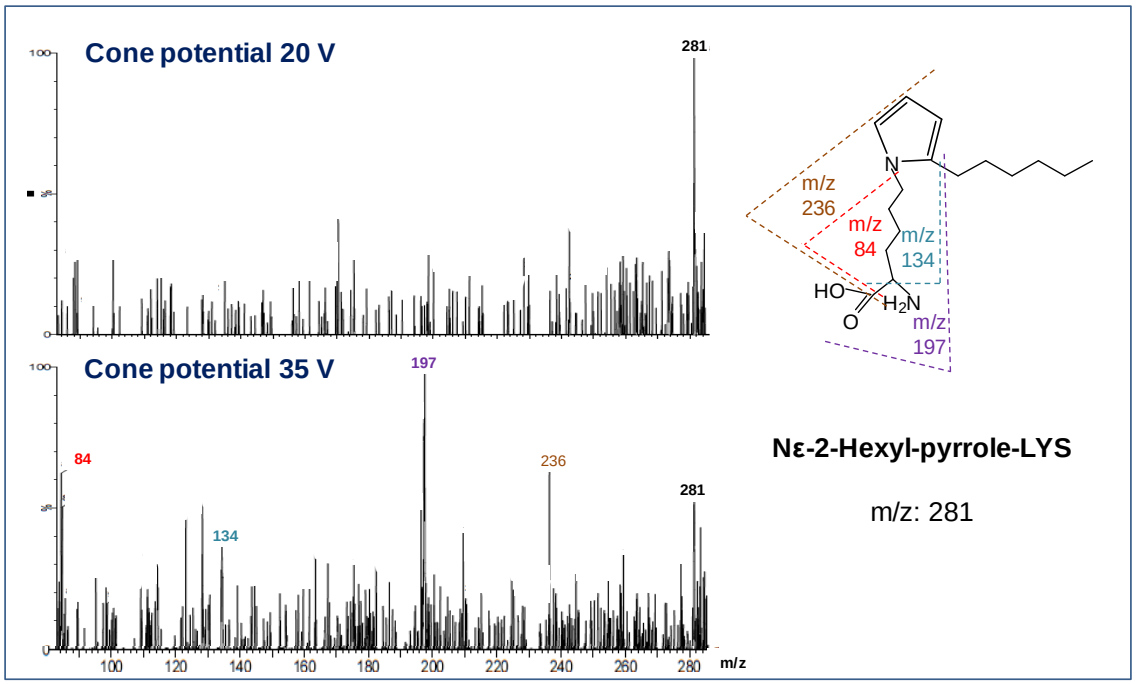
(*E*)-2-ALKENALS-LYSINE ADDUCTS



OXYGENATED α,β-UNSATURATED ALDEHYDES-LYSINE ADDUCTS



PYRROLE-TYPE LYSINE ADDUCT



Manuscript 6

INFLUENCE OF MINOR COMPONENTS ON LIPID BIOACCESSIBILITY AND OXIDATION DURING *IN VITRO* DIGESTION OF SOYBEAN OIL

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ABSTRACT

BACKGROUND: Minor components of edible oils could influence their evolution during *in vitro* digestion. This might affect the bioaccessibility of lipid nutrients and the safety of the ingested food. Bearing this in mind, the evolution of virgin and refined soybean oils, which are very similar in acyl group composition, has been studied throughout *in vitro* digestion using ^{1}H nuclear magnetic resonance (NMR) and solid-phase microextraction-gas chromatography /mass spectrometry, focusing on lipolysis and oxidation reactions. The fate of γ-tocopherol, the main antioxidant present in soybean oil, has also been analyzed with ^{1}HNMR

RESULTS: There were no noticeable differences in lipolysis between the two oils that were studied. The extent of oxidation during digestion, which was very low in both cases, was slightly higher in the virgin type, which showed lower tocopherols and squalene concentrations than the refined one, together with a considerable abundance of free fatty acids. This can be deduced both from the appearance after digestion of conjugated hydroperoxy- and hydroxy-dienes only in the virgin oil, and from its higher levels of volatile aldehydes and 2-pentyl-furan. Under *in vitro* digestion conditions, the formation of epoxides seemed to be favored over other oxidation products. Finally, although some soybean oil essential nutrients like polyunsaturated fatty acids exhibited no significant degradation after digestion, γ-tocopherol concentration diminished during this process, especially in the virgin oil.

CONCLUSION: Although the minor component composition of the soybean oils did not affect lipolysis during *in vitro* digestion, it influenced the extent of their oxidation and γ-tocopherol bioaccessibility.

Keywords: soybean oil, γ-tocopherol, *in vitro* digestion, lipolysis, oxidation, ^{1}H NMR, SPME-GC/MS

1. INTRODUCTION

The behaviour of lipids throughout the digestion process is of great interest as on the one hand food lipids provide essential nutrients and on the other because some lipids are very prone to oxidative degradation, which can affect both nutritional quality and safety of food[1.] In this context, attention can be paid to several matters occurring simultaneously in the gastrointestinal tract, such as lipid hydrolytic processes and oxidation reactions among others**.** The lipid hydrolysis degree reached, directly related with the bioaccessibility of lipophilic nutrients[2], can be affected by several factors which include: lipid concentration[3], the structure on which fatty acids are supported (triglycerides or partial glycerides), the length of the acyl group chains[3,4], the presence of free fatty acids[5] or the initial oxidation degree of the lipids[6,7]. Regarding oxidation reactions, their occurrence during the digestion process has been assessed by several authors, and their extent is also determined by various factors, like lipid and sample composition, initial oxidative status or food fat content[6-10]. As a consequence of lipid oxidation, not only can the bioaccessibility of lipid nutrients like some essential polyunsaturated fatty acids and certain compounds considered as antioxidants be reduced, but also toxic oxidation compounds can be generated[1]. These latter can exert their negative effects directly on the gastrointestinal tract, affecting the functionality of some biological compounds such as gastrointestinal detoxifying enzymes[11], and even throughout the body upon absorption. In the case of vegetable oils, which make a great contribution to the food lipids ingested, attention should also be given to their minor components, since these have an important influence on their oxidative stability and their behaviour under oxidative conditions[12]; amongst them, tocopherols, phytosterols, free fatty acids or metal ions can be mentioned. Their concentrations and relative proportions vary depending on the oil botanical origin[13], but also on oil refining and deodorization processes[14]. Actually, these latter operations can bring about virgin and refined oils of the same botanical origin with slightly different contents and proportions of minor compounds which can either increase the oxidative stability of bulk oils or emulsions, or on the contrary, reduce it, as reported in the case of free fatty acids[12,15]. Although there are many studies about the effect of minor components on oil oxidative stability[6,17], as far as we know, there is limited understanding of the influence that they can have on the reactions occurring during gastrointestinal digestion. In this regard, some studies have been conducted on the impact of tocopherols on lipid oxidation

during the *in vitro* digestion of rapeseed oil-in-water emulsions[18], and also of rapeseed[19] and cod liver[8] oils. However, these works have been performed either enriching commercial oils with α-tocopherol or comparing the behaviour of commercial and tocopherol-stripped oils, which is a different approach from that here proposed. Keeping all of the above in mind, the present work aims to study whether the pool of minor components present in commercial edible virgin and refined soybean oils, such as tocopherols and free fatty acids, can affect the hydrolytic processes and/or the occurrence of oxidation during *in vitro* digestion. Therefore, instead of focusing on one only specific compound or group of compounds, either antioxidant or prooxidant, this work takes into consideration all the minor components as a whole, thus offering a more realistic approach to the study of the edible oils digestion process. In addition, the influence of the digestion process on the stability and bioaccessibility of γ-tocopherol (γ-T), one of the main antioxidants present in soybean oil[13], has also been tackled. The techniques employed were Proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid-Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (SPME-GC/MS). ^{1}H NMR allows one to study simultaneously the lipolysis extent, the occurrence of oxidation reactions and the evolution of γ-T throughout *in vitro* digestion, providing a global picture of this process. SPME-GC/MS, in turn, constitutes a complementary and very useful tool that, thanks to its higher sensitivity and specificity, gives information about changes in volatile compounds that could not be detected by ^{1}H NMR.

2. MATERIALS AND METHODS

2.1. Samples subject of study

The samples subject of study were two commercial soybean oils: one of them virgin (VO) and the other refined (RO). Aside from minor differences in the molar proportions of the different kinds of acyl groups (see Table 1), the main divergence between these two oils consists in their content of minor components; the corresponding abundances, obtained by means of Direct Immersion (DI) SPME-GC/MS, following the methodology described by Alberdi-Cedeño, Ibargoitia, Cristillo, Sopelana and Guillén,[13] are shown in Table 2; some fragments of these total ion chromatograms can be observed in Figure S1 (see supporting information). As Table 2 shows, the concentrations of all the tocopherols and of squalene, another component attributed with

antioxidant ability,[20] are higher in RO than in VO. Free fatty acids, instead, are only detected in the virgin oil.

Table 1. Molar percentages of the several kinds of acyl groups and fatty acids in relation to the total of acyl groups plus fatty acids present in virgin and refined soybean oil samples before (VO and RO) and after (DVO and DRO) *in vitro* digestion. Different letters within each column indicate a significant difference among the samples ($p < 0.05$). Ln: linolenic; L: linoleic; O: oleic; S+M: saturated plus modified.

	Ln	L	O	S+M
VO	5.51 ± 0.15b	44.69 ± 2.33a	32.47 ± 0.14a	17.33 ± 2.60a
DVO	5.39 ± 0.12ab	44.24 ± 2.65a	32.27 ± 0.49a	18.09 ± 2.61a
RO	4.90 ± 0.44ab	47.61 ± 1.07a	32.09 ± 0.53a	15.39 ± 1.89a
DRO	4.76 ± 0.40a	46.97 ± 1.43a	31.94 ± 1.27a	16.33 ± 1.76a

Different letters within each column indicate a significant difference among the samples ($P < 0.05$). Ln: linolenic; L: linoleic; O: oleic; S+ M: saturated plus modified; AG+FA: acyl groups+fatty acids

Table 2. Abundances, expressed as arbitrary area units of the mass spectrum base peak (BP) of each compound extracted from the total ion chromatograms obtained by DI-SPME/GC-MS, divided by 10^6, of the main minor components expected to influence the evolution of the studied soybean oils during *in vitro* digestion, together with their respective molecular weights (MW). The asterisked compounds were acquired commercially and used as standards for identification purposes. All the values are given as the mean of two determinations ± standard deviation.

Compounds (MW)	BP	VSO	RSO
FREE FATTY ACIDS			
Total free fatty acids§	55	166.2 ± 7.3	nd
TOCOPHEROLS			
δ-Tocopherol (402)*	402	25.8 ± 2.3	34.9 ± 0.0
β-Tocopherol (416)*	416	1.9 ± 0.2	2.5 ± 2.0
γ-Tocopherol (416)*	416	67.5 ± 5.4	106.0 ± 2.0
α-Tocopherol (430)*	165	11.3 ± 0.9	14.9 ± 0.4
HYDROCARBONS			
Squalene (410)*	69	24.5 ± 1.5	33.2 ± 0.7

nd: not detected

§This total includes linoleic*, oleic* and linolenic* acids, whose mass spectra base peaks are 67, 55 and 79, respectively. However, given that all of them overlap, ion 55, which is common to all these unsaturated fatty acids, has been taken in order to quantify them altogether.

2.2. *In vitro* digestion

Samples (0.5 g) of the two oils were digested following the same procedure as in previous works,[6,7] based on the static *in vitro* gastrointestinal model developed by Versantvoort, Oomen, Van de Kamp, Rompelberg and Sips,[21] and slightly modified in our laboratory in order to reach a higher level of lipolysis.[22] This involves a three-step procedure to simulate digestive processes in the mouth, stomach, and small intestine, by sequentially adding the corresponding digestive juices (saliva, gastric juice, duodenal juice and bile), whose composition is given in Table S1 (see supporting information). The digestion experiment started by adding 6 mL of saliva to each of the oil samples. After 5 min of incubation, 12 mL of gastric juice were added and the mixture was rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. 1 hour after starting the gastric digestion, pH was set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring *in vivo*. After 2 h of gastric digestion, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice were added. Subsequently, pH was set between 6 and 7, and the mixture was rotated again at 40 rpm and incubated at 37±2ºC for 4 h. All the reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA): α-amylase from *Aspergillus oryzae* (10065, ~30 U/mg); pepsin from porcine gastric mucosa (P7125, ≥400 U/mg protein); amano lipase A from *Aspergillus niger* (534781, ≥120,000 U/g); pancreatin from porcine pancreas (P1750); lipase type II crude from porcine pancreas (L3126, 100-500 U/mg protein (using olive oil, 30 min incubation)) and bovine bile extract (B3883). Two digestion experiments, each including duplicate samples of the two oils, were performed. Blank samples corresponding to the mixture of juices submitted to digestive conditions were also taken for further analysis.

2.3. Lipid extraction of the digestates

Lipids of the digestates were extracted using dichloromethane as solvent (HPLC grade, Sigma-Aldrich) and following the methodology employed by Nieva-Echevarría, Goicoechea, Manzanos and Guillén,[23] slightly modified in a later study.[7]

2.4. Analysis by ^{1}H NMR 2.4.1. Operating conditions

The ^{1}H NMR spectra of the starting oils (VO and RO) and of the lipid extracts of their digestates (DVO and DRO) were acquired in quadruplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned lipid

samples (approximately 0.16 g) were dissolved in 400 μl of deuterated chloroform, which contained tetramethylsilane (TMS) as internal reference (Cortec, Paris, France). The acquisition conditions were the same used in previous studies.[24]

2.4.2. Identification of some oil components

The identification of the oil acyl groups, of γ-T and of the oxidation products formed throughout digestion was carried out on the basis of the signal assignments shown in Table S2 (see supporting information), made from bibliographic data and with the aid of standard compounds. These were γ-T, acquired from Sigma-Aldrich, and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica (Barcelona, Spain).

2.4.3. Quantification from ^{1}H NMR spectral data

2.4.3.1. Concerning the various types of glycerides

The molar percentages of triglycerides (TG%), diglycerides (1,2-DG% and 1,3-DG%), monoglycerides (2-MG% and 1-MG%) and glycerol (Gol%) in relation to the total of glyceryl structures present in the lipid samples were determined using the equations developed and validated in previous studies.[23,25] All these equations are given as supporting information (see equations [S1-S10]). Lipid bioaccessibility (LBA), another parameter concerning lipolysis extent, was calculated by using equations [S11] and [S12].

2.4.3.2. Concerning lipid composition

The molar percentages of linolenic (Ln%), linoleic (L%), oleic (O%), and saturated plus modified (S+M%) acyl groups (AG) or fatty acids (FA), in relation to the total moles of AG+FA present in the various lipid samples were estimated as in a previous study[7] by using equations [S13-S16]. In addition, the unsaturation degree of the studied oils was estimated by determining the area of olefinic protons (from signal "T" in Table S2) relative to that of the sum of all kinds of saturated protons (from signals "A"-"H" in Table S2).

2.4.3.3. Concerning oxidation compounds and γ-T

The concentrations of (*Z*,*E*)- and (*E*,*E*)-conjugated dienic systems supported on chains having either hydroperoxy or hydroxy groups, and of epoxides, expressed as millimoles per mole of

AG+FA present (mmol/mol AG+FA), were also estimated as in a previous study7 by using equation [S17]. This same approach was used to estimate the concentration of γ-T (equation [S18]).

2.5. Study by SPME-GC/MS

The extraction of the volatile components of the several samples (0.5 g in a 10 ml screw-cap vial) was accomplished automatically by using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). Given that the nature of the samples subjected to the digestion process (oil samples) is very different from that of the digested ones (basically aqueous samples), it is necessary to prepare mixtures of the non-digested oil samples with the digestive juices submitted to the digestion process, in the same proportions as in the digestates; this enables one to accurately assess the changes occurring throughout the *in vitro* digestion process. Therefore, the samples subject of study, which were analyzed in duplicate, were the following: i) the digestates of the two types of soybean oil samples (DVO and DRO); ii) the juices submitted to digestion conditions (DJ); and iii) the mixtures made up of starting oil samples and juices submitted to digestion conditions (VO+DJ and RO+DJ). The fiber used, coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm long), was acquired from Supelco (Sigma-Aldrich); this was inserted into the headspace of the sample and was maintained for 55 min at 50 ºC, after a pre-equilibration time of 5 min. The fiber containing the extracted components was desorbed for 10 min in the injection port (splitless mode with 5 min purge time) of a 7890A gas chromatograph equipped with a 5975C inert MSD with Triple Axis Detector (Agilent Technologies) and a computer operating with the ChemStation program. The column used, the operating conditions and the identification and semi-quantification procedures of the selected compounds were the same as in previous works.[6,7] With this aim, several commercial standards were used, acquired from Sigma-Aldrich (see supporting information).

2.6. Statistical analysis

The significance of the differences on the several determinations made among the samples was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

3. RESULTS AND DISCUSSION

This section will describe the results regarding the extent of lipolysis, the occurrence of lipid oxidation and the evolution of γ-T during the *in vitro* digestion of the samples subject of study.

3.1. Extent of lipolysis reached through the *in vitro* digestion of RO and VO oils

As Figure 1 reveals, the main glycerides present in the spectrum of RO oil are TG (signals "O" and "S", see Table S2), together with a much lower proportion of 1,2-DG (signal "J"); and the same is true for the virgin oil (VO), as deduced from Table 3, which shows the molar percentages of the several kinds of glycerides, including Gol, in the studied samples before and after digestion. After digestion, a decrease in the TG signals is observed in the ^{1}H NMR spectrum of DRO sample (see Figure 1), concomitant with both a great increase in the signals of 1,2-DG ("J", "P" and "R") and the appearance of new signals due to 2-MG (signals "K" and "Q"), 1-MG (signals "I", "L" and "N", this latter partially overlapped with "O") and 1,3-DG (signal "M", also overlapped with "O"). In agreement with that observed in Figure 1, Table 3 shows that the percentage of TG decreases sharply after digestion, its value being very similar in DVO and DRO samples (near 22%). Therefore, despite the presence of free fatty acids in VO might have affected lipolysis extent,[5] significant differences have not been noticed between the two studied oils. In addition to TG, the most abundant glycerides in the digested samples were 2-MG and 1,2-DG, while 1-MG and 1,3-DG, especially the latter, were in much lower proportions. According to some authors, the presence of these minor glycerides is probably a result of isomerization reactions of 2-MG[26] and 1,2-DG,[27] respectively. Finally, Gol, resulting from the complete hydrolysis of the different types of glycerides, is also present in a considerable proportion in the digestates, at a percentage of around 30% in both samples. These results are very similar to those reported in previous works.[6,7] To obtain a more biological approach to the results regarding the extent of lipolysis, lipid bioaccessibility parameter (LBA) can be employed. This indicates the proportion of absorbable molecules (FA and MG) with respect to the sum of AG+FA.[28] As Table 3 reveals, both in DVO and in DRO LBA reaches values near a 65% after digestion; these are of a similar order to those observed previously in digested sunflower and flaxseed oils.[6,7]

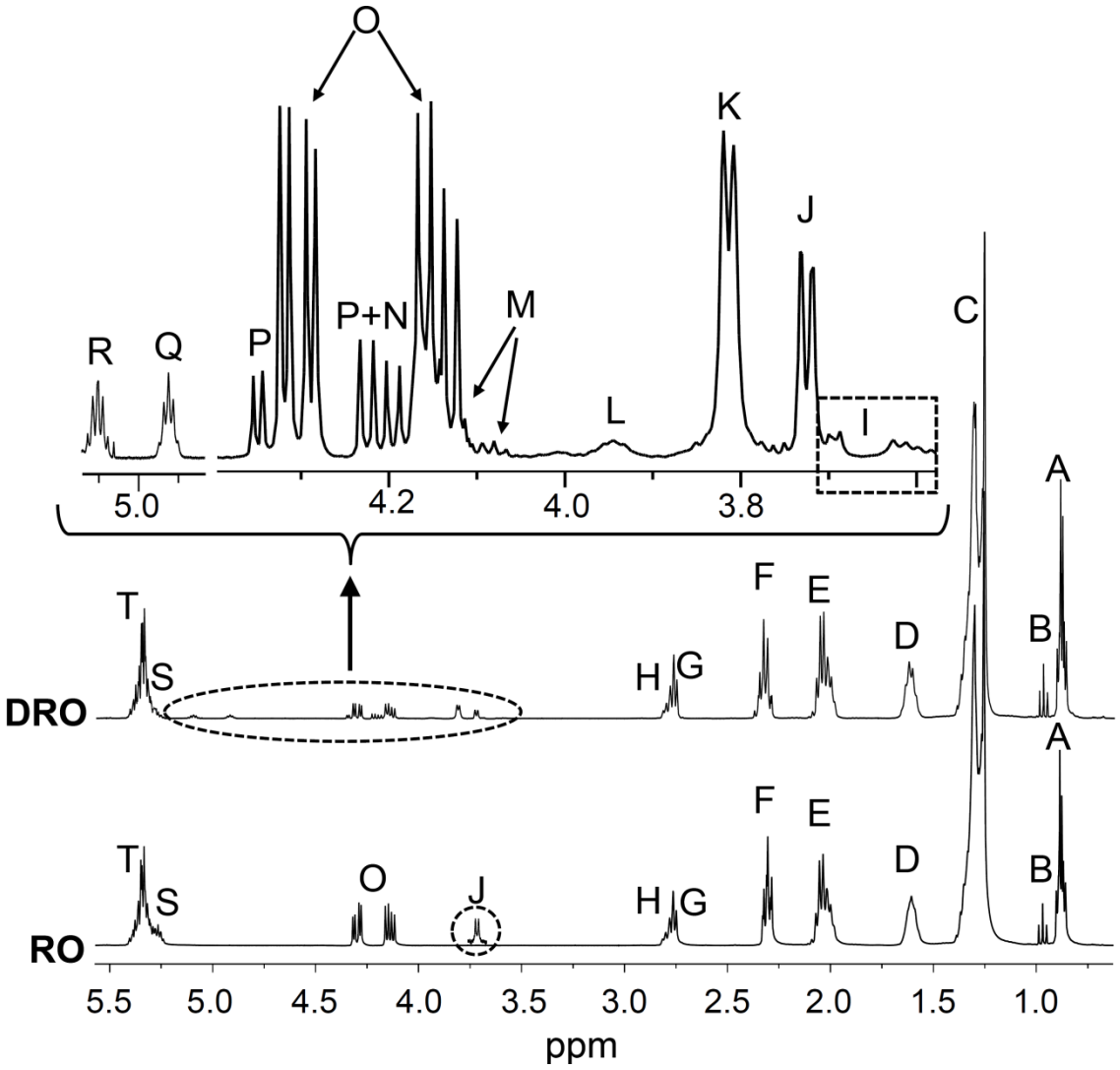

Figure 1. ^{1}H NMR spectra of the original refined oil (RO) and of the corresponding lipid extract obtained after the *in vitro* digestion process (DRO). The signal letters agree with those in Table S2.

Table 3. Molar percentages of triglycerides (TG%), diglycerides (1,2-DG% and 1,3-DG%), monoglycerides (2-MG% and 1-MG%) and glycerol (Gol%) in relation to the total number of glyceryl structures present in the samples, together with Lipid bioaccessibility (L_{BA}) parameter, in virgin and refined soybean oil samples, before (VO and RO) and after (DVO and DRO) *in vitro* digestion. Different letters within each column indicate a significant difference among the samples ($p < 0.05$).

	TG%	**1,2-DG%**	**1,3-DG%**	**2-MG%**	**1-MG%**	**Gol%**	**L_{BA}**
VO	99.56 ± 0.26b	0.26 ± 0.01a	-	-	-	0.18 ± 0.07a	0.27 ± 0.06a
DVO	22.80 ± 6.59a	14.52 ± 3.41b	3.13 ± 1.06a	23.24 ± 6.02a	6.12 ± 1.77a	30.20 ± 3.56b	65.44 ± 9.55b
RO	99.39 ± 0.06b	0.62 ± 0.03a	-	-	-	-	0.23 ± 0.04a
DRO	21.47 ± 8.11a	15.36 ± 1.94b	3.81 ± 1.48a	24.35 ± 7.27a	4.60 ± 0.41a	30.41 ± 4.48b	65.75 ± 10.38b

–, not detected; nd, not determined.

Different letters within each column indicate a significant difference among the samples ($P < 0.05$).

3.2. Assessment of lipid oxidation through *in vitro* digestion by ^{1}H NMR

In addition to lipolysis, which as mentioned above, is crucial for the bioaccessibility of lipophilic nutrients like some essential fatty acids, another process that can also affect the just mentioned issue, as well as the generation of directly bioaccessible toxic compounds is lipid oxidation. For this reason, in this section ^{1}H NMR has been used to assess the extent of lipid oxidation during *in vitro* digestion, both monitoring the changes in the proportions of the several kinds of AG or FA and observing the generation, or in case concentration changes, of primary and/or secondary oxidation products.

3.2.1. Changes in the proportions of the several kinds of AG+FA

The molar percentages of the several kinds of AG+FA in the lipid extracts obtained from the digested oil samples are shown in Table 1, together with those corresponding to the starting oils. In general, comparing them before and after the *in vitro* digestion, no significant changes are observed either in the virgin or in the refined oil.

3.2.2. Generation and concentration changes of primary and/or secondary oxidation products

Although VO and RO were acquired as fresh oils, in RO sample the tentative presence of epoxides was noticed in its ^{1}H NMR spectrum (see Figure 2, signal "c", partially overlapping with the left side band of *bis*-allylic protons signal, "H+G"); the corresponding concentration is given in Table 4, in mmol/mol AG+FA. As Figure 2 reveals, after the *in vitro* digestion of VO, the occurrence of oxidation is evidenced by the appearance in the ^{1}H NMR spectrum of DVO of small signals related to (*Z,E*)-conjugated dienes associated to both hydroperoxides ((*Z,E*)-CD-OOH) and hydroxy-compounds ((*Z,E*)-CD-OH) (signals "b" and "a", respectively), the concentration of the latter being slightly higher (see Table 4). These results agree with those of other authors who have also reported the generation of hydroperoxides after the *in vitro* digestion of other types of highly unsaturated oils like sunflower,[6] flaxseed[7] and cod liver.8 Regarding (*Z,E*)-CD-OH, their formation has also been observed in sunflower oil under conditions of low temperature and reduced oxygen availability,[29] as is the case of the simulated digestion process. Unlike DVO, the generation of conjugated diene systems was not detected in DRO sample. These findings suggest an influence of the composition in minor components of these two oils on their evolution during digestion, which makes VO more prone to oxidation than RO. This observation is reinforced by

the fact that the unsaturation degree of the refined oil is slightly higher than that of the virgin one (ratios of saturated to unsaturated protons of 9.88 in RO and of 10.29 in VO), since according to this, RO would oxidize somewhat easier than VO. Actually, as can be observed in Table 2, not only the concentration of γ-T but also those of the rest of tocopherols were lower in VO, while that of free fatty acids was higher. Regarding these latter compounds, it must be noticed that they are more prone to oxidation than TG[30] and, in addition, they can act as pro-oxidants.[15] In fact, a negative effect of free fatty acids on the oxidative stability of soybean oil was reported by Mistry and Min.[31] Concerning the epoxides initially present in RO sample, a significant increment in their concentration was monitored after digestion, as seen in Figure 2 and Table 4. Moreover, the generation of this type of oxidation compounds also took place in the virgin soybean oil (see also Figure 2 and Table 4). Although the exact identity of the epoxides detected remains unknown, it could be reasonably assumed that monoepoxides of linolenic groups, the most prone to oxidation amongst those present in soybean oil, might be generating this signal (see signal "c3" in Table S2).

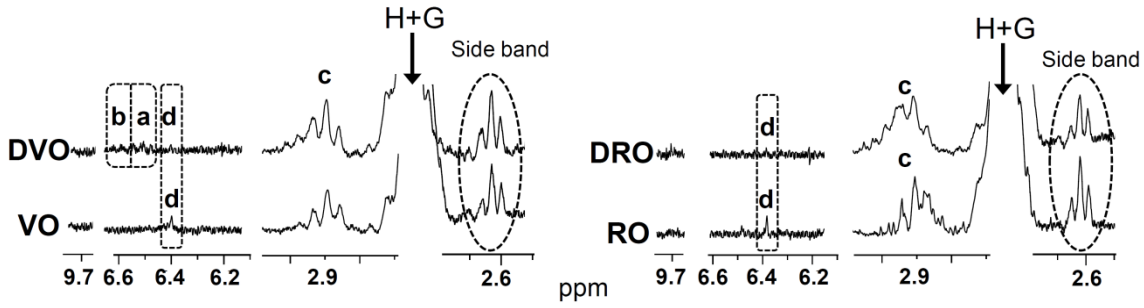

Figure 2. Enlargements of some regions of the ^{1}H NMR spectra of the original VO and RO, and of their corresponding lipid extracts obtained after the *in vitro* digestion process (DVO and DRO). The signal letters agree with those in Table S2, considering that 'c' includes signals 'c1-c4'. The plots corresponding to the same ^{1}H NMR spectral region are presented at a fixed value of absolute intensity, for them to be valid for comparative purposes.

Table 4. Concentration of the several kinds of oxidation compounds, expressed in mmol mol−1 AG+FA present in the virgin and refined oil samples, before (VO and RO) and after (DVO and DRO) *in vitro* digestion.

	(*Z*,*E*)-Hydroperoxy-dienes	(*Z*,*E*)-Hydroxy-dienes	Epoxides
VO	-	-	-
DVO	0.09 ± 0.04	0.30 ± 0.01	1.40 ± 0.32a
RO	-	-	3.41 ± 0.26b
DRO	-	-	5.62 ± 1.11c

–, not detected; nd, not determined. Different letters within each column indicate a significant difference among the samples ($P < 0.05$).

3.3. Assessment of lipid oxidation through the *in vitro* digestion by SPME-GC/MS

By employing SPME-GC/MS, specific oxidation products present in very low concentrations, not detectable by ^{1}H NMR, can be monitored, thus providing valuable information about the occurrence of oxidation, especially when this is low. Among all the volatile compounds, interest was focused on aldehydes and 2-pentyl-furan, well-known oxidation markers. The compounds detected in the starting samples and in their corresponding digestates, as well as in the digestive juices submitted to digestion, together with their abundances, are displayed in Figure 3. The data corresponding to the total abundance of each group of aldehydes are also shown in this figure. As can be observed, only a small number of aldehydes are present in the headspace of the reference samples of the two studied oils (VO+DJ and RO+DJ), among which (*E*)-2-butenal, mainly coming from digestive juices (see Figure 3B), and some n-alkanals like pentanal and hexanal (Figure 3A), stand out. In addition, some aldehydes that seem to come exclusively from soybean oil oxidation were also detected; these are (*E*)-2-heptenal (Figure 3B), generated from linoleic acyl groups degradation and, only in the case of VO+DJ sample, (*Z*,*E*)- and (*E*,*E*)-heptadienals (Figure 3C), mainly derived from linolenic groups.[32] After the digestion process, the abundances of all the above mentioned aldehydes increase in DVO. However, in DRO only the abundances of hexanal and (*E*)-2-heptenal rise after digestion, and to a lower extent than in DVO, while those of pentanal and (*E*)-2-butenal decrease. The diminution of these two latter aldehydes could be due to their reaction with proteinaceous components of the digestive juices during digestion.[33]

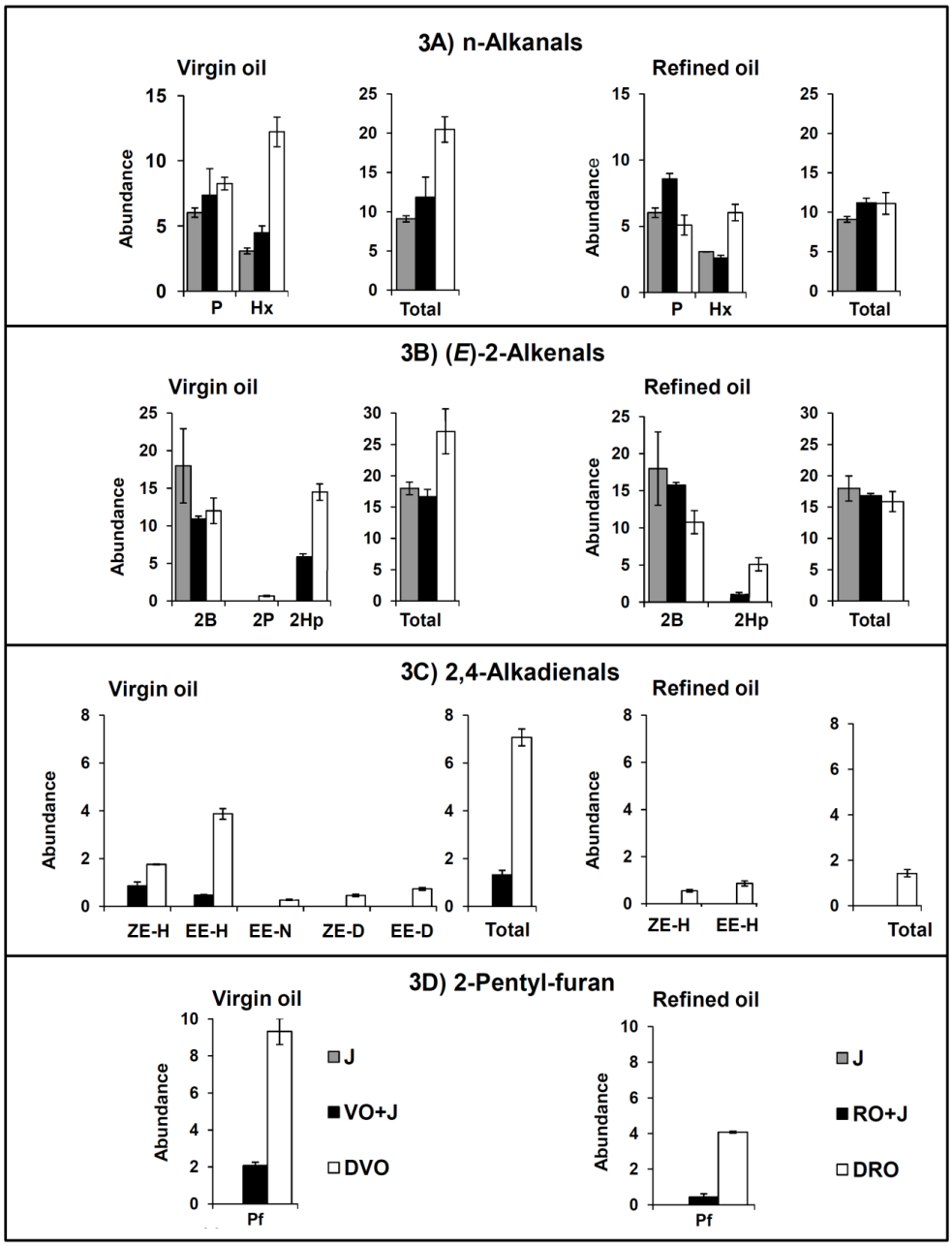

Figure 3. Bar graphics representing the abundance, expressed as arbitrary area units of the mass spectrum base peak of each compound (see supporting information) extracted from the total ion chromatograms obtained by SPME/GC-MS, divided by 10^6, of n-alkanals (3A), (*E*)-2-alkenals (3B), 2,4-alkadienals (3C) and 2-pentyl-furan (3D) in: the digestive juices subjected to digestion conditions (DJ), the original virgin and refined oils mixed with the digested juices (VO+DJ and RO+DJ) and the digestates of the virgin and refined oils (DVO and DRO), together with the total abundance of each group of aldehydes. Abbreviations employed: pentanal (P), hexanal (Hx), (*E*)-2-butenal (2B), (*E*)-2-pentenal (2P), (*E*)-2-heptenal (2Hp), (*Z*,*E*)-2,4-heptadienal (ZE-H), (*E*,*E*)-2,4-heptadienal (EE-H), (*E*,*E*)-2,4-nonadienal (EE-N), (*Z*,*E*)-2,4-decadienal (ZE-D), (*E*,*E*)-2,4-decadienal (EE-D) and 2-pentyl-furan (Pf).

This does not mean that other aldehydes than pentanal and (*E*)-2-butenal are not able to react with the amino acid residues of proteins, but that, in such a case, their generation rate might surpass that of the reaction. Furthermore, the generation of (*E*)-2-pentenal (Figure 3B) in DVO and of different types of 2,4-alkadienals in both oils (Figure 3C) was also noted. All the above mentioned confirms that a slight extent of lipid oxidation takes place during the digestion of both virgin and refined soybean oils that, in accordance with the results obtained by ^{1}H NMR (see Table 4 and Figure 2), is somewhat higher in the virgin one; this can be clearly deduced from the general increases observed both in the total amount of each group of aldehydes and in the concentration of 2-pentyl-furan, also present in the starting oils (Figure 3D). Therefore, while analysis by ^{1}H NMR does not allow one to notice the generation of aldehydes, this is evidenced by SPME-GC/MS, thus proving the usefulness of this technique in detecting very low levels of oxidation. At this point, it is worth pointing out that, when using *in vitro* digestion models like the one described in this work, the amount of oxygen, especially at the intestinal step, could be higher than that present *in vivo*, which might affect the extent of oxidation observed during digestion. However, few data are available relating not only to the levels of oxygen existing in the gastrointestinal tract and the factors influencing this oxygen content, but also to the influence of this parameter on lipid oxidation during digestion, as shown in the very recent review carried out by Nieva-Echevarría, Goicoechea and Guillén.[10] In any event, given that the conditions were the same for all the samples studied, this issue is not expected to influence the results of this work regarding the difference observed between the virgin and the refined soybean oils.

3.4. Evolution of γ-T during the *in vitro* digestion process

The occurrence of lipid oxidation affects the concentration of antioxidants in oils, since they degrade as a consequence of their involvement in reactions aimed at counteracting reactive oxygen species and/or free radicals.[34] Hence, considering that the development of oxidation reactions has been observed during the *in vitro* digestion of the studied oils, and that γ-T is the most abundant antioxidant in soybean oil (see Table 2), its concentration has been determined, by means of ^{1}H NMR, before and after digestion; this will allow an assessment of to what extent the oxidation undergone by soybean oil during digestion can influence its γ-T content. The first worthwhile issue is that, by contrast with what might be expected from bibliographic data on refined and

virgin oils,[14] the concentration of γ-T was lower in VO (0.52±0.05 mmol/mol AG+FA) than in RO (0.88±0.09 mmol/mol AG+FA), what is in agreement with the data obtained by means of DI-SPME/GC-MS (see Table 2). After digestion, only 0.11±0.01 mmol/mol AG+FA of γ-T remained in DVO and 0.30±0.06 mmol/mol AG+FA in DRO; these values, which account for about one fifth and one third of the γ-T initial concentration in the case of DVO and DRO, respectively, suggest a faint greater decrease of γ-T during the digestion of the virgin oil. The reduction in the intensity of the γ-T signal can be observed in Figure 2. These outcomes suggest that, as might be expected, the slightly higher oxidation degree observed during the *in vitro* digestion of the virgin oil is translated into a somewhat greater reduction of γ-T concentration in this oil, and consequently in its bioaccessibility. A degradation of tocopherols during *in vitro* digestion was also observed by Kenmogne-Domguia, Moisan, Viau, Genot and Meynier[35] in oil-in-water emulsions.

4. CONCLUSIONS

As far as we know, this is the first time that the evolutions of two commercial edible oils with practically the same composition in acyl groups but with different minor components profiles have been studied simultaneously during *in vitro* digestion. Although this process does not induce a significant degradation of polyunsaturated groups assessed by ^{1}H NMR, the analysis of both non-volatile and volatile oxidation products reveals that a certain degree of oxidation occurs, which seems to be influenced by oil composition in minor components. Thus, although differences in the lipolysis extent of the two studied oils are not observed, the lower concentration of tocopherols, and maybe of squalene, together with the presence of a considerable abundance of free fatty acids in the virgin soybean oil, are translated into a lower oxidative stability of this oil; this can be deduced from the generation of very small concentrations of hydroperoxy- and hydroxy-dienes in the virgin oil, as well as from higher concentrations of volatile aldehydes and 2-pentyl-furan. Moreover, the outcomes of this work suggest that, under gastrointestinal conditions, epoxidation reactions seem to be favoured over other types of oxidative reactions, since epoxides are generated in higher concentrations than other types of oxidation products. Therefore, these findings restate the importance not only of monitoring the generation of epoxides to accurately assess the extent of lipid oxidation, but also the need to delve into their generation pathways. It must also be noticed that although, as commented above, polyunsaturated groups hardly

exhibit variations after digestion, the concentration, and consequently, the bioaccessibility of γ-T are reduced during this process; this is especially noticeable in the virgin soybean oil, whose oxidative stability is lower than that of the refined one under *in vitro* digestion conditions. This serves to highlight the importance of the oxidative stability of the dietary lipids, not only to limit oxidative reactions in the gastrointestinal tract that can negatively affect food safety, but also to preserve their minor components with antioxidant potential.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

REFERENCES

1. Esterbauer H, Muskiet F and Horrobin DF. Cytotoxicity and genotoxicity of lipid-oxidation products. Am J Clin Nutr **57**:779S-786S (1993).
2. Nik AM, Wright AJ and Corredig M. Interfacial design of protein-stabilized emulsions for optimal delivery of nutrients. Food Funct **1**:141-148 (2010).
3. Li Y, Hu M and McClements DJ. Factors affecting lipase digestibility of emulsified lipids using an *in vitro* digestion model: Proposal for a standardised pH-stat method. Food Chem **126**:498-505 (2011).
4. Zhu X, Ye A, Verrier T and Singh H. Free fatty acid profiles of emulsified lipids during *in vitro* digestion with pancreatic lipase. Food Chem **139**:398-404 (2013).
5. Golding M and Wooster TJ. The influence of emulsion structure and stability on lipid digestion. Curr Opin Colloid Interface Sci **15**:90-101 (2010).
6. Nieva-Echevarría B, Goicoechea E, Manzanos MJ and Guillén MD. ^{1}H NMR and SPME-GC/MS study of hydrolysis, oxidation and other reactions occurring during *in vitro* digestion of non-oxidized and oxidized sunflower oil. Formation of hydroxy-octadecadienoates. Food Res Int **91**:171-182 (2017).

7. Nieva-Echevarría B, Goicoechea E and Guillén MD. Behaviour of non-oxidized and oxidized flaxseed oils, as models of omega-3 rich lipids, during *in vitro* digestion. Occurrence of epoxidation reactions. Food Res Int **97**:104-115 (2017).
8. Larsson K, Cavonius L, Alminger M and Undeland I. Oxidation of cod liver oil during gastrointestinal *in vitro* digestion. J Agric Food Chem **60**:7556-7564 (2012).
9. Steppeler C, Haugen JE, Rødbotten R and Kirkhus B. Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during *in vitro* digestion of cooked beef, pork, chicken, and salmon. J Agric Food Chem **64**:487-496 (2016).
10. Nieva-Echevarría B, Goicoechea E and Guillén MD. Food lipid oxidation under gastrointestinal digestion conditions: A review. Crit Rev Food Sci Nutr 1-18 (2018). DOI: 10.1080/10408398.2018.1538931.
11. Márquez-Ruiz G, García-Martínez MC and Holgado F. Changes and effects of dietary oxidized lipids in the gastrointestinal tract. Lipid Insights **2**:11-19 (2008).
12. Choe E and Min DB. Mechanisms and factors for edible oil oxidation. Compr Rev Food Sci Food Saf **5**:169-186 (2006).
13. Alberdi-Cedeño J, Ibargoitia ML, Cristillo G, Sopelana P and Guillén MD. A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components. Food Chem **221**:1135-1144 (2017).
14. Jung MY, Yoon SH and Min DB. Effects of processing steps on the contents of minor compounds and oxidation of soybean oil. J Am Oil Chem Soc **66**:118-120 (1989).
15. Waraho T, McClements DJ and Decker EA. Impact of free fatty acid concentration and structure on lipid oxidation in oil-in-water emulsions. Food Chem **129**:854-859 (2011).
16. Chen B, McClements DJ and Decker EA. Minor components in food oils: A critical review of their roles on lipid oxidation chemistry in bulk oils and emulsions. Crit Rev Food Sci Nutr **51**:901-916 (2011).
17. Kamal-Eldin A. Effect of fatty acids and tocopherols on the oxidative stability of vegetable oils. Eur J Lipid Sci Technol **108**:1051-1061 (2006).
18. Kenmogne-Domguia HB, Meynier A, Boulanger C and Genot C. Lipid oxidation in food emulsions under gastrointestinal-simulated conditions: the key role of endogenous tocopherols and initiator. Food Dig **3**:46-52 (2012).

19. Tarvainen M, Phuphusit A, Suomela JP, Kuksis A and Kallio H. Effects of antioxidants on rapeseed oil oxidation in an artificial digestion model analyzed by UHPLC-ESI-MS. J Agric Food Chem **60**:3564-3579 (2012).

20. Dessì MA, Deiana M, Day BW, Rosa A, Banni S and Corongiu FP. Oxidative stability of polyunsaturated fatty acids: Effect of squalene. Eur J Lipid Sci Technol **10**4:506-512 (2002). 21. Versantvoort CHM, Oomen AG, Van de Kamp E, Rompelberg CJ and Sips AJ. Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem Toxicol **43**:31-40 (2005).

22. Nieva-Echevarría B, Goicoechea E, Manzanos MJ and Guillén MD. A study by ^{1}H NMR on the influence of some factors affecting lipid *in vitro* digestion. Food Chem **211**:17-26 (2016).

23. Nieva-Echevarría B, Goicoechea E, Manzanos MJ and Guillén MD. Usefulness of ^{1}H NMR in assessing the extent of lipid digestion. Food Chem **179**:182-190 (2015).

24. Guillén MD and Ruiz A. Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups in vegetable oils. Eur J Lipid Sci Technol **105**:688-696 (2003).

25. Nieva-Echevarría B, Goicoechea E, Manzanos MJ and Guillén MD. A method based on ^{1}H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures. Food Res Int **66**:379-387 (2014).

26. Mattson FH and Volpenhein RA. Enzymatic hydrolysis at an oil/water interface. J Am Oil Chem Soc **43**:286-289 (1966).

27. De Groot WTM. Acyl migration solid phase isomerization of 1,2-diglycerides to 1,3-isomers. Lipids **7**:626-628 (1972).

28. Kenmogne-Domguia HB, Meynier A, Viau M, Llamas G and Genot C. Gastric conditions control both the evolution of the organization of protein-stabilized emulsions and the kinetic of lipolysis during *in vitro* digestion. Food Funct **3**:1302-1309 (2012). 29. Guillén MD and Goicoechea E. Oxidation of corn oil at room temperature: Primary and secondary oxidation products and determination of their concentration in the oil liquid matrix from ^{1}H nuclear magnetic resonance data. Food Chem **116**:183-192 (2009).

30. Shewfelt RL. Fish muscle lipolysis-a review. J Food Biochem **5**:79-100 (1981).

31. Mistry BS and Min DB. Effects of fatty acids on the oxidative stability of soybean oil. J Food Sci **52**:831-832 (1987).

32. Guillén MD and Uriarte PS. Aldehydes contained in edible oils of a very different nature after prolonged heating at frying temperature: Presence of toxic oxygenated α,β unsaturated aldehydes. Food Chem **131**:915-926 (2012).

33. Uchida K. Aldehyde adducts generated during lipid peroxidation modification of proteins. Free Radic Res **49**:896-904 (2015).

34. Shahidi F, Janitha PK and Wanasundara PD. Phenolic antioxidants. Crit Rev Food Sci Nutr **32**:67-103 (1992).

35. Kenmogne-Domguia HB, Moisan S, Viau M, Genot C and Meynier A. The initial characteristics of marine oil emulsions and the composition of the media inflect lipid oxidation during *in vitro* gastrointestinal digestion. Food Chem **152**:146-154 (2014).

SUPPLEMENTARY MATERIAL OF MANUSCRIPT 6

INFLUENCE OF MINOR COMPONENTS ON LIPID BIOACCESSIBILITY AND OXIDATION DURING *IN VITRO* DIGESTION OF SOYBEAN OIL

A.S. Martin-Rubio; P. Sopelana; María D. Guillén

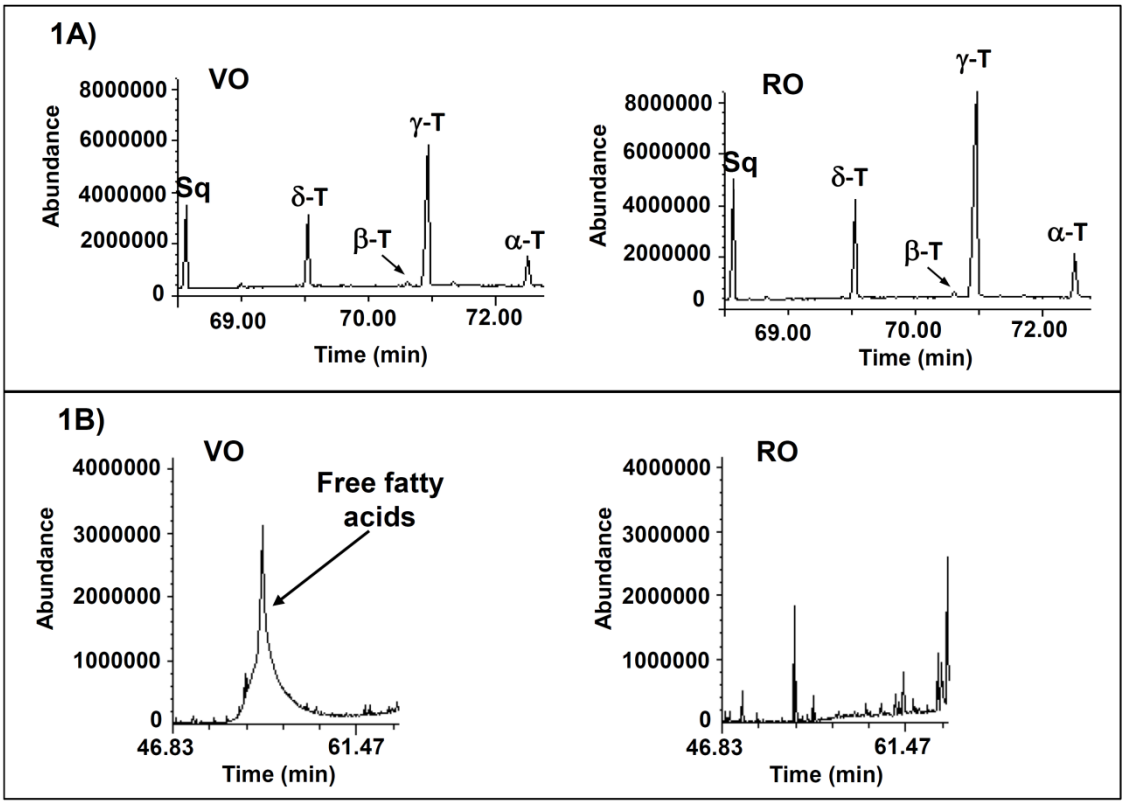


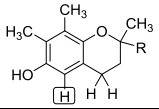
Figure S1. Fragments of the total ion chromatograms obtained by Direct Immersion SPME-GC/MS, according to the methodology described in the work of Alberdi-Cedeño, Ibargoitia, Cristillo, Sopelana and Guillén (Food Chem. 221: 1135-1144, 2017), of the virgin and refined oils (VO and RO, respectively). The indicated peaks in Figure S1A correspond to squalene (Sq), δ-tocopherol (δ-T), β-tocopherol (β-T), γ-tocopherol (γ-T) and α-tocopherol (α-T). The indicated broad peak in VO in Figure S1B corresponds to a mixture of linolenic, linoleic and oleic acids present in this oil.

Table S1. Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study.

Components	**Saliva**	**Gastric juice**	**Duodenal juice**	**Bile juice**
KCl (mmol/L)	12.02	11.06	7.57	5.05
NaCl (mmol/L)	5.10	47.09	119.98	89.99
$NaHCO_3$ (mmol/L)	20.17	-	40.33	68.86
NaH_2PO_4 (mmol/L)	7.40	0.22	-	-
NH_4Cl (mmol/L)	-	5.72	-	-
KH_2PO_4 (mmol/L)	-	-	0.59	-
Na_2SO_4 (mmol/L)	4.79	-	-	-
KSCN (mmol/L)	2.06	-	-	-
$MgCl_2$ (mmol/L)	-	-	0.53	-
$CaCl_2*2H_2O$ (mmol/L)	-	2.72	1.36	1.51
HCl (37%) (mL/L)	-	6.50	0.18	0.15
Urea (mmol/L)	3.33	1.42	1.67	4.16
Glucose (mmol/L)	-	3.61	-	-
Glucuronic acid (mmol/L)	-	0.10	-	-
Uric acid (mmol/L)	0.09	-	-	-
Glucoseamine hydrochloride (mmol/L)	-	1.53	-	-
Bovine serum albumin (g/L)	-	1.00	1.00	1.80
Mucin (g/L)	0.025	3.00	-	-
A. oryzae α-amylase (g/L)	0.29	-	-	-
A. niger lipase (U/mL)	-	100	-	-
Pepsin (g/L)	-	2.50	-	-
Pancreatin (g/L)	-	-	9.00	-
Lipase type II from porcine pancreas (g/L)	-	-	1.50	-
Bovine bile extract (g/L)	-	-	-	18.75
pH	6.8±0.2	1.6±0.3	8.1±0.2	8.2±0.2

Table S1. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of the main protons of glycerides, fatty acids and some oxidation compounds present in the samples before and after *in vitro* digestion. TG: triglycerides; DG: diglycerides; MG: monoglycerides. The signal letters agree with those given in Figures 2 and 3.

Signal	Chemical shift (ppm)	Multiplicity	Functional group	
			Type of protons	Compound
Main acyl groups (AG) and fatty acids (FA)[a,b]				
A	0.88	t	–C**H**$_3$	saturated and monounsaturated **ω-9 AG** and **FA**
	0.89	t	–C**H**$_3$	linoleic **AG** and **FA**
B	0.97	t	–C**H**$_3$	linolenic **AG** and **FA**
C	1.19–1.42	m[*]	–(C**H**$_2$)$_n$–	**AG** and **FA**
D	1.61	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **TG**
	1.62	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **1,2-DG**
	1.63	m	–OCO–CH$_2$–C**H**$_2$–, COOH–CH$_2$–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	1.64	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **2-MG**
E	1.92–2.15	m[**]	–C**H**$_2$–CH=CH–	**AG** and **FA**
F	2.26–2.36	dt	–OCO–C**H**$_2$–	**AG** in **TG**
	2.33	m	–OCO–C**H**$_2$–	**AG** in **1,2-DG**
	2.35	t	–OCO–C**H**$_2$–, COOH–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	2.38	t	–OCO–C**H**$_2$–	**AG** in **2-MG**
G	2.77	t	=HC–C**H**$_2$–CH=	Linoleic **AG** and **FA**
H	2.80	t	=HC–C**H**$_2$–CH=	Linolenic **AG** and **FA**
I	3.65	ddd	ROCH$_2$–CHOH–C**H**$_2$OH	glyceryl group in **1-MG**
J	3.73	m[***]	ROCH$_2$–CH(O*R*')–C**H**$_2$OH	glyceryl group in **1,2-DG**
K	3.84	m[***]	HOC**H**$_2$–CH(O*R*)–C**H**$_2$OH	glyceryl group in **2-MG**
L	3.94	m	ROCH$_2$–C**H**OH–CH$_2$OH	glyceryl group in **1-MG**
M	4.05–4.21	m	ROC**H**$_2$–C**H**OH–C**H**$_2$OR'	glyceryl group in **1,3-DG**
N	4.18	ddd	ROC**H**$_2$–CHOH–CH$_2$OH	glyceryl group in **1-MG**
O	4.22	dd,dd	ROC**H**$_2$–CH(O*R*')–C**H**$_2$OR''	glyceryl group in **TG**
P	4.28	ddd	ROC**H**$_2$–CH(O*R*')–CH$_2$OH	glyceryl group in **1,2-DG**

Q	4.93	m	$HOCH_2–C\underline{\mathbf{H}}(OR)–CH_2OH$	glyceryl group in **2-MG**
R	5.08	m	$ROCH_2–C\underline{\mathbf{H}}(OR')–CH_2OH$	glyceryl group in **1,2-DG**
S	5.27	m	$ROCH_2–C\underline{\mathbf{H}}(OR')–CH_2OR''$	glyceryl group in **TG**
T	5.28–5.46	m	$–C\underline{\mathbf{H}}=C\underline{\mathbf{H}}–$	**AG** and **FA**
			Signals related to oxidation compounds	
Conjugated dienic systems[c,d]				
–	5.44	ddd	$–C\underline{\mathbf{H}}=C\underline{\mathbf{H}}–C\underline{\mathbf{H}}=C\underline{\mathbf{H}}–$	(*Z*,*E*)-conjugated double bonds associated with hydroxy group (OH) in octadecadienoic **AG** and **FA**
-	5.66	dd		
-	5.97	t		
a	6.49	dd		
-	5.51	dtm	$–C\underline{\mathbf{H}}=C\underline{\mathbf{H}}–C\underline{\mathbf{H}}=C\underline{\mathbf{H}}–$	(*Z*,*E*)-conjugated double bonds associated with hydroperoxy group (OOH) in octadecadienoic **AG** and **FA**
-	5.56	ddm		
-	6.00	ddtd		
b	6.58	dddd		
Epoxides				
Epoxy-derivatives				
c1	2.88[e]	m	$-C\underline{\mathbf{H}}O\underline{\mathbf{H}}C-$	(*Z*)-9,10-epoxystearate
c2	2.9[f]		$-C\underline{\mathbf{H}}O\underline{\mathbf{H}}C-$	monoepoxy-octadecenoate groups
c3	2.94****	m	$-C\underline{\mathbf{H}}O\underline{\mathbf{H}}C-$	(*Z*)-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid
Epoxy-hydroxy-derivatives				
c4	2.93[g]	dt	$-C\underline{\mathbf{H}}OHC-CHOH-CH=CH-$	*threo*-11-hydroxy-(E)-12,13-epoxy-(Z)-9-octadecenoate
			γ-Tocopherol	
d	6.36****	s		γ-tocopherol[h]

Abbreviations: t: triplet; m: multiplet; d: doublet. *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***This signal shows different

multiplicity if the spectrum is acquired from the pure compound or taking part in the mixture; ****Assignment made with the aid of standard compounds.

[a]Assignments of AG in TG taken from Guillén, M. D., & Ruiz, A. (2003). *Journal of the Science of Food and Agriculture, 83*, 338–346.

[b]Assignments of AG in partial glycerides (DG and MG) and of FA taken from Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). *Food Research International, 66*, 379-387.

[c]Data taken from Goicoechea, E., & Guillén, M. D. (2010). *Journal of Agricultural and Food Chemistry, 58*, 6234-6245 (conjugated (*Z*,*E*)- and (*E*,*E*)-hydroperoxy-dienes).

[d]Data taken from Dong, M., Oda, Y., & Hirota, M. (2000). *Bioscience, Biotechnology and Biochemistry, 64*, 882-886 (conjugated (*Z*,*E*)-hydroxy-dienes).

[e]Data taken from Du, G., Tekin, A., Hammond, E. G., & Woo, L. K. (2004). *Journal of the American Oil Chemists' Society, 81*, 477–80.

[f]Data taken from Aerts, H. A. J., & Jacobs, P. A. (2004). *Journal of the American Oil Chemists' Society, 81*, 841-846.

[g]Data taken from Garssen, G. J., Veldink, G. A., Vliegenthart, J. F., & Boldingh, J. (1976). *The FEBS Journal, 62*, 33-36.

[h]Assignment taken from Baker, J. K., & Myers, C. W. (1991). *Pharmaceutical Research, 8*, 763-770.

Quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and/or in the lipid extracts of the digestates, and of Lipid Bioaccessibility

Bearing in mind that the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generate it, and that the proportionality constant is the same for all kinds of protons, the area of some spectral signals can be employed to quantify a wide variety of compounds, as detailed below.

A. Lipolytic products and lipid bioaccessibility

The number of moles (N) of fatty acids and all the glycerides present in the lipid samples were expressed as follows:

$N_{2\text{-}MG}=Pc*A_K/4$ [eq. S1]
$N_{1\text{-}MG}=Pc*A_L$ [eq. S2]
$N_{1,2\text{-}DG}=Pc*(A_{I+J}-2A_L)/2$ [eq. S3]
$N_{TG}=Pc*(2A_{4.26\text{-}4.38}-A_{I+J}+2A_L)/4$ [eq. S4]
$N_{1,3\text{-}DG}=Pc*(A_{4.04\text{-}4.38}-2A_{4.26\text{-}4.38}-2A_L)/5$ [eq. S5]
$N_{FA}=(Pc*A_F-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2$ [eq. S6]
$N_{Gol}=(N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2N_{2\text{-}MG}-2N_{1\text{-}MG})/3$ [eq. S7]

where Pc is the proportionality constant existing between the area of the ^{1}H NMR signals and the number of protons that generate them, A_K, A_L, A_{I+J} and A_F are the areas of the corresponding signals indicated in Table S2 and Figure 1, and $A_{4.26\text{-}4.38}$ and $A_{4.04\text{-}4.38}$ represent the areas of the signals between 4.26 and 4.38 ppm, and between 4.04 and 4.38 ppm, respectively.

Using these equations, the molar percentages of the different kinds of glycerides in relation to the total number of moles of glyceryl structures present (NT_{GS}) were determined as follows:

$NT_{GS}=N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{Gol}$ [eq. S8]
$G\%=100N_G/NT_{GS}$ [eq. S9]

where G is each kind of glyceride (TG, 1,2-DG, 1,3-DG, 2-MG and 1-MG) and N_G the respective number of moles.

$Gol\%=100N_{Gol}/NT_{GS}$ [eq. S10]

Likewise, the Lipid Bioaccessibility parameter was calculated as follows:

$L_{BA}\%=100(N_{1\text{-}MG}+N_{2\text{-}MG}+N_{FA})/NT_{AG+FA}$ [eq. S11]
$NT_{AG+FA}=Pc*A_F/2$ [eq. S12]

where NT_{AG+FA} is the total number of moles of AG plus FA present.

B. Molar percentages of the several kinds of acyl groups (AG) and fatty acids (FA)

The molar percentages of linolenic (Ln%), linoleic (L%), oleic (O%) and saturated plus modified (S+M%) AG or FA, in relation to the total number of moles of AG plus FA (NT_{AG+FA}) present in the starting oils and in the lipid extracts of the corresponding digestates were estimated as follows:

$Ln\% = 100*(Pc*A_H/4)/NT_{AG+FA}$ [eq. S13]

$L\% = 100*(Pc*A_G/2)/NT_{AG+FA}$ [eq. S14]

$O\% = U\% - L\% - Ln\%$ [eq. S15]

$(S+M)\% = 100 - U\%$ [eq. S16]

where A_E, A_H and A_G are the areas of signals E, H and G indicated in Table S2 and Figure 1. It must be noted that due to partial overlapping of signals H and G, a previous correction of both areas must be undertaken to properly assess the area corresponding to each one of them. For this purpose, trilinolein and trilinolenin, acquired from Sigma-Aldrich, were used as references.

C. Oxidation compounds and γ-tocopherol

The concentration of the several kinds of oxidation compounds, as well as that of γ-tocopherol, expressed as millimoles per mole of the sum of AG+FA present, was estimated by using the following equations:

$[OP] = [(A_{OP}/n)/(A_F/2)]*1000$ [eq. S17]

$[\gamma\text{-T}] = [A_{\gamma T}/(A_F/2)]*1000$ [eq. S18]

where A_{OP} and $A_{\gamma T}$ are the areas of the signals selected for the quantification of each oxidation product (OP) and of γ-T, respectively (see Table S2), and n the number of protons that generate each signal. In the case of epoxides (signals "c1-c4" in Table S2), the overlapped area due to the side band of *bis*-allylic protons signals G and H must be subtracted. Although the epoxy-compounds given in Table S1 can contribute to signal "c" either with one ("c4") or with two protons ("c1-c3"), it has been assumed that all contribute with two protons.

Standard compounds used for the identification of volatile compounds by SPME-GC/MS

Pentanal (base peak: 86), hexanal (100), (*E*)-2-pentenal (84), (*E*)-2-heptenal (112), (*Z*,*E*)-2,4-heptadienal (110), (*E*,*E*)-2,4-heptadienal (110), (*E*,*E*)-2,4-nonadienal (138), (Z,E)-2,4-decadienal (152), (*E*,*E*)-2,4-decadienal (152) and 2-pentyl-furan (138).

MANUSCRIPT 7

THE KEY ROLE OF OVALBUMIN IN LIPID BIOACCESSIBILITY AND OXIDATION PRODUCT PROFILE DURING THE *IN VITRO* DIGESTION OF SLIGHTLY OXIDIZED SOYBEAN OIL

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Food and Function

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ABSTRACT

The behaviour of slightly oxidized virgin and refined soybean oils during *in vitro* digestion was studied by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry. The main objectives were to analyze lipolysis extent and oxidation during digestion, and to assess the impact of two different proportions of ovalbumin on both processes. At the same time γ-tocopherol fate was monitored, when possible, by ^{1}H NMR. The results reveal that the initial oxidation degree of the oils negatively influences the lipolysis extent, reducing the bioaccessibility of the major oil components, which include some essential fatty acids. Although the low ovalbumin proportion tested does not significantly affect lipolysis, this is greatly enhanced when ovalbumin is added at a high level, improving lipid bioaccessibility. It has also been shown that oxidation does not seem to have occurred to a great enough extent during digestion for it to be detected from polyunsaturated acyl group degradation. Moreover, the changes observed in the oxidation product profile of the starting oils after digestion can be considered to be due mainly to the transformation of the initially present hydroperoxides, whose concentration diminishes in the digested samples to give hydroxy-dienes, epoxides and aldehydes. In presence of a high ovalbumin proportion, hydroperoxide reduction to hydroxy-dienes is favoured and lower levels of aldehydes and epoxides are observed. This latter could be due to a diminution in their generation and/or to their reaction with ovalbumin. A high proportion of this protein in the system also increases γ-tocopherol bioaccessibility.

1. INTRODUCTION

Knowledge of the processes undergone by food components during digestion represents a great challenge due to their health-related implications and to the multitude of factors that can influence the course of such processes. In this regard, lipids deserve special attention since they are vehicles of both major and minor essential nutrients that are exposed to different types of reactions along the gastrointestinal tract. These may include hydrolysis, oxidation or interactions with other food components,[1-4] which will finally determine not only the bioaccessibility of these nutrients but also the nature of the compounds present in the food bolus.

With regard to oxidation reactions, their occurrence throughout the digestion process has been demonstrated by several authors.[5-8] As a consequence, the nutritional quality and safety of food can be adversely affected, since not only losses of essential lipophilic nutrients like polyunsaturated fatty acids or certain antioxidants can occur,[3,6,9] but also compounds with negative biological implications, such as oxygenated α,β-unsaturated aldehydes,[10] could be generated[5]. In this context, the unsaturation degree of lipids and their oxidation extent have been found to affect the oxidation progress throughout digestion.[3,6,7,9]

Another element that can influence the evolution of digested lipids in the gastrointestinal tract is the presence of other nutrients, among them proteins. In this sense, Nieva-Echevarría, Goicoechea and Guillén[4] investigated the effect of two proteins (ovalbumin and soy protein isolate) on the *in vitro* digestion process of slightly oxidized sunflower and flaxseed oils, using quite a high proportion of proteins to lipids, in order to simulate the ratio that might exist between these two types of nutrients in a model food system. It was observed that the presence of protein during the *in vitro* digestion of edible oils affects the extent of lipolysis and of oxidation reactions taking place during this process; this can be explained both by the emulsifying properties of this type of compounds[11] and by the antioxidant ability of proteins and peptides,[12] which might favour and limit, respectively, both types of reactions. However, the impact of proteins on oxidation reactions also depends on their composition, since iron-containing proteins (heme proteins) could promote oxidation during digestion.[13]

Bearing in mind all the above, and with the aim of contributing towards building a solid body of knowledge concerning the complex process of lipid digestion, which is directly relevant to the health effects of dietary lipids, the study under *in vitro* digestion

conditions of slightly oxidized virgin and refined soybean oils was tackled in this work. These oils were selected as examples of linoleic-rich vegetable oils but, unlike the sunflower oil previously studied,[6] with a certain proportion of linolenic acyl groups and a different profile in minor components when compared with the latter.[14] The main difference between these two oils in their unoxidized state laid in their composition in minor components[15]. In this context, the fate during *in vitro* digestion of γ-tocopherol (γ-T), the main tocopherol present in soybean oil,[14] was monitored when possible. The use of slightly oxidized oils will show the extent to which their initial lipid degradation status can affect the progress of lipolysis and oxidation during *in vitro* digestion. In addition, the influence on these processes of the presence of two fairly different proportions of ovalbumin, a non-heme protein widely employed as an ingredient in many food formulations, was also addressed.

The techniques employed were Proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (SPME-GC/MS). ^{1}H NMR allows one to study simultaneously the lipolysis degree, the oxidation extent and the evolution of γ-T through the *in vitro* digestion of the selected oils. This technique is able to provide information about oxidation products that can be either bound or unbound to acyl groups. SPME-GC/MS, in turn, constitutes a complementary and very useful tool that, thanks to its higher sensitivity and specificity, makes it possible to obtain further information in relation to the occurrence of oxidation and even to confirm the results obtained by using ^{1}H NMR.

2. MATERIALS AND METHODS

2.1. Samples subjected to *in vitro* digestion

The samples subjected to *in vitro* digestion were two slightly oxidized commercial soybean oils: one virgin (VSx) and the other refined (RSx). They were obtained after submitting the corresponding fresh oils to accelerated storage conditions, as in previous works.[16,17] To this aim, 10 g of each of the fresh oils were weighed in glass Petri dishes of 80 mm diameter and placed in a convection oven (Memmert GmbH+Co, Schwabach, Germany) at 70ºC with circulating air for 4 and 5 days in the case of the virgin and the refined oils respectively, in order to obtain oils with a similar oxidation degree.

In addition, samples were prepared by mixing each of these two slightly oxidized oils with two different proportions of ovalbumin; 0.26 g of ovalbumin per g of oil in the samples with the low level of ovalbumin (VSx+LO and RSx+LO) and 2.6 g of

ovalbumin per g of oil in the ones with the high ovalbumin proportion (VSx+HO and RSx+HO), this latter corresponding to a dose previously used in another study.[4] Food grade ovalbumin was acquired from a protein manufacturer (Apasa SA, Astigarraga, Spain).

2.2. *In vitro* digestion

Samples (0.5 g) of the two oils, both in the absence and in presence of ovalbumin, were digested following the same procedure as in previous works,[6,7] based on the static *in vitro* gastrointestinal model developed by Versantvoort, Oomen, Van de Kamp, Rompelberg and Sips[18] and slightly modified in our laboratory in order to reach a higher level of lipolysis.[19] This *in vitro* digestion model involves a three-step procedure to simulate digestive processes in the mouth, stomach, and small intestine, by sequentially adding the corresponding digestive juices. More details about the *in vitro* digestion procedure can be found in the supplementary material.

All the reagents used were acquired from Sigma-Aldrich (St. Louis, MO, USA). Two digestion experiments, each including duplicate samples of the two studied oils, were performed. Blank samples corresponding to the mixture of juices submitted to digestive conditions were also taken for further analysis.

2.3. Lipid extraction of the digestates

Lipids of the digestates were extracted using dichloromethane as solvent (HPLC grade, Sigma-Aldrich) and following the same methodology as in previous studies,[20] which involves a three-stage liquid-liquid extraction process with 20 ml of dichloromethane each. Afterwards, to ensure a complete protonation of fatty acids and/or the dissociation of the potential salts formed, the remaining water phase was acidified to pH 2 with HCl (37%) and a second extraction was carried out, also in three steps.[6,7] Given that in the case of the samples with a high proportion of ovalbumin a strong emulsion is formed when mixing the digested sample with the extraction solvent, which makes it difficult to separate the aqueous and lipid phases, the extraction was performed with the aid of a centrifuge in order to break up this emulsion. For this purpose, a Sigma 3K30 centrifugal machine working at 10,000 rpm was used (Sigma Laboratory Centrifuges, Germany), each extraction step lasting 10 min. This same extraction procedure was used for all the samples, without any differences in extraction efficiencies achieved when using either centrifugation or extraction with separating funnels in the case of the samples without ovalbumin and with a low proportion of this

protein. All the dichloromethane extracts of each sample were mixed and the solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. Afterwards, these extracts were stored at -80 ºC until their analysis.

2.4. Analysis by ^{1}H NMR

2.4.1. Operating conditions

The ^{1}H NMR spectra of the starting oils (VSx and RSx), of the lipid extracts of their corresponding digestates (DVSx and DRSx), and of the extracts obtained from the digestates of the oil samples containing a low proportion of ovalbumin (D(VSx+LO) and D(RSx+LO)), and a high proportion of this protein (D(VSx+HO) and D(RSx+HO)) were acquired in quadruplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned lipid samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform, which contained tetramethylsilane (TMS) as internal reference (Cortec, Paris, France). The acquisition conditions were the same as those used in previous studies.[21,22]

2.4.2. Identification of some lipid components

The identification of the oil acyl groups, of partial glycerides, of γ-T and of the oxidation products present in the various samples was carried out on the basis of the signal assignments shown in Table S1 (see supplementary material), made from bibliographic data and with the aid of several standard compounds. These were: γ-T, acquired from Sigma-Aldrich, and *cis*-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid, acquired from Cymit Quimica (Barcelona, Spain).

2.4.3. Quantification from ^{1}H NMR spectral data

Bearing in mind that the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generate it, and that the proportionality constant is the same for all kinds of protons, the area of some spectral signals can be employed to quantify: i) the molar percentages of the different types of glycerides; ii) the molar concentrations of linolenic and linoleic acyl groups+fatty acids, referring to the total of acyl groups+fatty acids; iii) the molar concentration of γ-T, referring to the total of acyl groups+fatty acids; and iv) the molar concentrations of several oxidation compounds present in the starting oils and/or in the lipid extracts of the digestates, referring to the total of acyl groups+fatty acids, following the procedures indicated below.

***Various types of glycerides and glycerol*.** The molar percentages of triglycerides (TG%), diglycerides (1,2-DG% and 1,3-DG%), monoglycerides (2-MG% and 1-MG%) and glycerol in relation to the total number of glyceryl structures present in the lipid samples were determined using the equations developed and validated in previous studies.[20,23] All these equations are given as supplementary material (see equations [S1-S10]).

Another parameter relating to lipolysis extent can also be determined from the data relative to the different types of glycerides, namely lipid bioaccessibility (L_{BA}). This takes into account only the molecules that are directly absorbable, monoglycerides and fatty acids, in relation to the total number of acyl groups+fatty acids present in the sample. This parameter was calculated by using equations [S11 and S12].

***Lipid composition*.** The concentrations of linolenic and linoleic acyl groups+fatty acids were estimated in millimoles per mole of the sum of acyl groups+fatty acids (mmol/mol AG+FA), both in the starting oils and in the lipid extracts of the digested samples, by using equations [S13 and S14].

***Oxidation compounds*.** The molar concentrations of the different types of oxidation products present in the starting oils and in the lipid extracts of the digested samples, referring to the total of acyl groups+fatty acids and expressed as mmol/mol AG+FA, were also estimated as in a previous study[4] by using equation [S15].

It must be noticed that, as shown in Figure S1 for D(VSx+HO) (see supplementary material), in the case of the samples digested with a high ovalbumin proportion, signals coming from the protein used are perceived in the ^{1}H NMR spectra of their corresponding digestates. Some of these overlap with the signals used to estimate both the concentration of epoxides giving signal at 2.9 ppm (see signals "e1-e4" in Table S1) and the molar percentages of 1,3-DG and TG (see signals "M" and "O", respectively), so their contribution must be subtracted, especially in the case of epoxides. For this purpose, ovalbumin was added to the digestive juices after undergoing the digestion process and this mixture was extracted in the same way as the rest of digested samples; the relative areas of the different ovalbumin signals can be determined from the corresponding ^{1}H NMR spectrum, free of lipids. This enables one to subtract the area of the signals overlapping with those of lipid components in the spectra of the extracts obtained from the digested oil samples taking as a reference the signals that do not overlap with one another (see Figure S1). It is worth noticing that while some signals

coming from ovalbumin also overlap with those of *bis*-allylic protons (signals "H+G"), their area is very small in relation to that of the latter, and so can be ignored.

γ-T. The concentration of γ-T was estimated from signal "h" (see Table S1), in the same way as described for oxidation compounds (see equation [S16]).

2.5. Study of oxidation during *in vitro* digestion by Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry

2.5.1. Solid Phase Microextraction (SPME)

The extraction of the volatile components of the several digestates (0.5 g in 10 ml screw-cap vials) was carried out automatically by using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA), in the same way as in previous works.[4,6,7]

Given that the nature of the samples subjected to the digestion process (oil samples) is very different from that of the digestates (with a basically aqueous matrix), it is necessary to prepare mixtures of the non-digested oils with the digestive juices after being submitted to the digestion process, in the same proportions as in the digestates; this enables one to make an adequate assessment by SPME-GC/MS of the changes taking place throughout the *in vitro* digestion process. Therefore, the samples subject of study, which were analyzed in duplicate, were the following: i) the digestates both of the slightly oxidized soybean oil samples (DVSx and DRSx), and of the slightly oxidized oil samples plus ovalbumin at the two levels of concentration (D(VSx+LO), D(RSx+LO), D(VSx+HO) and D(RSx+HO)); ii) the digestive juices after being submitted to digestion conditions (DJ); and iii) the mixtures made up of the starting oils and DJ (VSx+DJ and RSx+DJ).

The fiber used, coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm long), was acquired from Supelco (Sigma-Aldrich); this was inserted into the headspace of the sample and was maintained for 55 min at 50 ºC, after a pre-equilibration time of 5 min.

2.5.2. Gas chromatography/Mass Spectrometry (GC/MS) study

Operating conditions. The fiber containing the extracted components was desorbed for 10 min in the injection port (splitless mode with 5 min purge time) of a 7890A gas chromatograph equipped with a 5975C inert MSD with Triple Axis Detector (Agilent Technologies) and a computer operating with the ChemStation program. The column used was a fused-silica capillary column (60 m long x 0.25 mm inner diameter x 0.25

μm film thickness, from Agilent J&W Advanced Capillary GC Columns), coated with a non-polar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The operating conditions were as follows: the oven temperature was set initially at 50º C (5 min hold) and increased to 290 ºC at 4 ºC/ min (2 min hold); the temperatures of the ion source and of the quadrupole mass analyser were kept at 230 ºC and 150 ºC respectively; helium was used as carrier gas at a pressure of 18.611 psi; injector temperature was held at 250 ºC; mass spectra were recorded at an ionization energy of 70 eV, and the data acquisition mode employed was scan. In order to avoid carry-over problems between samples, after each run the fiber was submitted to heating at 250 ºC for 20 min in the Fiber Cleaning and Conditioning Station of the CombiPAL autosampler.

A reference sample of known composition was periodically analyzed in order to verify not only the extraction efficiency and repeatability of the SPME fiber but also the performance of the equipment.

***Identification of the compounds present in the headspace of the samples*.** Most of the components were identified by using commercial standards, acquired from Sigma-Aldrich. These were: pentanal (base peak: 86), hexanal (100), heptanal (114), octanal (128), nonanal (142), (*E*)-2-pentenal (84), (*E*)-2-hexenal (98), (*E*)-2-heptenal (112), (*E*)-2-octenal (126), (*E*)-2-nonenal (140), (*Z*,*E*)-2,4-heptadienal (110), (*E*,*E*)-2,4-heptadienal (110), (*Z*,*E*)-2,4-nonadienal (138), (*E*,*E*)-2,4-nonadienal (138), (*Z*,*E*)-2,4-decadienal (152), (*E*,*E*)-2,4-decadienal (152) and 2-pentyl-furan (138).

When standards were not available, matching of the mass spectra with those obtained from scientific literature or from a commercial library at higher than 85% (Wiley W9N08, Mass Spectral Database of the National Institute of Standards and Technology (NIST)), was taken as identification criterion.

Semi-quantification of the compounds present in the headspace of the samples. This was based on the area counts of the base peak (Bp) of the mass spectrum of each compound divided by 10^6. When the Bp of a compound overlapped with the same ion peak of the mass spectrum of another compound, an alternative ion peak was selected for the semi-quantification of the former. The area counts thus determined are useful for the comparison of the abundance of each compound in the different samples.

2.6. Statistical analysis

The significance of the differences between the various determinations made among the samples was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Characterization of the starting oil samples

As mentioned in section 2.1, the samples selected for the study were two slightly oxidized soybean oils: one virgin and the other refined. The concentrations of their polyunsaturated acyl groups (linolenic and linoleic), and of some oxidation compounds, all of them determined by ^{1}H NMR and expressed in mmol/mol AG+FA, are given in Tables 1 and 2, respectively. Table 2 reveals that both oils have a low oxidation level, their concentration of hydroperoxides (signals "c" and "b" in Figure 1) being very low, with values of 11.16 ± 0.22 mmol/mol AG+FA in the virgin oil and 11.97 ± 1.21 mmol/mol AG+FA in the refined one. Both samples also have small concentrations of epoxides (see signal "e" in Figure 1) and aldehydes are not detected in the ^{1}H NMR spectra of any of these oils. A small amount of hydroxy (*Z*,*E*)-conjugated dienes can also be noticed in the virgin oil (signal "a" in Figure 1). The concentrations of tocopherols and squalene are higher in the oxidized refined oil than in the oxidized virgin oil, and the opposite is true for free fatty acids (see Table S2 in the supplementary material).

Table 1. Concentrations of polyunsaturated AG+FA, expressed as mmol/mol AG+FA, in the slightly oxidized virgin and refined soybean oil samples, before (VSx and RSx) and after *in vitro* digestion in the absence of ovalbumin (DVSx and DRSx), with a low proportion of ovalbumin added (D(VSx+LO) and D(RSx+LO)) and with a high proportion of ovalbumin (D(VSx+HO) and D(RSx+HO)). Different letters within each column indicate a significant difference among the samples corresponding to the same type of oil (virgin or refined) ($p < 0.05$). AG+FA: acyl groups+fatty acids.

	Linolenic	**Linoleic**
VSx	51.4 ±1.7a	431.3 ± 2.9a
DVSx	49.8 ± 3.2a	424.9 ± 12.5a
D(VSx+LO)	50.0 ± 1.0a	422.7 ± 8.4a
D(VSx+HO)	50.7 ± 2.6a	426.1 ± 3.6a
RSx	44.3 ± 1.8a	459.5 ± 12.7a
DRSx	42.5 ± 3.0a	451.4 ± 13.3a
D(RSx+LO)	42.7 ± 2.8a	452.3 ± 0.7a
D(RSx+HO)	43.8 ± 2.1a	454.2 ± 2.2a

3.2.1. Lipolysis in the samples digested without ovalbumin

As one would expect, the main glyceride structures present in the non-digested oils were TG, together with a much lower proportion of 1,2-DG. This can be observed in Table 3, which shows the molar percentages of the several kinds of glycerides and of glycerol, in all the studied samples before and after digestion, in relation to the total number of glyceryl structures. As this table reveals, the molar percentage of TG greatly diminishes after digestion, reaching values of 30.47 and 31.38% in the digested virgin and refined oil samples, respectively. These figures are of a similar order to those obtained in earlier works conducted with slightly oxidized samples of other highly unsaturated oils like sunflower[6] and flaxseed;[7] and the same can be said of the relative proportions of the different types of partial glycerides generated during the lipolytic process. Of these, 2-MG and 1,2-DG are the most abundant, while the molar percentages of 1-MG and 1,3-DG are much lower.

3.2. Extent of lipolysis through the *in vitro* digestion

However, the TG molar percentages in the digested samples are higher than those found when the same unoxidized soybean oils were digested, which were close to 22%.[15] The lower hydrolysis extent found in the oxidized oils, which is in agreement with the findings of other authors,[24,25] could be explained by decreased lipase activity

Table 2. Concentrations of the several kinds of oxidation compounds, expressed in mmol/mol of acyl groups+fatty acids, present in the virgin and refined slightly oxidized soybean oil samples, before (VSx and RSx) and after *in vitro* digestion in the absence of ovalbumin (DVSx and DRSx), with a low proportion of ovalbumin added (D(VSx+LO) and D(RSx+LO)) and with a high proportion of ovalbumin (D(VSx+HO) and D(RSx+HO)). Different letters within each column indicate a significant difference among the samples corresponding to the same type of oil (virgin or refined) ($p < 0.05$). CD-OOH: conjugated hydroperoxy-dienes; CD-OH: conjugated hydroxy-dienes.

	(*Z*,*E*)-CD-OOH	**(*E*,*E*)-CD-OOH**	**Total CD-OOH**	**(*Z*,*E*)-CD-OH**	**(*E*,*E*)-CD-OH**	**Total CD-OH**	**Epoxides**	**n-Alkanals**
VSx	5.19 ± 0.31c	5.97 ± 0.13c	11.16 ± 0.22c	1.16 ± 0.29a	-	1.16 ± 0.29a	1.42 ± 0.05a	-
DVSx	3.60 ± 0.28b	2.64 ± 0.26b	6.24 ± 0.38b	1.54 ± 0.09b	1.99 ± 0.15a	3.53 ± 0.19b	5.03 ± 1.23b	0.71 ± 0.21b
D(VSx+LO)	3.62 ± 0.39b	2.55 ± 0.34b	6.17 ± 0.70b	1.84 ± 0.05b	2.03 ± 0.10a	3.87 ± 0.15b	4.93 ± 0.98b	0.49 ± 0.11b
D(VSx+HO)	1.74 ± 0.41a	0.82 ± 0.09a	2.56 ± 0.49a	4.19 ± 0.38c	3.79 ± 0.33b	7.97 ± 0.44c	3.83 ± 0.29b	0.28 ± 0.04a
RSx	5.87 ± 0.63c	6.09 ± 0.62c	11.97 ± 1.21c	-	-	-	3.63 ± 0.14a	-
DRSx	4.67 ± 0.80bc	2.53 ± 0.18b	7.20 ± 0.95b	1.42 ± 0.15a	2.10 ± 0.36a	3.52 ± 0.44a	6.95 ± 0.69c	0.58 ± 0.07b
D(RSx+LO)	4.37 ± 0.04b	2.48 ± 0.12b	6.85 ± 0.17b	1.63 ± 0.19a	2.54 ± 0.18a	4.16 ± 0.26a	6.53 ± 0.41c	0.52 ± 0.11b
D(RSx+HO)	1.68 ± 0.13a	1.05 ± 0.14a	2.73 ± 0.21a	4.16 ± 0.48b	3.92 ± 0.41b	8.08 ± 0.89b	5.54 ± 0.55b	0.23 ± 0.04a

-: not detected

due to the incipient oxidation level of the samples. In fact, it has been reported that hydroperoxides, present in the oxidized oil samples of this work (see section 3.1), could react with amino acid residues of proteins,[26,27] which could lead to a decrease in their functionality in the case of enzymes**.** Thus, decreased activity of several digestive enzymes, including porcine pancreatic lipase, which is the main lipase used in this study, due to their interaction with lipid hydroperoxides has been reported by some researchers.[28-30] In addition, other authors[31] have described that the presence of dimers and polymers generated due to lipid oxidation impairs TG hydrolysis by negatively affecting the activity of pancreatic lipase; however, this type of oxidation products has not been determined in the present work.

The lower lipolysis degree achieved in the digestates of the oxidized oil samples here studied when compared with those of their respective fresh oil samples[15] is also noticed in the lower molar percentage of glycerol in the former (23.12 and 20.14% in the virgin and in the refined oil samples, respectively) than in the latter (near 30%).

3.2.2. Lipolysis in the samples containing ovalbumin

The addition of a low proportion of ovalbumin (0.26 g ovalbumin per g of oil) to the slightly oxidized soybean oil samples does not cause significant variations in the lipolytic process, as can be seen in Table 3. However, in the samples containing a high level of this protein (2.6 g per g of oil), a very high lipolysis degree is achieved; this can be observed in Table 3, which shows percentages of remaining TG of 5.26% and 7.27% in the digestates of the virgin and the refined oil samples, respectively. The great increment observed in lipolysis could be due to the emulsifying effect of the ovalbumin added,[32] possibly increased during *in vitro* digestion due to proteolysis;[33] this is expected to improve the dispersion of the oil into the aqueous phase and to reduce the oil droplets size, thus increasing the lipid area exposed to the action of lipases.

It must also be highlighted that the lipolysis extent in the samples digested with a high ovalbumin proportion was considerably higher even than that achieved after the *in vitro* digestion of the same unoxidized soybean oils.[15] Therefore, in the light of these findings, one could conclude that the presence of this high level of ovalbumin clearly favours lipid digestion by improving lipolysis and, in consequence, the bioaccessibility of some lipophilic nutrients. These outcomes confirm those results observed in previous works concerning the effect of the same ovalbumin proportion here tested on the lipolysis degree of slightly oxidized sunflower and flaxseed oils.[4,6,7]

Another noteworthy fact about the samples digested with a high proportion of ovalbumin is the important surge in the molar percentage of 2-MG in comparison with the rest of the digested samples. This reveals that, despite the high extent of TG hydrolysis observed in these samples, the increased lipolytic process does not seem to go beyond 2-MG; consequently, a great increase in glycerol molar percentage is not observed (see Table 3). The buffer capacity of ovalbumin might be involved in this finding, since it has been reported that a more alkaline pH in aqueous media can restrict the isomerization reactions of 2-MG to 1-MG;[34] this would limit the MG hydrolysis to give rise to glycerol and fatty acids, since pancreatic lipase acts specifically on 1-MG.[35] In this sense, it is worth mentioning that a higher pH was found during the gastric stage of the *in vitro* digestion process of the samples with a high proportion of ovalbumin in comparison with the rest of the samples. This potential influence of pH, coupled with the higher hydrolysis of TG, and by extension, of 1,2-DG, might explain the accumulation of 2-MG; however, the influence of other factors should not be discarded either. This high proportion of 2-MG could in turn also contribute towards favouring the lipolytic process to a certain extent since, according to some authors,[36] monoglycerides are highly surface-active products that can help to decrease the mean droplet diameter.
With respect to the molar percentages of 1,2-DG and 1,3-DG, these are somewhat higher and lower, respectively, than in the samples digested without ovalbumin and with a small proportion of this protein. These outcomes might be a consequence of the enhanced lipolysis degree reached when digestion is performed in presence of a high ovalbumin level.

3.3. Lipid bioaccessibility

Of the parameters employed to evaluate the extent of lipolysis, lipid bioaccessibility (L_{BA}) can be considered of particular interest, since it indicates the proportion of absorbable molecules (fatty acids and MG) with respect to the sum of acyl groups+fatty acids.[3] While the addition of a low proportion of ovalbumin does not affect this parameter (see Table 3), when a high level of ovalbumin is present in the medium, L_{BA} increases considerably, reaching values of 81.50% and 77.79% in the digestates of the virgin and of the refined oil samples, respectively. This indicates that the presence of this high proportion of protein can improve the absorption of dietary lipids, mainly due to the greatly increased 2-MG molar percentage.

3.4. Assessment by ^{1}H NMR of lipid oxidation during *in vitro* digestion

3.4.1. Study of the changes undergone by polyunsaturated acyl groups+fatty acids throughout *in vitro* digestion

The molar concentrations of the polyunsaturated groups in relation to the total of acyl groups+fatty acids in all the digested samples are shown in Table 1, together with those corresponding to the starting oils. Comparing the data before and after the *in vitro* digestion process, both in the absence and in presence of the two different ovalbumin proportions, statistically significant changes are not noticed. This reveals that, despite the initial oxidative degradation of the soybean oils here studied, oxidation occurrence during *in vitro* digestion cannot be inferred from acyl group degradation. However, this does not mean that some oxidation, undetectable through variations in the concentrations of polyunsaturated groups determined from ^{1}H NMR spectral data, has not taken place.

3.4.2. Study of the changes in the oxidation product profile caused by *in vitro* digestion

***Evolution of hydroperoxide concentration*.** The *in vitro* digestion process causes a diminution in the concentration of hydroperoxides initially present in the oil samples, monitored through their associated conjugated dienes, this being more pronounced for the hydroperoxy-(*E*,*E*)-isomers than for the (*Z*,*E*)-ones (see Figure 1 and Table 2). This does not necessarily mean that hydroperoxides are not generated during *in vitro* digestion, but that the rate of transformation overtakes that of formation. The same is observed when the samples are digested in presence of ovalbumin. However, while a low proportion of this protein does not significantly affect the hydroperoxide levels found after digestion, a more pronounced decline in the concentration of conjugated (*Z*,*E*)-, and especially of (*E*,*E*)-hydroperoxy-dienes occurs in the samples digested with a high ovalbumin proportion in comparison with the rest of digested samples. At this point, it is worth noticing that conflicting results can be found about the stability and evolution of hydroperoxides in the gastrointestinal tract, as discussed by Márquez-Ruiz and coworkers.[24] Thus, according to some authors,[37,38] hydroperoxides decompose in the stomach giving rise to other oxidation products, while other researchers have proved that hydroperoxides can reach the intestine and then be absorbed.[39] In our case, although hydroperoxide concentration diminishes during digestion, some part of them remains in the digestates.

Table 3. Molar percentages of triglycerides (TG%), diglycerides (1,2-DG% and 1,3-DG%), monoglycerides (2-MG% and 1-MG%) and glycerol (Gol%) in relation to the total number of glyceryl structures present in the lipid samples, together with lipid bioaccessibility (L_{BA}) parameter, in the slightly oxidized virgin and refined soybean oil samples, before (VSx and RSx) and after *in vitro* digestion in the absence of ovalbumin (DVSx and DRSx), with a low proportion of ovalbumin added (D(VSx+LO) and D(RSx+LO)), and with a high proportion of ovalbumin (D(VSx+HO) and D(RSx+HO)). Different letters within each column indicate a significant difference among the samples corresponding to the same type of oil (virgin or refined) ($p < 0.05$).

	TG%	**1,2-DG%**	**1,3-DG%**	**2-MG%**	**1-MG%**	**Gol%**	**L_{BA}**
VSx	99.50 ± 0.05c	0.29 ± 0.02a	-	-	-	nd	nd
DVSx	30.47 ± 4.11b	14.15 ± 2.25b	3.01 ± 1.42b	22.84 ± 3.53a	6.41 ± 0.60b	23.12 ± 4.31a	58.09 ± 6.49a
D(VSx+LO)	29.56 ± 3.00b	15.35 ± 1.34bc	2.98 ± 0.68b	22.96 ± 2.34a	4.31 ± 1.24a	24.84 ± 1.86a	58.22 ± 3.93a
D(VSx+HO)	5.26 ± 0.08a	17.86 ± 0.14cd	1.99 ± 0.25a	39.17 ± 1.69b	6.75 ± 0.87b	28.97 ± 0.78a	81.50 ± 0.01b
RSx	99.40 ± 0.08c	0.62 ± 0.03a	-	-	-	nd	nd
DRSx	31.38 ± 4.51b	14.70 ± 1.40bc	4.26 ± 1.17b	22.67 ± 1.23a	6.86 ± 0.88b	20.14 ± 5.20a	55.98 ± 5.94a
D(RSx+LO)	30.80 ± 3.391b	16.29 ± 1.50bc	4.11 ± 1.84b	23.77 ± 1.69a	4.92 ± 0.67ab	20.10 ± 5.33a	55.60 ± 5.45a
D(RSx+HO)	7.27 ± 0.03a	20.88 ± 0.12d	1.53 ± 0.01a	38.37 ± 0.16b	5.32 ± 0.04ab	26.63 ± 0.28a	77.79 ± 0.12b

-: not detected; nd: not determined

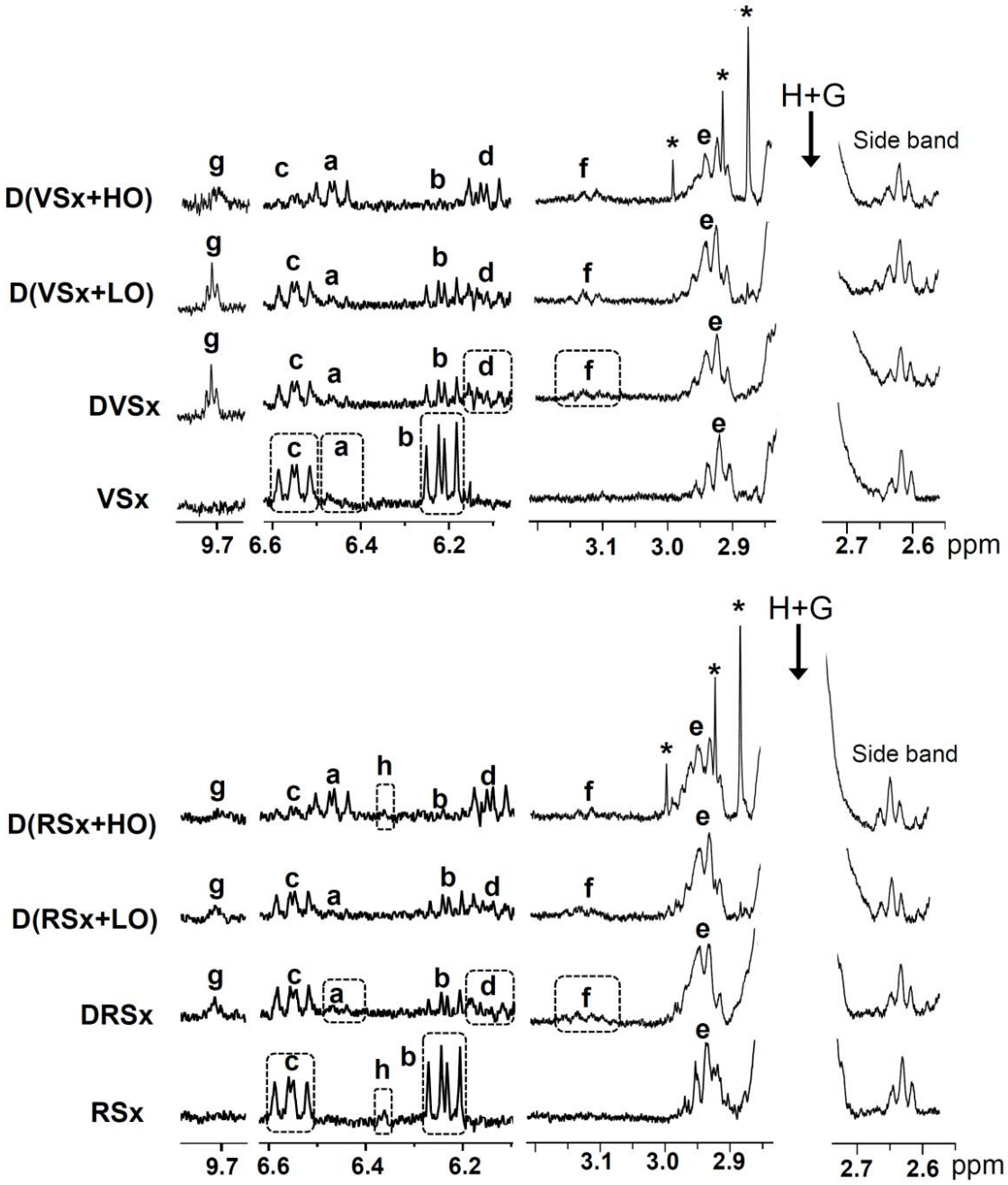

Figure 1. Enlargements of some regions of the ^{1}H NMR spectra of the slightly oxidized virgin and refined oils (VSx and RSx), and of the lipid extracts obtained after their *in vitro* digestion process in the absence of ovalbumin (DVSx and DRSx), as well as in the presence of a low (D(VSx+LO) and D(RSx+LO)) and a high proportion of ovalbumin (D(VSx+HO) and D(RSx+HO)). The signal letters agree with those in Table S1, considering that "e" includes signals "e1-e4" and "f" signals "f1+f2". Signals marked with an asterisk are considered to come from the ovalbumin sample used. The plots corresponding to the same ^{1}H NMR spectral region are presented at a fixed value of absolute intensity, for them to be valid for comparative purposes.

Formation of conjugated hydroxy-dienes. The generation of both conjugated (*Z,E*)- and (*E,E*)-hydroxy-dienes during digestion is observed in all the studied samples (see Figure 1, signals "a" and "d", and Table 2). This suggests that a small part of the hydroperoxides present in the starting oils is reduced to hydroxy-dienes during digestion. Actually, the reduction of hydroperoxides to hydroxides throughout this process has also been reported previously both *in vitro*[6] and *in vivo*.[37-39] While an increase of a similar order is observed in the concentration of conjugated hydroxy-dienes in the digestates of oils without or with a low proportion of ovalbumin, this increase is noticeably greater in the digestate of the oil with a high ovalbumin level. This confirms previous findings[4] and evidences the reducing effect of this protein.

Generation of epoxides. The concentration of epoxides exhibits an increment after digestion in all the studied samples (see Table 2), of a similar order in both types of soybean oils. This is due to an increase in the levels of the epoxides initially present in the starting oils, but also to the formation of other types of compounds, also tentatively identified as epoxides, which give signal "f" (see Figure 1). Therefore, although hydroperoxides and aldehydes are generally employed to assess the occurrence of lipid oxidation under diverse conditions including gastrointestinal ones,[8,9] epoxides should not be ignored as oxidation markers; this reinforces previous findings of this research group, since the generation of epoxides under *in vitro* digestion conditions had also been observed in slightly oxidized flaxseed oil.[7]

In line with all the above mentioned, the generation of epoxides during digestion does not seem to be influenced by a low ovalbumin concentration. However, a less pronounced increment of the level of epoxides is noticed when a high proportion of ovalbumin is present in the system (see Table 2).

Generation of aldehydes. As could be expected, taking into account that a depletion in hydroperoxide concentration is observed in the digested samples, aldehydes are generated during digestion. This is evidenced by the appearance of signals of n-alkanals in the spectra of all the digested samples (see signal "g" in Figure 1); their estimated concentrations are displayed in Table 2. The generation of n-alkanals during the *in vitro* digestion of slightly oxidized flaxseed oil was also reported in a previous study.[7] Although the concentration of aldehydes found after digestion is not significantly affected by the low ovalbumin proportion tested, similarly to that commented on epoxides, lower levels of saturated aldehydes are observed after the digestion of the

samples containing a high ovalbumin proportion. It is worthwhile mentioning that, as Table 2 shows, in all cases aldehydes are present in lower concentrations than epoxides. In summary, the *in vitro* digestion of the slightly oxidized soybean oils and of these oils with a low ovalbumin level provokes very similar changes in the oxidation product profile of the starting oils. By contrast, when this protein is present in a high concentration, the reduction of hydroperoxides to hydroxy-dienes seems to be favoured over other reactions leading to the generation of epoxides and aldehydes. However, taking into account that not only hydroperoxides but also epoxides and saturated aldehydes can react with some amino acid residues of proteins,[26,27,40] a potential reaction of all these compounds with the amino acid residues of ovalbumin should not be ruled out as contributing to their lower concentration increase in comparison with the rest of digested samples.

3.5. Assessment of lipid oxidation through *in vitro* digestion by SPME-GC/MS

The SPME-GC/MS analysis of the samples provides information about specific volatile compounds that can reveal the occurrence of different types of reactions, including oxidation. Among the several classes of volatile compounds present in the studied oils, interest was focused on aldehydes and 2-pentyl-furan. The compounds detected in the various digested samples and their respective abundances, expressed as arbitrary area units of the mass spectra base peak (see section 2.5.2), are displayed in Figure 2, together with the data related to the reference samples prepared from the starting oils (see section 2.5.1), and to the digestive juices after being submitted to digestion conditions. The total abundances of each group of aldehydes are shown in Table 4.

As Figure 2 shows, the reference samples already contain aldehydes, both saturated (n-alkanals) and unsaturated ((*E*)-2-alkenals and 2,4-alkadienals). Among n-alkanals, hexanal (Hx) stands out both in the virgin and in the refined oils due to its high level (Figures 2A and 2B, respectively), probably as a consequence of some linoleic group oxidation[41]. Regarding (*E*)-2-alkenals and 2,4-alkadienals, the most abundant ones (apart from (*E*)-2-butenal, 2B, coming basically from the digestive juices) are (*E*)-2-octenal (2O), (*E*)-2-heptenal (2Hp), (*E*,*E*)-2,4-nonadienal (EE-N) and (*E*,*E*)-2,4-decadienal (EE-D), mainly arising from the degradation of linoleic groups, as well as 2,4-heptadienals (ZE-H and EE-H), generated due to linolenic acyl group oxidation[41]. In addition to aldehydes, 2-pentyl-furan was also present in the samples studied.

When it comes to the digested samples, it can be observed in Figure 2 that both in the samples digested without ovalbumin and with a low proportion of this protein, concentration of most n-alkanals increases significantly after *in vitro* digestion. However, for hexanal and nonanal (in this latter case only in the refined oil) the increase is less pronounced in the samples with a low ovalbumin level. By contrast, when a high ovalbumin proportion is present in the system, hexanal is the only n-alkanal that exhibits a concentration increase after digestion, while the rest either remain unchanged or show a decrease in relation to the non-digested samples (see Figure 2). In fact, the total concentration of saturated aldehydes in the samples digested with a high ovalbumin proportion is lower than in the rest of digested samples (see Table 4). These findings follow the lines of those above obtained by ^{1}H NMR.

With regard to unsaturated aldehydes, the concentration of most (*E*)-2-alkenals and of all 2,4-alkadienals also increases after digestion in the samples digested without ovalbumin (see Figure 2), although the global concentration increment of each group of unsaturated aldehydes is not as marked as in the case of the saturated ones (see Table 4). It must be highlighted that although ^{1}H NMR analysis does not allow one to notice the generation of unsaturated aldehydes, it is evidenced by SPME-GC/MS; this might be explained by their lower abundance in comparison with that of n-alkanals in the digested samples (see Table 4).

The same trend is observed in the samples digested with a low proportion of ovalbumin, where, as in the case of some n-alkanals, the overall increases of both types of unsaturated aldehydes are not so generally marked as in the samples digested without ovalbumin (see Table 4). However, in contrast to these findings, in the samples digested with a high ovalbumin proportion, the concentrations of (*E*)-2-alkenals and 2,4-alkadienals generally diminishes after digestion (see Figure 2 and Table 4); this reinforces the idea noted above that reactions between aldehydes and ovalbumin take place during digestion. In this respect, the reaction of (*E*)-2-alkenals with proteins is a well documented issue.[42] A reduction in aldehyde concentration during the *in vitro* digestion of other types of slightly oxidized highly unsaturated oils in presence of a high protein proportion was also observed in a previous work[4], but in that case data concerning the non-digested samples were not reported, so a direct comparison between these and the samples digested in presence of protein could not be made.

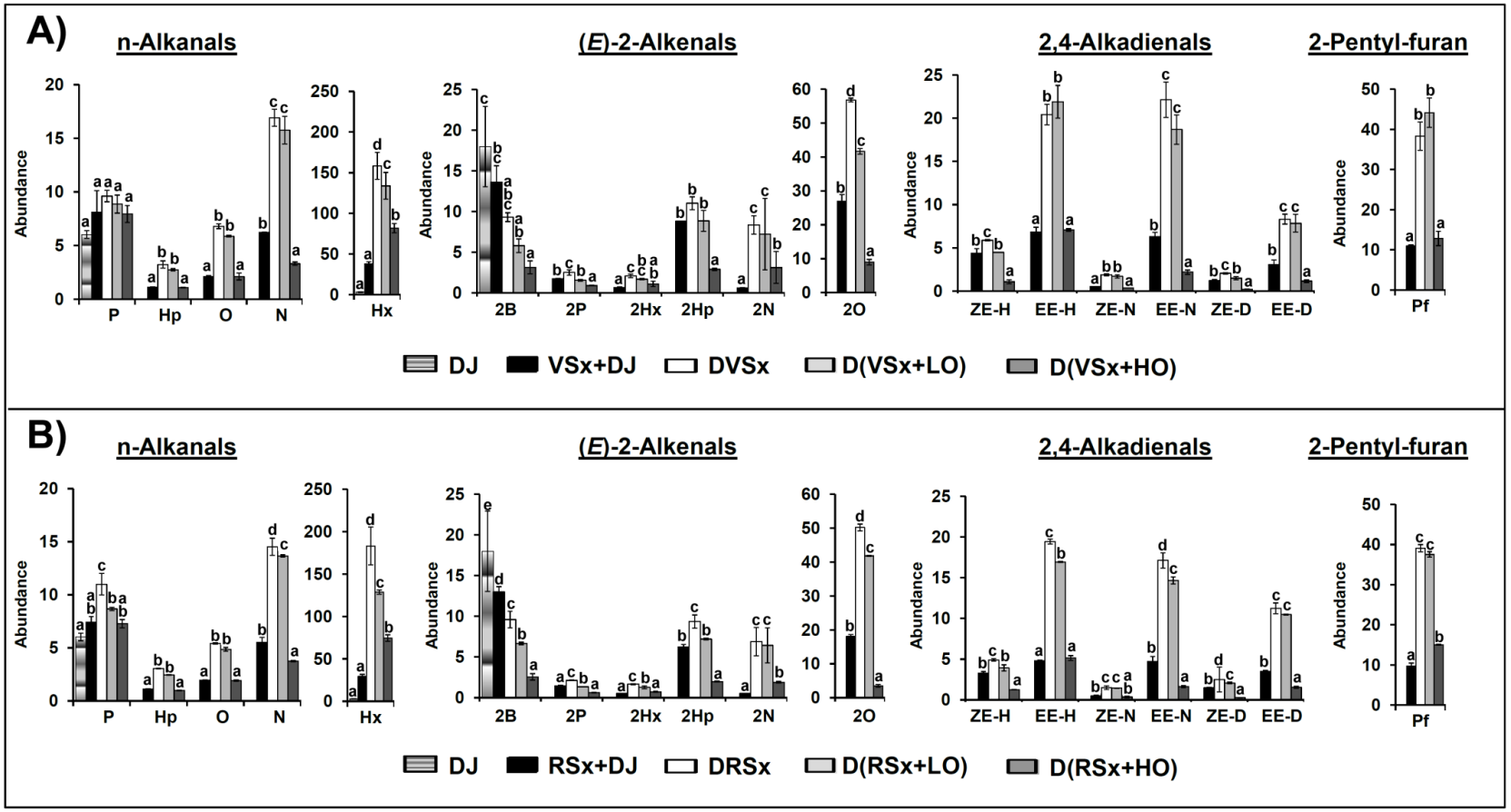

Figure 2. Bar graphics representing the abundance, expressed as arbitrary area units of the mass spectrum base peak of each compound (see section 2.5.2) extracted from the Total Ion Chromatograms obtained by SPME/GC-MS, divided by 10^6, of n-alkanals, (*E*)-2-alkenals, 2,4-alkadienals and 2-pentyl-furan in: the digestive juices subjected to digestion conditions (DJ), the slightly oxidized virgin (A) and refined (B) oils mixed with the DJ (VSx+DJ and RSx+DJ), the slightly oxidized virgin and refined oils digested in the absence of ovalbumin (DVSx and DRSx), with a low proportion of ovalbumin (D(VSx+LO) and D(RSx+LO)) and with a high proportion of ovalbumin (D(VSx+HO) and D(RSx+HO)). Different letters within the bars relative to the same compound indicate a significant difference among the samples ($p < 0.05$). Abbreviations employed: pentanal (P), hexanal (Hx), heptanal (Hp), octanal (O), nonanal (N), (*E*)-2-butenal (2B), (*E*)-2-pentenal (2P), (*E*)-2-hexenal (2Hx), (*E*)-2-heptenal (2Hp), (*E*)-2-octenal (2O), (*E*)-2-nonenal (2N), (*Z*,*E*)-2,4-heptadienal (ZE-H), (*E*,*E*)-2,4-heptadienal (EE-H), (*Z*,*E*)-2,4-nonadienal (ZE-N), (*E*,*E*)-2,4-nonadienal (EE-N), (*Z*,*E*)-2,4-decadienal (ZE-D), (*E*,*E*)-2,4-decadienal (EE-D) and 2-pentyl-furan (Pf).

Comparing the effect of the high proportion of ovalbumin on the concentration of the various classes of aldehydes after digestion, and taking as a reference their respective levels in the samples digested without ovalbumin, it is noticeable that, in general, a more pronounced decrease is observed for the unsaturated aldehydes (see Table 4); this seems to agree with the greater reactivity that could be expected from this type of aldehydes in comparison with the saturated ones. Thus, the level of each aldehyde after the digestion process will depend on the balance between its generation and its ability to react with the proteins present in the medium.

In line with observations made for aldehyde evolution, the concentration of 2-pentyl-furan also increases after digestion, to a similar extent in the samples digested without ovalbumin and with a low ovalbumin proportion (see Figure 2). However, in agreement with previous findings,[4] the level of this compound is considerably lower in the samples digested with a high proportion of ovalbumin. Taking into account that, as far as we know, the ability of 2-pentyl-furan to react with proteins has not been described, this might be interpreted as that the presence of a high proportion of ovalbumin has exerted a certain antioxidant effect during digestion.

Table 4. Total abundances of n-alkanals, (*E*)-2-alkenals and 2,4-alkadienals in: the digestive juices subjected to digestion conditions (DJ), the slightly oxidized virgin and refined oils mixed with DJ (VSx+DJ and RSx+DJ), and the slightly oxidized virgin and refined oils digested in the absence of ovalbumin (DVSx and DRSx), with a low proportion of ovalbumin added (D(VSx+LO) and D(RSx+LO)) and with a high proportion of ovalbumin (D(VSx+HO) and D(RSx+HO)). Different letters within each column indicate a significant difference among the samples corresponding to the same type of oil (virgin or refined) ($p < 0.05$).

	n-Alkanals	**(*E*)-2-Alkenals**	**2,4-Alkadienals**
DJ	9.1 ± 0.4a	18.0 ± 4.9a	-
VSx+DJ	55.4 ± 4.2b	52.3 ± 2.2b	22.3 ± 2.3b
DVSx	195.0 ± 18.9d	90.1 ± 3.7c	60.7 ± 4.0c
D(VSx+LO)	167.1 ± 20.8d	66.7 ± 7.3b	56.1 ± 5.0c
D(VSx+HO)	96.2 ± 6.8c	20.1 ± 3.5a	12.1 ± 0.8a
RSx+DJ	45.4 ± 2.3b	39.7 ± 5.1b	18.3 ± 1.1b
DRSx	217.0 ± 22.3e	79.8 ± 3.7d	56.7 ± 3.8d
D(RSx+LO)	158.2 ± 2.5d	64.7 ±2.7c	49.5 ± 1.1c
D(RSx+HO)	88.5 ± 3.8c	11.3 ± 0.8a	10.2 ± 0.6a

-: not detected

3.6. Evolution of γ-tocopherol during the *in vitro* digestion process monitored by ^{1}H NMR

The main tocopherol in soybean oil is γ-T.[14] However, in these oxidized oils its presence could only be detected by ^{1}H NMR in the refined one (see signal "h" in Figure 1, due to one of the protons present in the γ-T chromanol ring), its concentration being low: 0.63±0.06 mmol/mol AG+FA. After digestion, γ-T is only detected in the ^{1}H NMR spectrum of the sample digested with a high proportion of ovalbumin, although in a very low concentration (0.07±0.01 mmol/mol AG+FA). This finding indicates, on the one hand, that, in agreement with previous results,[15] the bioaccessibility of γ-T diminishes during *in vitro* digestion, and on the other that the presence of a high proportion of ovalbumin in the system preserves the γ-T content of the starting oil to a certain extent, possibly due to a decrease in oxidative reactions.

3.7. Final remarks

In order to make an approximate assessment of the overall oxidation extent during the *in vitro* digestion of the two types of slightly oxidized soybean oils studied, the total amounts of oxidation products considered to be supported on long acyl group or fatty acid chains (hydroperoxides, hydroxy-dienes and epoxides), were compared before and after this process. According to these data, increments in the total concentration of the considered oxidation products of about 1 and 2 mmol/mol AG+FA in the case of the virgin and the refined oil samples, respectively, were observed after digestion. This suggests that, despite significant variations in acyl groups not being noticed after digestion (see Table 1), some oxidation seems to have taken place during this process.

Although a clear development of oxidative reactions was noticed during the *in vitro* digestion of slightly oxidized flaxseed oil in a previous works,[7] it must be taken into account that it is more prone to oxidation than soybean oil simply due to its composition in acyl groups,[43] although their composition in minor components might also have some influence. However, the results found after the *in vitro* digestion of slightly oxidized sunflower oil[6] are not very different from those here observed.

It is worth noticing that the small differences in the minor component profile of the slightly oxidized virgin and refined soybean oils relative to tocopherol, squalene and free fatty acid contents (see Table S2) do not seem to be enough to provoke noticeable variations in their behaviour under *in vitro* digestion conditions.

4. CONCLUSIONS

The results of this work reveal that the initial oxidation degree of the soybean oil samples studied leads to a lower lipolysis extent in comparison with non-oxidized oils studied before, reducing the bioaccessibility of the oil major components, which include some essential fatty acids like ω-3 ones. This lipolysis degree is not significantly affected by the lowest proportion of ovalbumin tested; however, the addition of a high ovalbumin level greatly enhances the action of lipolytic enzymes, improving lipid bioaccessibility.

Regarding oxidation, this does not seem to have occurred to a great enough extent for it to be detected from polyunsaturated acyl group degradation. Moreover, although the slight increase in the total concentration of oxidation products considered to be supported on acyl group or fatty acid chains after digestion might indicate some oxidation occurrence, the changes observed in the oxidation product profile of the digested samples seem to be mainly due to the transformation of the hydroperoxides initially present in the oils, to give hydroxy-dienes, epoxides and aldehydes. The fact that after digestion epoxides exhibit a greater concentration increase than aldehydes evidences that the assessment of lipid oxidation should not be based on only one group of oxidation products, but would require the determination of as many types of oxidation compounds as possible, among which epoxides should be included. While the presence of a low ovalbumin proportion during digestion does not significantly affect oxidation compound evolution, a high level of this protein favours the reduction of hydroperoxides to hydroxy-dienes over other type of reactions leading to the generation of epoxides and aldehydes. Notwithstanding, the decrease observed in the concentrations of hydroperoxides, epoxides and aldehydes might also be influenced by their reaction with the protein, and indeed, it has been evidenced by SPME-GC/MS in the case of aldehydes.

Therefore, it is evident that ovalbumin, and possibly other proteins, can play a key role in the digestion of lipids, especially in those showing a slight oxidation degree, increasing their absorbable fraction. In addition, the presence of a high proportion of ovalbumin not only increases γ-T bioaccessibility, but also contributes to reducing the concentration in the gastrointestinal tract of certain types of oxidation products that could exert negative effects, such as aldehydes and epoxides.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. G. Fave, T. C. Coste and M. Armand, Physicochemical properties of lipids: new strategies to manage fatty acid bioavailability, *Cell. Mol. Biol.*, 2004, **50**, 815-832.
2. J. Kanner and T. Lapidot, The stomach as a bioreactor: Dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants, *Free Radic. Biol. Med.*, 2001, **31**, 1388-1395.
3. H. B. Kenmogne-Domguia, A. Meynier, C. Boulanger and C. Genot, Lipid oxidation in food emulsions under gastrointestinal-simulated conditions: The key role of endogenous tocopherols and initiator, *Food Dig.*, 2012, **3**, 46-52.
4. B. Nieva-Echevarría, E. Goicoechea and M. D. Guillén, Effect of the presence of protein on lipolysis and lipid oxidation occurring during *in vitro* digestion of highly unsaturated oils, *Food Chem*., 2017, **235**, 21-33.
5. K. Larsson, L. Cavonius, M. Alminger and I. Undeland, Oxidation of cod liver oil during gastrointestinal *in vitro* digestion, *J. Agric. Food Chem*., 2012, **60**, 7556-7564.
6. B. Nieva-Echevarría, E. Goicoechea, M. J. Manzanos and M. D. Guillén, ^{1}H NMR and SPME-GC/MS study of hydrolysis, oxidation and other reactions occurring during *in vitro* digestion of non-oxidized and oxidized sunflower oil. Formation of hydroxy-octadecadienoates, *Food Res. Int*., 2017, **91**, 171-182.
7. B. Nieva-Echevarría, E. Goicoechea and M. D. Guillén, Behaviour of non-oxidized and oxidized flaxseed oils, as models of omega-3 rich lipids, during *in vitro* digestion. Occurrence of epoxidation reactions, *Food Res. Int*., 2017, **97**, 104-115.
8. C. Steppeler, J. E. Haugen, R. Rødbotten and B. Kirkhus, Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during *in vitro*

digestion of cooked beef, pork, chicken, and salmon, *J. Agric. Food Chem*., 2016, **64**, 487-496.

9. H. B. Kenmogne-Domguia, S. Moisan, M. Viau, C. Genot and A. Meynier, The initial characteristics of marine oil emulsions and the composition of the media inflect lipid oxidation during *in vitro* gastrointestinal digestion, *Food Chem*., 2014, **152**, 146-154.
10. M. D. Guillén and E. Goicoechea, Toxic oxygenated α,β-unsaturated aldehydes and their study in foods: a review, *Crit. Rev. Food Sci. Nutr.*, 2008, **48**, 119-136.
11. R. S. Lam and M. T. Nickerson, Food proteins: a review on their emulsifying properties using a structure-function approach, *Food Chem.*, 2013, **141**, 975-984.
12. R. J. Elias, S. S. Kellerby and E. A. Decker, Antioxidant activity of proteins and peptides, *Crit. Rev. Food Sci. Nutr*., 2008, **48**, 430-441.
13. T. Van Hecke, J. Vanden Bussche, L. Vanhaecke, E. Vossen, J. Van Camp and S. De Smet, Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during *in vitro* digestion, *J. Agric. Food Chem.*, 2014, **62**, 1980-1988.
14. J. Alberdi-Cedeño, M. L. Ibargoitia, G. Cristillo, P. Sopelana and M. D. Guillén, A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components, *Food Chem*., 2017, **221**, 1135-1144.
15. A. S. Martin-Rubio, P. Sopelana and M. D. Guillén. Influence of minor components on lipid bioaccessibility and oxidation during *in vitro* digestion of soybean oil, *J. Sci. Food Agric*., 2019, DOI 10.1002/jsfa.9734.
16. E. Goicoechea and M. D. Guillén, Analysis of hydroperoxides, aldehydes and epoxides by ^{1}H nuclear magnetic resonance in sunflower oil oxidized at 70 and 100 C, *J. Agric. Food Chem*., 2010, **58**, 6234-6245.
17. M. D. Guillén and A. Ruiz, Oxidation process of oils with high content of linoleic acyl groups and formation of toxic hydroperoxy- and hydroxyalkenals. A study by ^{1}H nuclear magnetic resonance. *J. Sci. Food Agric*., 2005, **85**, 2413-2420.
18. C. H. Versantvoort, A. G. Oomen, E. Van de Kamp, C. J. Rompelberg and A. J. Sips, Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food, *Food Chem. Toxicol*., 2005, **43**, 31-40.

19. B. Nieva-Echevarría, E. Goicoechea, M. J. Manzanos and M. D. Guillén, A study by ^{1}H NMR on the influence of some factors affecting lipid *in vitro* digestion, *Food Chem*., 2016, **211**, 17-26.

20. B. Nieva-Echevarría, E. Goicoechea, M. J. Manzanos and M. D. Guillén, Usefulness of ^{1}H NMR in assessing the extent of lipid digestion, *Food Chem*., 2015, **179**, 182-190.

21. M. D. Guillén and A. Ruiz, Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups, *Eur. J. Lipid Sci. Technol*., 2003, **105**, 688-696.

22. M. D. Guillén and P. S. Uriarte, Contribution to further understanding of the evolution of sunflower oil submitted to frying temperature in a domestic fryer: study by ^{1}H Nuclear Magnetic Resonance, *J. Agric. Food Chem*., 2009, **57**, 7790-7799.

23. B. Nieva-Echevarría, E. Goicoechea, M. J. Manzanos and M. D. Guillén, A method based on ^{1}H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures, *Food Res. Int*., 2014, **66**, 379-387.

24. G. Márquez-Ruiz, M. C. García-Martínez and F. Holgado, Changes and effects of dietary oxidized lipids in the gastrointestinal tract, *Lipid Insights*, 2008, **2**, 11-19.

25. F. J. Sánchez-Muniz, R. Arroyo, J. M. Sánchez-Montero and C. Cuesta, *In vitro* digestibility study of thermally oxidised palm oleins, *Food Sci. Technol. Int.*, 2000, **6**, 449-456.

26. H. W. Gardner. Lipid hydroperoxide reactivity with proteins and amino acids: a review, *J. Agric. Food Chem*., 1979, **27**, 220-229.

27. K. M. Schaich, Co-oxidation of proteins by oxidizing lipids, *Lipid Oxidation Pathways*, 2008, **2**, 181-272.

28. S. Matsushita and M. Kobayashi, Effect of linoleic acid hydroperoxides on pepsin activity, *Agric. Biol. Chem.*, 1970, **34**, 825-829.

29. S. Matsushita, M. Kobayashi, and Y. Nitta, Inactivation of enzymes by linoleic acid hydroperoxides and linoleic acid, *Agric. Biol. Chem.*, 1970, **34**, 817-824.

30. S. Matsushita, Specific interactions of linoleic acid hydroperoxides and their secondary degraded products with enzyme proteins, *J. Agric. Food Chem.*, 1975, **23**, 150-154.

31. G. Márquez-Ruiz, G. Guevel and M. C. Dobarganes, Applications of chromatographic techniques to evaluate enzymatic hydrolysis of oxidized and polymeric triglycerides by pancreatic lipase *in vitro*, *J. Am. Oil Chem. Soc*., 1998, **75**, 119-126.
32. Y. Mine, T. Noutomi and N. Haga, Emulsifying and structural properties of ovalbumin, *J. Agric. Food Chem*., 1991, **39**, 443-446.
33. C. Chen, Y. J. Chi, M. Y. Zhao, and W. Xu, Influence of degree of hydrolysis on functional properties, antioxidant and ACE inhibitory activities of egg white protein hydrolysate, *Food Sci. Biotechnol.*, 2012, **21**, 27-34.
34. F. H. Mattson and R. A. Volpenhein, Synthesis and properties of glycerides, *J. Lipid Res*., 1962, **3**, 281-296.
35. F. H. Mattson and R. A. Volpenhein, The digestion and absorption of triglycerides, *J. Biol. Chem*., 1964, **239**, 2772-2777.
36. A. M. Nik, A. J. Wright and M. Corredig, Interfacial design of protein-stabilized emulsions for optimal delivery of nutrients, *Food Funct.*, 2010, **1**, 141-148.
37. K. Kanazawa and H. Ashida, Catabolic fate of dietary trilinoleoylglycerol hydroperoxides in rat gastrointestines, *Biochim. Biophys. Acta*, 1998, **1393**, 336-348.
38. K. Kanazawa and H. Ashida, Dietary hydroperoxides of linoleic acid decompose to aldehydes in stomach before being absorbed into the body, *Biochim. Biophys. Acta*, 1998, **1393**, 349-361.
39. K. Nakatsugawa and T. Kaneda, Absorption and metabolism of methyl linoleate hydroperoxides in rats, *J. Jpn. Oil Chem. Soc*., 1983, **32**, 361-366.
40. K. Ishino, T. Shibata, T. Ishii, Y. T. Liu, S. Toyokuni, X. Zhu, L. M. Sayre and K. Uchida, Protein N-acylation: H_2O_2-mediated covalent modification of protein by lipid peroxidation-derived saturated aldehydes, *Chem. Res. Toxicol*., 2008, **21**, 1261-1270.
41. M. D. Guillén and P. S. Uriarte, Aldehydes contained in edible oils of a very different nature after prolonged heating at frying temperature: Presence of toxic oxygenated α,β unsaturated aldehydes, *Food Chem*., 2012, **131**, 915-926.
42. K. Uchida, Aldehyde adducts generated during lipid peroxidation modification of proteins, *Free Radic. Res*., 2015, **49**, 896-904.

43. M. D. Guillén and A. Ruiz, Monitoring the oxidation of unsaturated oils and formation of oxygenated aldehydes by proton NMR. *Eur. J. Lipid Sci. Technol*., 2005, **107**, 36-47.

SUPPLEMENTARY MATERIAL OF MANUSCRIPT 7

THE KEY ROLE OF OVALBUMIN IN LIPID BIOACCESSIBILITY AND OXIDATION PRODUCT PROFILE DURING THE *IN VITRO* DIGESTION OF SLIGHTLY OXIDIZED SOYBEAN OIL

A.S. Martin-Rubio; P. Sopelana; María D. Guillén

Some details of the in vitro digestion procedure

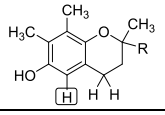
The digestion experiment started by adding 6 mL of saliva to each of the oil samples. After 5 min of incubation, 12 mL of gastric juice were added and the mixture was rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. 1 hour after starting the gastric digestion, pH was set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring *in vivo*. After 2 h of gastric digestion, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice were added. Subsequently, pH was set between 6 and 7, and the mixture was rotated again at 40 rpm and incubated at 37±2ºC for 4 h.

The enzymes used for the preparation of digestive juices, acquired from Sigma-Aldrich (St. Louis, MO, USA), were the following: α-amylase from *Aspergillus oryzae* (10065, ~30 U/mg); pepsin from porcine gastric mucosa (P7125, ≥400 U/mg protein); amano lipase A from *Aspergillus niger* (534781, ≥120,000 U/g); pancreatin from porcine pancreas (P1750) and lipase type II crude from porcine pancreas (L3126, 100-500 U/mg protein (using olive oil, 30 min incubation)).

Table S1. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of the main protons of glycerides, fatty acids, some oxidation compounds and γ-tocopherol, present in the samples before and after *in vitro* digestion. TG: triglycerides; DG: diglycerides; MG: monoglycerides. The signal letters agree with those given in Figures 1 and S1.

Signal	Chemical shift (ppm)	Multiplicity	Functional group	
			Type of protons	Compound
Main acyl groups (AG) and fatty acids (FA)[a,b]				
A	0.88	t	–C**H**$_3$	saturated and monounsaturated **ω-9 AG** and **FA**
	0.89	t	–C**H**$_3$	linoleic **AG** and **FA**
B	0.97	t	–C**H**$_3$	linolenic **AG** and **FA**
C	1.19–1.42	m*	–(C**H**$_2$)$_n$–	**AG** and **FA**
D	1.61	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **TG**
	1.62	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **1,2-DG**
	1.63	m	–OCO–CH$_2$–C**H**$_2$–, COOH–CH$_2$–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	1.64	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **2-MG**
E	1.92–2.15	m**	–C**H**$_2$–CH=CH–	**AG** and **FA**
F	2.26–2.36	dt	–OCO–C**H**$_2$–	**AG** in **TG**
	2.33	m	–OCO–C**H**$_2$–	**AG** in **1,2-DG**
	2.35	t	–OCO–C**H**$_2$–, COOH–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	2.38	t	–OCO–C**H**$_2$–	**AG** in **2-MG**
G	2.77	t	=HC–C**H**$_2$–CH=	Linoleic **AG** and **FA**
H	2.80	t	=HC–C**H**$_2$–CH=	Linolenic **AG** and **FA**
I	3.65	ddd	ROCH$_2$–CHOH–C**H**$_2$OH	glyceryl group in **1-MG**
J	3.73	m***	ROCH$_2$–CH(O*R*')–C**H**$_2$OH	glyceryl group in **1,2-DG**
K	3.84	m***	HOC**H**$_2$–CH(O*R*)–C**H**$_2$OH	glyceryl group in **2-MG**
L	3.94	m	ROCH$_2$–C**H**OH–CH$_2$OH	glyceryl group in **1-MG**
M	4.05–4.21	m	ROC**H**$_2$–C**H**OH–C**H**$_2$OR'	glyceryl group in **1,3-DG**
N	4.18	ddd	ROC**H**$_2$–CHOH–CH$_2$OH	glyceryl group in **1-MG**
O	4.22	dd,dd	ROC**H**$_2$–CH(O*R*')–C**H**$_2$OR''	glyceryl group in **TG**
P	4.28	ddd	ROC**H**$_2$–CH(O*R*')–CH$_2$OH	glyceryl group in **1,2-DG**
Q	4.93	m	HOCH$_2$–C**H**(O*R*)–CH$_2$OH	glyceryl group in **2-MG**
R	5.08	m	ROCH$_2$–C**H**(O*R*')–CH$_2$OH	glyceryl group in **1,2-DG**
S	5.27	m	ROCH$_2$–C**H**(O*R*')–CH$_2$OR''	glyceryl group in **TG**
T	5.28–5.46	m	–C**H**=C**H**–	**AG** and **FA**
Oxidation compounds				

Conjugated dienic systems[c,d,e]				
–	5.44	ddd	–C**H**=C**H**–C**H**=C**H**–	(*Z*,*E*)-conjugated double bonds associated with hydroxy group (OH) in octadecadienoic **AG** and **FA**
-	5.66	dd		
-	5.97	t		
a	6.49	dd		
-	5.47	ddm	–C**H**=C**H**–C**H**=C**H**–	(*E*,*E*)-conjugated double bonds associated with hydroperoxy group (OOH) in octadecadienoic **AG** and **FA**
-	5.76	dtm		
-	6.06	ddtd		
b	6.27	ddm		
-	5.51	dtm	–C**H**=C**H**–C**H**=C**H**–	(*Z*,*E*)-conjugated double bonds associated with hydroperoxy group (OOH) in octadecadienoic **AG** and **FA**
-	5.56	ddm		
-	6.00	ddtd		
c	6.58	dddd		
-	5.58	dd	–C**H**=C**H**–C**H**=C**H**–	(*E*,*E*)-conjugated double bonds associated with hydroxy group (OH) in octadecadienoic **AG** and **FA**
-	5.71	dd		
-	6.03	dd		
d	6.18	dd		
Epoxides				
Epoxy-derivatives				
e1	2.88[f]	m	-C**H**O**H**C-	(*Z*)-9,10-epoxystearate monoepoxy-octadecenoate groups
e2	2.9[g]		-C**H**O**H**C-	
e3	2.94[****]	m	-C**H**O**H**C-	(*Z*)-12,13-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid
Epoxy-hydroxy-derivatives				
e4	2.93[h]	dt	-C**H**OHC-CHOH-CH=CH-	*threo*-11-hydroxy-(*E*)-12,13-epoxy-(Z)-9-octadecenoate
f1	3.09[i]/3.097[j]	dd	-CHO**H**C-CH=CH-CHOH-	9-hydroxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate
Epoxy-hydroperoxy-derivatives				
f2	3.11[i]	dd	-CHO**H**C-CH=CH-CHOOH-	9-hydroperoxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate[k]

Aldehydes				
g	9.75[l]	t	-C**H**O	n-alkanals
		γ-Tocopherol[m]		
h	6.36****	s		

Abbreviations: t: triplet; m: multiplet; d: doublet. *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***This signal shows different multiplicity if the spectrum is acquired from the pure compound or taking part in the mixture; ****Assignment made with the aid of standard compounds.

[a]Assignments of AG in TG taken from M. D. Guillén and A. Ruiz, *J. Sci. Food Agric.*, 2003, **83**, 338-346.

[b]Assignments of AG in partial glycerides (DG and MG) and of FA taken from B. Nieva-Echevarría, E. Goicoechea, M. J. Manzanos and M. D. Guillén, *Food Res. Int.*, 2014, **66**, 379-387.

[c]Data taken from E. Goicoechea and M. D. Guillén, *J. Agric. Food Chem.*, 2010, **58**, 6234-6245 (conjugated (*Z,E*)- and (*E,E*)-hydroperoxy-dienes).

[d]Data taken from M. Dong, Y. Oda and M. Hirota, *Biosci., Biotech. Biochem.*, 2000, **64**, 882-886 (conjugated (*Z,E*)-hydroxy-dienes).

[e]Data taken from P. Tassignon, P. De Waard, T. De Rijk, H. Tournois, D. de Wit and L. De Buyck, *Chem. Phys. Lipids*, 1994, **71**, 187-196 (conjugated (*E,E*)-hydroxy-dienes).

[f]Data taken from G. Du, A. Tekin, E. G. Hammond and L. K. Woo, *J. Am. Oil Chem. Soc.*, 2004, **81**, 477-480.

[g]Data taken from H. A. J. Aerts and P. A. Jacobs, *J. Am. Oil Chem. Soc.*, 2004, **81**, 841-846.

[h]Data taken from G. J. Garssen, G. A. Veldink, J. F. Vliegenthart and J. Boldingh, *FEBS J.*, 1976, **62**, 33-36.

[i]Data taken from H. W. Gardner, D. Weisleder and R. Kleiman, *Lipids*, 1978, **13**, 246-252.

[j]Data taken from P. A. Van Os Cornelis, J. F. G. Vliegenthart, C. G. Crawford and H. W. Gardner, *Biochim. Biophys. Acta*, 1982, **713**, 173-176.

[k]δ-Ketols (hydroxy-keto-derivatives) could also contribute to this signal (H. W. Gardner, R. Kleiman and D. Weisleder, *Lipids*, 1974, **9**, 696-706).

[l]Data taken from M. D. Guillén and A. Ruiz. *Eur. J. Lipid Sci. Technol.*, 2004, **106**, 680-687.

[m]Assignment taken from J. K. Baker and C. W. Myers. *Pharm. Res.*, 1991, **8**, 763-770.

Quantification from ^{1}H NMR spectral data of several compounds present in the starting oil samples and/or in the lipid extracts of the digestates, and of Lipid Bioaccessibility

A. Lipolytic products and Lipid Bioaccessibility

The number of moles (N) of all the glycerides and fatty acids present in the lipid samples were expressed as follows:

$N_{2\text{-}MG}=Pc*A_K/4$ [eq. S1]
$N_{1\text{-}MG}=Pc*A_L$ [eq. S2]
$N_{1,2\text{-}DG}=Pc*(A_{I+J}-2A_L)/2$ [eq. S3]
$N_{TG}=Pc*(2A_{4.26\text{-}4.38}-A_{I+J}+2A_L)/4$ [eq. S4]
$N_{1,3\text{-}DG}=Pc*(A_{4.04\text{-}4.38}-2A_{4.26\text{-}4.38}-2A_L)/5$ [eq. S5]
$N_{FA}=Pc*(A_F-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2$ [eq. S6]
$N_{Gol}=(N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2N_{2\text{-}MG}-2N_{1\text{-}MG})/3$ [eq. S7]

where Pc is the proportionality existing between the area of the ^{1}H NMR signals and the number of protons that generate them, A_K, A_L, A_{I+J} and A_F are the areas of the corresponding signals indicated in Table S1, and $A_{4.26\text{-}4.38}$ and $A_{4.04\text{-}4.38}$ represent the areas of the signals between 4.26 and 4.38 ppm, and between 4.04 and 4.38 ppm, respectively (see Figure S1). Gol: glycerol.

Using these equations, the molar percentages of the different kinds of glycerides in relation to the total number of moles of glyceryl structures present (NT_{GS}) were determined as follows:

$NT_{GS}=N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{Gol}$ [eq. S8]
$G\%=100N_G/NT_{GS}$ [eq. S9]

where G is each kind of glyceride (TG, 1,2-DG, 1,3-DG, 2-MG and 1-MG) and N_G the respective number of moles.

$Gol\%=100N_{Gol}/NT_{GS}$ [eq. S10]

Likewise, the Lipid Bioaccessibility parameter was calculated as follows:

$L_{BA}\%=100(N_{1\text{-}MG}+N_{2\text{-}MG}+N_{FA})/NT_{AG+FA}$ [eq. S11]
$NT_{AG+FA}=Pc*A_F/2$ [eq. S12]

where NT_{AG+FA} is the total number of moles of AG plus FA present.

B. Polyunsaturated acyl groups and fatty acids

The concentrations of linolenic (Ln) and linoleic (L) AG and FA, expressed as millimoles per mole of the sum of AG+FA present in either the starting oils or the lipid extracts of the digested samples were estimated by using the following equations:

$$[Ln] = [(A_H/4)/(A_F/2)]*1000 \quad \text{[eq. S13]}$$

$$[L] = [(A_G/2)/(A_F/2)]*1000 \quad \text{[eq. S14]}$$

where A_H and A_G are the areas of signals H and G indicated in Table S1. It must be noted that due to partial overlapping of signals H and G, a previous correction of both areas must be carried out to properly assess the area corresponding to each one of them. For this purpose, trilinolenin and trilinolein were used as references.

Finally, it should be pointed out that signal F is due to methylenic protons bonded to carbon atoms in *alpha* position in relation to carbonyl/carboxyl groups of AG and FA, modified or not, as well as to carbonyl groups of other compounds formed during oxidation such as aldehydes. However, as the oxidation level of both the non-digested and the *in vitro* digested samples is very low, the inclusion in this signal of methylenic protons in *alpha* position in relation to carbonyl groups different from those of AG and FA does not affect the calculations in which A_F is included, because the concentration of aldehydes is negligible in relation with that of AG+FA.

C. Oxidation compounds and γ-tocopherol

The concentration of the several kinds of oxidation compounds, as well as that of γ-tocopherol, expressed as millimoles per mol of the sum of AG+FA present, was estimated by using the following equations:

$$[OP] = [(A_{OP}/n)/(A_F/2)]*1000 \quad \text{[eq. S15]}$$

$$[\gamma\text{-T}] = [(A_{\gamma T}/n)/(A_F/2)]*1000 \quad \text{[eq. S16]}$$

where A_{OP} and $A_{\gamma T}$ are the areas of the signals selected for the quantification of each oxidation product (OP) and of γ-T, shown in Table S1, and n the number of protons that generate each signal. In the case of the epoxides giving signal at approximately 2.9 ppm (signals "e1-e4" in Table S1), the overlapped area due to the side band of *bis*-allylic protons signals G and H must be subtracted. Although the epoxy-

compounds included in signal "e" can contribute with two ("e1-e3") or one ("e4") protons, it has been assumed that all contribute with two protons. This type of epoxides has been quantified together with those giving signal at approximately 3.1 ppm (see signals "f1+f2" in Table S1).

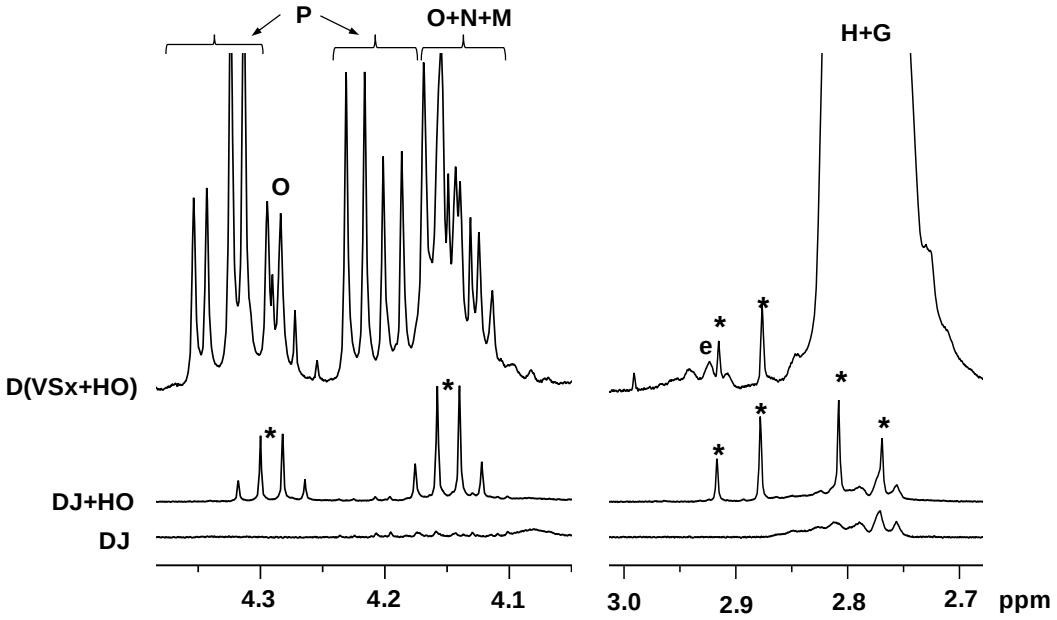


Figure S1. Enlargement of some spectral regions of the ^{1}H NMR spectra of the lipid extracts of: the digestive juices subjected to digestion conditions (DJ); the digestive juices subjected to digestion conditions mixed with ovalbumin at the high proportion tested (DJ+HO); and the slightly oxidized virgin soybean oil digested in presence of the high ovalbumin proportion (D(VSx+HO)). The signal letters agree with those in Table S1, considering that signal "e" includes signals "e1 to e4". Signals marked with an asterisk are considered to come from the ovalbumin sample used.

Levels of some minor components in the oxidized oils studied, determined by Direct Immersion Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (DI SPME-GC/MS), according to the methodology described by J. Alberdi-Cedeño, M. L. Ibargoitia, G. Cristillo, P. Sopelana and M. D. Guillén, Food Chem., 2017, 221, 1135-1144.

Table S2. Abundances, expressed as arbitrary area units of the mass spectrum base peak (BP) of each compound, extracted from the total ion chromatograms obtained by DI SPME-GC/MS, divided by 10^6, of the main minor components of the studied soybean oils, together with their respective molecular weights (MW).

Compounds (MW)	**BP**	**VSx**	**RSx**
Free fatty acids			
Total free fatty acids†	55	136.8 ± 19.9	-
Tocopherols			
δ-Tocopherol (402)‡	402	11.0 ± 0.4	15.8 ± 1.3
β-Tocopherol (416)‡	416	0.5 ± 0.1	1.2 ± 0.1
γ-Tocopherol (416)‡	416	8.4 ± 1.1	34.7 ± 4.7
α-Tocopherol (430)‡	165	0.3 ± 0.1	0.5 ± 0.1
Hydrocarbons			
Squalene (410)‡	69	22.9 ± 1.7	31.5 ± 0.1

-: not detected

†This total includes linoleic, oleic and linolenic acids, whose mass spectra base peaks are 67, 55 and 79, respectively. However, given that all of them overlap, ion 55, common to all these fatty acids, has been used to quantify them altogether.

‡Standard compounds were acquired commercially and used for identification purposes.

MANUSCRIPT 8

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^{1}H NMR STUDY OF THE *IN VITRO* DIGESTION OF HIGHLY OXIDIZED SOYBEAN OIL, FOCUSING ON THE BIOACCESSIBILITY OF MAJOR LIPID NUTRIENTS AND OF SOME OXIDATION COMPOUNDS. EFFECT OF THE PRESENCE OF OVALBUMIN.

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Prepared for submission

ABSTRACT

The bioaccessibility after *in vitro* digestion of major lipid nutrients and oxidation compounds present in highly oxidized soybean oils was studied by ^{1}H NMR, focusing attention on lipolysis extent, oxidation occurrence and fate of oxidation compounds. Moreover, the effect of two ovalbumin proportions on these processes was also addressed. Lipid bioaccessibility is negatively affected by both high initial oil oxidation level and by the occurrence of oxidation during digestion, which cause a reduction in lipolysis and in the concentration of polyunsaturated groups. While hydroperoxide concentration decreases considerably during digestion, epoxides, keto-dienes, dihydroxyderivatives and n-alkanals persist to a great extent, some of them showing increased concentration after digestion. Conversely, α,β-unsaturated aldehydes, especially the very reactive and toxic oxygenated ones, diminish, probably due to their reaction with proteins of the digestive fluids. While a low ovalbumin proportion hardly affects lipolysis and oxidation during digestion, at a high level it slightly increases lipolysis, diminishes oxidation and reduces the levels of oxidation compounds, especially of aldehydes.

Keywords: *in vitro* digestion, ^{1}H NMR, bioaccessibility, oxidation, lipolysis, hydroperoxides, hydroxy-dienes, epoxides, aldehydes, keto-dienes, hydroxy-derivatives

1. INTRODUCTION

The storage and processing of edible oils and fats can lead to their oxidation, which entails the degradation of main and minor lipid components, and the subsequent generation of a very broad range of oxidation products that can adversely affect food quality and safety (Esterbauer, 1993). The oxidation process, and thus the nature, amount and relative proportions of the compounds generated, can vary depending on different factors such as lipid composition, temperature, time or aeration, among others (Martínez-Yusta, Goicoechea & Guillén, 2014). Thus, when oxidation occurs at low or medium temperatures, lipid degradation, in an initial stage, gives rise to hydroperoxides. The breakdown of these primary oxidation products may generate a wide variety of compounds, such as different kinds of aldehydes, epoxides, ketones or alcohols. It is worth noticing that although most oxidized lipids in foods are expected to come from high temperature processes, as is the case, for example, of frying oils and fats (Kalogeropoulos, Salta, Chiou & Andrikopoulos, 2007; Marmesat, Velasco & Dobarganes, 2008), exposure to oxidation products can also be produced through non-heated vegetable oils widely consumed in different parts of the world like soybean, olive and sunflower (Brühl, Weisshaar & Matthäus, 2016; Martin-Rubio, Sopelana & Guillén, 2019). Therefore, a wide variety of oxidation products could be ingested through diet and reach the gastrointestinal tract, where they might react with different biological components, especially of the mucosa (Kanazawa, Ashida, Minamoto, Danno & Natake, 1988), and also be absorbed, thus reaching different targets. Actually, the *in vivo* and/or *in vitro* absorption of hydroperoxy-, hydroxy- and epoxy-fatty acids, as well as of 4-hydroxy-(*E*)-2-alkenals has already been described (Awada et al., 2012; Penumetcha, Khan & Parthasarathy, 2000; Wilson, Fernie, Scrimgeour, Lyall, Smyth & Riemersma, 2002).

In this context, the fate of the several types of oxidation products that can be present in dietary lipids and their bioaccessibility during digestion deserve interest due to the toxicity of some of them; among these, oxygenated α,β-unsaturated aldehydes (Guillén & Goicoechea, 2008) and certain monoepoxides coming from both linoleic (Greene, Williamson, Newman, Morisseau & Hammock, 2000) and oleic groups (Liu, Cheng, Li, Wang & Liu, 2018) can be cited. However, while the toxic effect of the above mentioned types of aldehydes is well known, the impact of epoxides on human health needs further research; according to Brühl and coworkers (2016), epoxidized fatty acids

are suspected of being linked with different diseases, such as cardiac failure or respiratory distress syndrome, among others.

Notwithstanding, in spite of the relevance of this topic, it could be said that very little is known about the evolution of oxidation products during digestion. In addition, in some cases dissenting results can be found, for example, relative to the persistence of hydroperoxides in the gastrointestinal tract (Kanazawa & Ashida, 1998; Nakatsugawa & Kaneda, 1983). With regard to secondary oxidation compounds, some studies have been conducted to assess the fate of aldehydes (Awada et al., 2012; Goicoechea et al., 2008; Goicoechea, Brandon, Blokland & Guillén, 2011) and of some epoxides (Chalvardjian, Morris & Holman, 1962; Wilson et al., 2002). Nevertheless, most of these works focused on determining the absorbed fraction of only a few compounds like 4-hydroxy-(*E*)-2-hexenal (Awada et al., 2012) and some ^{13}C-labelled monoepoxy- and diepoxy-triglycerides (Wilson et al., 2002), so the information provided is very limited.

With all the above in mind, the molecular processes of oxidized lipids occurring in digestion, the fate of lipid oxidation compounds which may impact on human health, and the influence of a high lipid oxidation degree on the bioaccessibility of this type of food components, will be addressed. To this aim, the evolution during *in vitro* digestion of highly oxidized virgin and refined soybean oils, coming from a prolonged accelerated storage process and containing a wide variety of oxidation products will be studied. Attention will focus on the bioaccessibility of both some oil components and oxidation products, which will be assessed by analyzing the extent of lipolysis, the occurrence of oxidation during digestion, the fate of different classes of lipid oxidation products already present in the samples subject of study and the generation of additional oxidation compounds, if any.

Furthermore, taking into account that the presence of other nutrients like proteins can modify both hydrolysis extent and the occurrence of oxidation during *in vitro* digestion (Nieva-Echevarría, Goicoechea & Guillén, 2017a), the effect of two different proportions of ovalbumin during digestion will also be addressed. The technique employed to accomplish the goals of this work was ^{1}H NMR, which permits one to study lipolysis extent as well as the advance and/or the occurrence of oxidation during the digestion process, in a global way.

The study of the evolution of lipids and their oxidation products during digestion, and of the parameters influencing this evolution can be considered an issue of primary

interest in furthering the intricate task of assessing how dietary lipids can affect human health. Indeed, some authors have already highlighted the need to deepen our knowledge about which classes of dietary lipid oxidation products decompose in the gut *versus* which ones survive transit to intestines, thus being available for absorption in the intestinal lumen (Márquez-Ruiz, García-Martínez, & Holgado, 2008; Schaich, Xie & Bogusz, 2017).

2. MATERIALS AND METHODS

2.1. Samples subject of study

The samples subject of study were two soybean oils, one virgin and the other one refined, both in an advanced degree of oxidation, containing both primary and a wide variety of secondary oxidation products. In order to obtain these samples, 10 g of both the virgin and the refined oils were weighed in glass Petri dishes of 80 mm diameter and submitted to an accelerated storage process at 70 ºC in a convection oven with aeration (Memmert GmbH+Co, Schwabach, Germany) for 8 and 9 days, respectively, in order to obtain samples with a similar oxidation degree; these were designated as VSX and RSX.

In addition, samples were prepared by mixing each one of the highly oxidized oils with food grade ovalbumin, acquired from a protein manufacturer (Apasa SA, Astigarraga, Spain). Two different proportions of ovalbumin were tested: 0.26 g per g of oil (low level of ovalbumin: LO) and 2.6 g per g of oil (high ovalbumin proportion: HO). The virgin oil samples were designated as VSX+LO and VSX+HO, and those prepared from the refined oil RSX+LO and RSX+HO.

2.2. *In vitro* gastrointestinal digestion

All the samples above mentioned (0.5 g of oil in all cases) were digested following the same procedure as in previous works (Nieva-Echevarría, Goicoechea, Manzanos & Guillén, 2017b; Nieva-Echevarría, Goicoechea & Guillén, 2017c), based on the static *in vitro* gastrointestinal model developed by Versantvoort, Oomen, Van de Kamp, Rompelberg and Sips (2005) and slightly modified in our laboratory in order to reach a higher level of lipolysis (Nieva-Echevarría, Goicoechea, Manzanos & Guillén, 2016). This *in vitro* digestion model involves a three-step procedure to simulate digestive processes in the mouth, stomach, and small intestine, by adding sequentially the corresponding digestive juices, whose composition is given in Table S1 (see

supplementary material). More details of the above procedure are also given in the supplementary material.

All the reagents used were acquired from Sigma-Aldrich. Two digestion experiments, each including duplicate samples of all the oil systems studied, were performed. Blank samples corresponding to the juices submitted to digestive conditions were also taken for further analysis.

2.3. Lipid extraction of the digestates

Lipids of the digestates were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich) and following the same methodology as in previous studies (Nieva-Echevarría et al., 2017b,c). Given that a strong emulsion is formed when mixing the digested sample with the extraction solvent in the case of the samples with a high proportion of ovalbumin, making it difficult to separate the aqueous and lipid phases, this extraction was performed with the aid of a centrifuge in order to break up this emulsion. For this purpose, a Sigma 3K30 centrifugal machine working at 10,000 rpm was used (Sigma Laboratory Centrifuges, Germany), each extraction step lasting 10 min. All the CH_2Cl_2 extracts of each sample were mixed and the solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. Afterwards, these extracts were stored at -80 ºC until their analysis.

In order to verify that the methodology used was suitable to efficiently extract the different types of oxidation products present in the undigested oils, it must be noted that samples of the starting oxidized oils were mixed with the digestive juices submitted to the digestion process, and then extracted following the same procedure mentioned above, complete recoveries being achieved in all cases.

2.4. Analysis by ^{1}H NMR

2.4.1. Operating conditions

The ^{1}H NMR spectra of the starting oils (VSX and RSX) and of the lipid extracts of the oil samples digested without ovalbumin (DVSX and DRSX), with a low proportion of ovalbumin (D(VSX +LO) and D(RSX+LO)) and with a high proportion of this protein (D(VSX+HO) and D(RSX+HO)) were acquired in quadruplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned lipid samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform that contained tetramethylsilane (TMS) as internal reference (Cortec, Paris, France).

The acquisition conditions were the same as those used in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2009).

2.4.2. Identification of some compounds

The identification of the different types of oxidation products present in the various samples was carried out on the basis of the proton signal assignments shown in Table S2 (see supplementary material), made from bibliographic data and with the aid of several standard compounds, also given in the supplementary material.

2.4.3. Quantification from ^{1}H NMR spectral data

Bearing in mind that the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generate it, and that the proportionality constant is the same for all kinds of protons, the area of some spectral signals was used to quantify: i) the molar percentages of the different types of glycerides and of glycerol; ii) the molar concentrations of linolenic and linoleic acyl groups+fatty acids referring to the total of acyl groups+fatty acids; and iii) the molar concentrations of the several classes of oxidation compounds present in the starting oils and in the lipid extracts of the various digested samples, referring to the total of acyl groups+fatty acids.

Concerning the various types of glycerides and glycerol. The molar percentages of triglycerides (TG%), diglycerides (1,2-DG% and 1,3-DG%), monoglycerides (2-MG% and 1-MG%) and glycerol (Gol%) in relation to the total number of glyceryl structures present in the lipid samples were determined using the equations developed and validated in previous studies (Nieva-Echevarría, Goicoechea, Manzanos & Guillén, 2014; Nieva-Echevarría, Goicoechea, Manzanos & Guillén, 2015). All these equations are given as supplementary material (see equations [S1-S10]). The chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of the main protons of glycerides and fatty acids are displayed in Table S2.

From the data relative to the different types of glycerides, another parameter related to lipolysis extent can also be determined: lipid bioaccessibility (L_{BA}), which takes into account only the molecules that are directly absorbable: MG and fatty acids (FA), in relation to the total number of acyl groups+fatty acids (AG+FA) present in the sample. This parameter was calculated by using equations [S11 and S12].

Concerning lipid composition. The concentrations of linolenic (Ln) and linoleic (L) AG+FA were estimated in millimoles per mole of the sum of AG+FA (mmol/mol

AG+FA) present both in the starting oils and in the lipid extracts of the various digested samples, by using equations [S13 and S14].

Concerning oxidation compounds. The concentrations of the different types of oxidation compounds present both in the starting oils and in the extracts of the digested samples, expressed in mmol/mol AG+FA, were also estimated as in a previous study (Nieva-Echevarría et al., 2017a) by using equation [S15]. In the case of the samples digested with a high ovalbumin proportion, signals attributable to the ovalbumin samples are perceived in the ^{1}H NMR spectra of their corresponding digestates, which overlap with those of some oil components (see Figure 1). Therefore, some corrections must be made, following the procedure described in the supplementary material.

2.5. Statistical analysis

The significance of the differences on the several determinations made among the samples was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Composition of the starting oil samples

Despite having been submitted to a prolonged accelerated storage process (see section 2.1), the oils subject of study (VSX and RSX) still contain polyunsaturated acyl groups (linoleic and linolenic) able to undergo oxidation during the *in vitro* digestion process; their respective concentrations are given in Table 1, expressed in mmol/mol AG+FA. In relation to the glyceryl structures in which acyl groups are supported, TG account for by far the highest proportion, even though very low molar percentages of 1,2-DG are also noticed in both samples; this can be observed in Table 2.

As a consequence of the previous thermoxidation process, VSX and RSX oils contain a wide variety of oxidation products; these are detailed in Table 3, together with their concentrations, expressed in mmol/mol TG. In addition, the enlargements of several ^{1}H NMR spectral regions of these samples where the signals of the various types of oxidation compounds appear are displayed in Figure 1. As Table 3 shows, both oils contain hydroperoxides supporting conjugated (*Z*,*E*)- and (*E*,*E*)-dienes (see signals "c" and "b", respectively, in Figure 1), the latter in higher concentrations than the former.

Together with hydroperoxides, several kinds of secondary oxidation products are found in samples VSX and RSX, of which epoxides constitute the major group. These

latter include several types of compounds, such as the tentatively identified (*E*)-epoxystearates, derived from oleic groups (see signal "d" in Figure 1). In addition, other epoxides giving signals either at 2.9 ppm or/and at 3.1 ppm (see signals "e" and "f", respectively, in Figure 1), are also present in the starting oils, the latter being the most abundant. Regarding signal "e", this could be due to (*Z*)-epoxystearates coming from oleic AG+FA (see signal "e1" in Table S2), to (*E*)-epoxy-keto-enes derived from linoleic groups (see signals "e4" and "e5" in Table S2), which also give another detectable signal (see signal "g" in Table S2 and Figure 1), and/or to other types of epoxy-compounds coming from polyunsaturated AG+FA (see signals "e2", "e3" and "e6" in Table S2). As far as signal "f" is concerned, some of the compounds contributing to it could be epoxy-hydroperoxy-enes coming from linoleic AG+FA (see signal "f3" in Table S2), since (*E*)-12,13-epoxy-9-hydroperoxy-10(*E*)-octadecenoate gives, among other signals, one double doublet at 3.11 ppm and another one at 5.85 ppm (Gardner, Weisleder & Kleiman, 1978), and signals that might match this latter are also observed in the spectra of samples VSX and RSX (see signal "i" in Figure 1); however, in our case, more than one isomer might be present, which would give rise to a more complex pattern of signals.

In addition, small amounts of (*Z,E*)- and (*E,E*)-keto-dienes (signals "m" and "l" in Figure 1) were present in SVX and RSX oils, as well as of other types of compounds tentatively identified as hydroxy-derivatives. The signals given by these latter appear very close to those of epoxides (see Figure 1, signals "j" and "k"), and they could be tentatively attributed to dihydroxy-derivatives like leukotoxin and/or isoleukotoxin diols, and to monohydroxy-derivatives, respectively (see Table S2), or to very closely related compounds.

Finally, samples VSX and RSX also exhibit several classes of aldehydes (see Table 3), among which 4-hydroperoxy-(*E*)-2-alkenals, 4-hydroxy-(*E*)-2-alkenals and (*E*)-2-alkenals, (signals "r", "q" and "n", respectively, in Figure 1) are the most abundant, followed by n-alkanals, (*E,E*)-2,4-alkadienals and 4,5-epoxy-2-alkenals (signals "s", "o" and "p").

It must be noticed that other oxidation compounds additional to the aforementioned ones will certainly be present in the starting oils, but they cannot be identified for the moment.

Table 1. Molar concentrations of the two kinds of polyunsaturated acyl groups+fatty acids, referring to the total of acyl groups+fatty acids present in the highly oxidized virgin and refined soybean oil samples, before (VSX and RSX) and after *in vitro* digestion in the absence of ovalbumin (DVSX and DRSX), with a low proportion of ovalbumin added (D(VSX+LO) and D(RSX+LO)), and with a high proportion of this protein (D(VSX+HO) and D(RSX+HO)). Different letters within each column indicate a significant difference among the samples corresponding to the same type of oil ($p < 0.05$).

	Linolenic	**Linoleic**
VSX	10.24 ± 0.44b	174.41 ± 2.34c
DVSX	7.03 ± 0.97a	148.38 ± 9.45a
D(VSX+LO)	7.14 ± 0.63a	147.76 ± 2.90a
D(VSX+HO)	7.59 ± 0.58a	161.73 ± 3.59b
RSX	7.10 ± 0.46b	167.73 ± 5.86b
DRSX	3.94 ± 1.07a	134.13 ± 9.32a
D(RSX+LO)	3.51 ± 0.42a	134.84 ± 3.62a
D(RSX+HO)	6.93 ± 0.68b	159.11 ± 7.50b

3.2. *In vitro* bioaccessibility of some compounds initially present in samples VSX and RSX after digestion

In this subsection the *in vitro* bioaccessibility of several types of compounds present in the starting oil samples is assessed. This will be influenced, among other factors, by the extent of lipolysis reached after the *in vitro* digestion process, which determines the fraction of bioaccessible glycerides and fatty acids, and also by other molecular processes taking place during digestion. Of these, the occurrence of oxidation could be cited; this can lead to the degradation of unsaturated acyl groups, together with other transformations undergone by the oxidation compounds either initially present in the samples, or newly formed during digestion, which determine the oxidation product profile of the digestates. Therefore, all the above mentioned issues (lipolysis, polyunsaturated acyl group degradation and changes in the oxidation compound profile after digestion) will be addressed.

Table 2. Molar percentages of triglycerides (TG%), diglycerides (1,2-DG% and 1,3-DG%), monoglycerides (2-MG% and 1-MG%) and glycerol (Gol%) in relation to the total number of glyceryl structures present in the oxidized virgin and refined soybean oil samples, together with lipid bioaccessibility (L_{BA}) parameter, before (VSX and RSX) and after *in vitro* digestion in the absence of ovalbumin (DVSX and DRSX), with a low proportion of ovalbumin added (D(VSX+LO) and D(RSX+LO)), and with a high proportion of this protein (D(VSX+HO) and D(RSX+HO)). Different letters within each column indicate a significant difference among the samples corresponding to the same type of oil ($p < 0.05$).

	TG%	**1,2-DG%**	**1,3-DG%**	**2-MG%**	**1-MG%**	**Gol%**	**L_{BA}**
VSX	99.01 ± 0.18b	0.50 ± 0.00a	-	-	-	nd	nd
DVSX	35.62 ± 0.60a	13.23 ± 1.76b	3.46 ± 1.09a	19.02 ± 1.35a	8.25 ± 0.85b	20.43 ± 2.61a	53.26 ± 2.34a
D(VSX+LO)	34.26 ± 0.88a	14.74 ± 0.76bc	2.78 ± 0.92a	21.90 ± 2.60a	7.26 ± 0.2b	18.39 ± 3.83a	53.39 ± 0.64a
D(VSX+HO)	30.21 ± 3.60a	16.58 ± 0.22c	2.96 ± 0.94a	28.90 ± 2.59b	5.59 ± 0.65a	15.77 ± 0.37a	56.77 ± 2.94a
RSX	99.08 ± 0.08d	0.90 ± 0.06a	-	-	-	nd	nd
DRSX	35.02 ± 0.47c	13.23 ± 1.18b	2.98 ± 0.29a	20.92 ± 1.04a	7.27 ± 1.47a	20.59 ± 1.50a	54.18 ± 0.51a
D(RSX+LO)	34.09 ± 0.11b	14.34 ± 0.55b	2.51 ± 0.02a	23.95 ± 1.87ab	7.44 ± 0.22a	17.68 ± 1.40a	54.68 ± 0.50a
D(RSX+HO)	31.51 ± 0.49a	17.01 ± 1.59c	3.22 ± 1.35a	27.16 ± 0.75c	4.35 ± 1.16b	16.74 ± 1.86a	55.13 ± 0.81a

-: not detected; nd: not determined

3.2.1. Lipolysis extent

3.2.1.1. In the samples digested without ovalbumin

As can be observed in Table 2, the percentage of TG remaining after the *in vitro* digestion process is very similar in samples DVSX and DRSX (around 35%) and the same can be said of the molar percentages of partial glycerides that either appear or exhibit an increase after digestion. This is the case of 2-MG, the most abundant ones after TG, with molar percentages between 19 and 21%, and 1,2-DG, in proportions of 13%. Furthermore, glycerol was released from the total hydrolysis of glyceride structures, reaching percentages of around 20% in both samples. 1-MG and 1,3-DG, stated as coming from the isomerization of 2-MG (Mattson & Volpenhein, 1966) and 1,2-DG (De Groot, 1972), respectively, were in lower proportions, especially the latter (around 7-8% and 3%, respectively). This distribution of the different types of glycerides matches well with that observed in previous works conducted with both fresh and slightly oxidized oils (Nieva-Echevarría et al., 2017b,c; Martin-Rubio et al., 2019). However, on the basis of the TG and glycerol percentages found in samples DVSX and DRSX (around 35 and 20%, respectively), it could be said that the overall lipolysis extent is lower than that observed after the *in vitro* digestion of the same unoxidized virgin and refined soybean oils (around 22% for TG and 30% for glycerol) (Martin-Rubio et al., 2019), thus reinforcing previous works (Nieva-Echevarría et al., 2017b,c).

However, something that attracts attention is that the lipolysis extent in samples DVSX and DRSX is only somewhat lower than that found in another study performed with these same virgin and refined soybean oils but with a lower oxidation degree (unpublished results). In that study, lower concentrations of hydroperoxides and practically no secondary oxidation products were detected by ^{1}H NMR. Therefore, despite the advanced oxidation degree of the samples of this study in comparison with that of the previous ones, the differences in the lipolysis extent between these two groups of samples are not very marked.

At this point, it must be mentioned that although it seems to be generally assumed that oxidation products can negatively affect the functionality of proteins, and thus the activity of enzymes, there are, as far as we know, only a few works in which the impact of specific oxidation products on the activity of digestive enzymes has been studied

(Gamage & Matsushita, 1973; Gamage, Mori & Matsushita, 1973; Matsushita & Kobayashi, 1970; Matsushita, Kobayashi & Nitta, 1970; Matsushita, 1975). Moreover, of all the cited studies, only the one carried out by Matsushita in 1975 includes pancreatic lipase. This researcher, who studied the effect of linoleic acid primary and secondary oxidation products separately, and also the effect of safflower oil TG hydroperoxides on the activity of RNAse, trypsin, pepsin and pancreatic lipase, showed that some enzymes were more inactivated by primary than by secondary oxidation products, and vice versa. In this regard, it is worth pointing out that pancreatic lipase was hardly inhibited by linoleic acid secondary oxidation products, and something more by safflower oil TG hydroperoxides. Moreover, only a small difference between the inhibition degrees caused by concentrations of TG hydroperoxides of 1 and 10 μmol/ml of lipase solution was observed. However, in the case of linoleic acid hydroperoxides, the lipase inhibition extent was considerably higher and, although the difference between the effects of a lower and a higher hydroperoxide concentration on lipase activity were more marked than that observed for safflower TG hydroperoxides, these were not proportional to their concentration either. Notwithstanding, it must be noticed that all these effects were observed after 20 min of incubation at 37 ºC, which is a much shorter time than that corresponding to the *in vitro* digestion process here studied.

In addition to the susceptibility of each enzyme to inactivation by specific oxidation products, another factor that could affect enzyme performance is the polarity of the system, which is higher in more oxidized samples due to the presence of a greater amount of compounds supporting oxygenated groups, which could facilitate lipolysis (Sánchez-Muniz, Benedí, Bastida, Olivero-David & González-Muñoz, 2011). In this sense, according to Arroyo, Sánchez-Muniz, Cuesta, Sinisterra and Sánchez-Montero (1997), the polar compounds formed during lipid thermal treatment could act as surfactants favouring the formation of a microemulsion, and in turn, the action of pancreatic lipase.

Therefore, all the above mentioned, which includes opposing effects that could be counteracting one another during digestion, could help to explain to some extent the small differences in lipolysis degree observed between the highly oxidized oils studied in this work and the slightly oxidized ones studied previously (unpublished results).

3.2.1.2. In the samples digested with different proportions of ovalbumin

As Table 2 shows, the presence of a low ovalbumin level hardly affects the molar percentages of the several kinds of glycerides when compared to the samples digested without ovalbumin, although a slight increment in the 2-MG molar percentage and a small glycerol proportion decrease are noticed. This could be due, at least in part, to a reduction in isomerization reactions of 2-MG to 1-MG, which are considered necessary to achieve complete lipolysis due to the specificity of pancreatic lipase (Mattson & Volpenhein, 1964). Notwithstanding, given that both 2-MG and 1-MG are absorbable molecules, the small changes observed do not affect lipid bioaccessibility (see L_{BA} parameter in Table 2).

However, when ovalbumin is present in a high proportion, lipolysis is enhanced to a certain extent (see samples D(VSX+HO) and D(RSX+HO) in comparison with DVSX and DRSX in Table 2); this is evidenced by a decreased amount of remaining TG and increased molar percentages of 1,2-DG and especially of 2-MG. The more pronounced TG hydrolysis might be explained by the emulsifying ability of ovalbumin and its hydrolysis products (Chen, Chi, Zhao & Xu, 2012; Mine, Noutomi & Haga, 1991), which would diminish the size of the oil droplets, increasing the lipid area exposed to the lipases present in the aqueous phase (Li, Hu & McClements, 2011). In addition, the potential reaction of some oxidation products with ovalbumin instead of with lipases, might also contribute to somewhat improving lipolysis extent. Regarding the increment in 1,2-DG and 2-MG proportions mentioned, which is accompanied by a decrease in the molar percentages of 1-MG and glycerol (see Table 2), this might be due to a buffering effect of the high proportion of ovalbumin. This could give rise to a more elevated pH during the gastric step of the *in vitro* digestion than in the rest of digested samples, causing a reduction in the isomerization reactions of 1,2-DG and 2-MG (Mattson & Volpenhein, 1964), and consequently in the hydrolysis of 1-MG to glycerol. Moreover, any change in the pH existing during digestion might also affect both the activity of digestive enzymes and their inactivation degree by oxidation products (Matsushita & Kobayashi, 1970), since variations in pH can provoke changes in the conformational structure of proteins, modifying the surface exposed groups susceptible to reacting with the compounds present in the surrounding medium (Gamage, Mori & Matsushita, 1973). As far as L_{BA} is concerned, data in Table 2 reveals that, in agreement with that observed in the proportions of the different kinds of glycerides and of glycerol, only

small increases are found in this parameter when a high proportion of ovalbumin is present in the system.

The small effect of this high concentration of ovalbumin on the molar percentages of TG and on L_{BA} found after digestion contrasts with the great increase in both parameters observed in presence of the same proportion of ovalbumin during the *in vitro* digestion of less oxidized soybean oil samples (unpublished results) (from approximately 31% of TG to less than 8%, and from 56-58% to around 80% in the case of L_{BA}), and also of slightly oxidized sunflower and linseed oils (Nieva-Echevarría et al., 2017a). In this regard, although as commented above, a high proportion of ovalbumin could exert an emulsifying effect throughout digestion, the same effect might also favour the reaction of the oxidation products present in the oil with active sites of the lipases, thus counteracting to a certain extent the potential increase in their activity due to a reduction in droplet size. In addition, the emulsifying ability of ovalbumin could also be negatively influenced by the potential reaction of oxidation products either with ovalbumin and/or with proteases like trypsin and pepsin, which can hydrolyze ovalbumin and increase its emulsifying properties (Chen et al., 2012). Another possibility could be that the polymerization degree of the samples also hindered the emulsifying effect of ovalbumin. Either way, although the potential influence of all these factors, and of others not considered, makes it difficult to explain the results observed, their comparison with the outcomes of the previous study carried out with slightly oxidized soybean oil samples (unpublished results) suggests that the high oxidation degree of the oils here studied causes a loss in the efficiency of ovalbumin in improving lipolysis.

3.2.2. Assessment of oxidation occurrence during digestion through the monitoring of polyunsaturated group degradation

Given that polyunsaturated groups are the most prone to oxidation, their concentrations, expressed in mmol/mol AG+FA, were also determined after the *in vitro* digestion process; the corresponding values are displayed in Table 1 (see samples DVSX and DRSX), together with those of the undigested oils. This reveals clear and important diminutions in the concentrations of linolenic and linoleic AG+FA after digestion, more pronounced for linolenic (reduction of more than 30% after digestion) than for linoleic groups (15-20% of reduction after digestion).

While the presence of a low ovalbumin proportion in the samples subjected to digestion does not affect the evolution of polyunsaturated AG+FA during this process, a less pronounced decrease in their concentrations is observed in the samples digested with a high ovalbumin proportion than in the rest of digested samples, (see Table 1), thus reinforcing previous results obtained with other types of slightly oxidized oils (Nieva-Echevarría et al., 2017a). This suggests that, at the higher concentration tested, ovalbumin seems to exert an antioxidant effect during digestion, and indeed the antioxidant ability of ovalbumin and its hydrolysates has already been described (Abeyrathne, Lee, Jo, Nam & Ahn, 2014).

These results contrast with the findings relative to the digestion of these same soybean oils but with a lower degree of oxidation (unpublished results), where significant variations in the concentrations of polyunsaturated acyl groups were not observed after digestion, either in the absence or in presence of the same two ovalbumin proportions here tested. This reveals that oxidation occurs to a greater extent when the initial oxidative status of the oil samples is greater.

3.2.3. Changes in the oxidation compound profile after digestion

The concentration of the different kinds of oxidation products in DVSX and DRSX samples are shown in Table 3, and the changes, or in some cases appearance, of their corresponding ^{1}H NMR spectral signals, in Figure 1.

3.2.3.1. In the samples digested without ovalbumin

***Evolution of hydroperoxides, monitored through their associated conjugated dienes*.** The concentration of both (*Z,E*)- and (*E,E*)-hydroperoxy-dienes decreases after digestion (see signals "c" and "b", respectively, in Figure 1 and Table 3), the diminution being more pronounced for the (*E,E*)-isomers than for their (*Z,E*)-counterparts. This means that part of the hydroperoxides evolve during *in vitro* digestion, even though a potential reaction with proteins present in the digestive juices should not be discarded (Gardner, 1979). However, these findings do not indicate that hydroperoxide generation has not occurred during digestion, but that their rate of transformation is higher than that of formation, if any.

***Generation of hydroxy-dienes*.** In addition to the drop in the initial hydroperoxide levels, the appearance of very small signals of conjugated (*Z,E*)-dienes supporting hydroxy-groups (signal "a" in Figure 1), probably coming from the reduction of hydroperoxides, can also be noticed in the spectra of the lipid extracts of DVSX and

DRSX samples. Although the generation of (*E,E*)-hydroxy-dienes has also been noticed after the *in vitro* digestion of slightly oxidized sunflower (Nieva-Echevarría et al., 2017b) and slightly oxidized soybean oils (unpublished results), the existence of overlapping signals in the same spectral region in the samples of this study does not allow one to evaluate the concentration of this type of compounds. The generation of linoleic acid hydroxides after administering linoleic acid hydroperoxides intragastrically to rats was also observed by Kanazawa and Ashida (1998).

Changes in epoxide profile. Regarding (*E*)-epoxystearates, Figure 1 shows that a slight shifting of its signal ("d") to lower fields in relation to the undigested oils occurs in the ^{1}H NMR spectra of the extracts of samples DVSX and DRSX; this causes its partial overlap with the signals of *bis*-allylic protons ("H+G", see Table S2), making it somewhat difficult to accurately estimate the concentration of this type of epoxides; for this reason, they have not been quantified.

With respect to the epoxides giving signal "e", a very small decrease in their concentration can be observed after digestion (see samples DVSX and DRSX in Table 3). This finding contrasts to a certain extent with that found in previous works performed with fresh virgin and refined soybean oils (Martin-Rubio et al., 2019), where the generation of this type of epoxides was noticed after digestion. This difference might be explained because, although epoxide generation could take place during the *in vitro* digestion of SVX and RSX oils, some of the epoxides already present in these samples could give rise to other types of compounds, in such a way that the rate of transformation could be higher than that of potential formation. Among these transformations, it would be possible that the epoxy group react with amino acid residues of proteins present in the digestive fluids (Gardner, 1979; Schaich, 2008).

As far as epoxides giving signal "f" are concerned, their concentration decreases after digestion to a greater extent than that of the above mentioned (see Table 3). This seems to be due, at least in part, to the drop in the concentration of the tentatively identified epoxy-hydroperoxy-enes, deducible from the reduction in the intensity of signal "i" (see Figure 1). This could be due to their evolution bringing about other compounds, including their reaction with protein amino acid residues, since this class of compounds bears two reactive groups (epoxy and hydroperoxy).

When it comes to (*E*)-epoxy-keto-enes, their concentration significantly increases after digestion in a statistically significant way (see Figure 1, signals "g" and "h", and

Table 3). Taking into account that 12,13(*E*)-epoxy-9-hydroperoxy-10(*E*)-octadecenoic acid could be an intermediate in the formation of 12,13(*E*)-epoxy-9-keto-10(*E*)-octadecenoic (Gardner & Kleiman, 1981), part of the epoxy-hydroperoxy-enes initially present in the samples might have given rise to epoxy-keto-enes, thus contributing to the increased concentration of these latter. Notwithstanding, epoxy-keto-enes could also proceed directly from hydroperoxy-dienes, since the epoxyketo-ene derivative has been reported to be a major product of the decomposition of linoleic acid hydroperoxides under acidic conditions (Gardner, Weisleder & Nelson, 1984), which exist during the gastric stage of the *in vitro* digestion process. In this regard, Kanazawa and Ashida (1998) also described the generation of epoxy-keto-enes coming from the decomposition of linoleic acid hydroperoxides in the stomach of rats, although the compounds found by these authors were different from those detected here.

Changes in the concentration of keto-dienes. As Table 3 reveals, this type of compounds exhibits a concentration increase in samples DVSX and DRSX, probably due to their generation from the hydroperoxides initially present.

Changes in the concentration of compounds tentatively considered as hydroxy-derivatives. As Table 3 shows, the concentration of the compounds tentatively identified as dihydroxy-derivatives (giving signal "j") hardly varies after digestion, exhibiting only a very small decrease. These outcomes contrast to some extent with those posed by some authors, according to whom diols could be generated from epoxy compounds under acidic conditions in gastric medium (Giuffrida, Destaillats, Robert, Skibsted & Dionisi, 2004). Taking into account that these diols might come from linoleic monoepoxides, this finding would match with the scarce variations observed after digestion in the levels of epoxides giving signal "e".

Regarding the other type of tentative hydroxy-derivatives detected in samples VSX and RSX, giving signal "k" (see Table 3), it was not possible to estimate their concentration in samples DVSX and DRSX, since this signal (see Figure 1) overlaps with that of 1-MG (see signal "I" in Table S2), much more abundant in the digested samples.

***Changes in the concentration of aldehydes*.** Both Figure 1 and Table 3 show significant increments in the concentration of n-alkanals after digestion. Along the same lines, the generation of saturated aldehydes was also observed in previous works carried out with slightly oxidized samples both of linseed oil (Nieva-Echevarría et al., 2017c)

and of virgin and refined soybean oils (unpublished results). Similarly, the generation of hexanal was noticed by Kanazawa and Ashida (1998) after administering linoleic acid hydroperoxides intragastrically to rats. By contrast, the concentrations of the rest of aldehydes decrease, this diminution being very sharp for the sum of 4-hydroperoxy- and 4-hydroxy-(*E*)-2-alkenals, and for 4,5-epoxy-2-alkenals, less pronounced for (*E*)-2-alkenals and practically null for (*E,E*)-2,4-alkadienals. This decline in the levels of most unsaturated aldehydes could be due to their reaction with proteins (Uchida, 2003), which are present in the digestive juices. This possibility has also been pointed out by other authors (Steppeler, Haugen, Rødbotten & Kirkhus, 2016; Van Hecke, Vossen, Bussche, Raes, Van Hecke and De Smet, 2014) regarding 4-hydroxy-(*E*)-2-alkenals, and in fact, the greatest concentration diminution is observed for the oxygenated α,β-unsaturated aldehydes (4-hydroperoxy-(*E*)-2-alkenals, 4-hydroxy-(*E*)-2-alkenals and 4,5-epoxy-2-alkenals), which are the most reactive among those detected, although they are still present in the digestates. This latter finding agrees with those of Goicoechea and coworkers (2008, 2011), who analyzed the fate of volatile aldehydes during the *in vitro* digestion of a mixture of thermodegraded sunflower oil and a standard meal, observing that part of them, including the oxygenated α,β-unsaturated ones, were detectable after this process.

In this context, the expected lower reactivity of n-alkanals in comparison with that of α,β-unsaturated aldehydes, together with a potentially greater generation during digestion, could contribute to explaining why saturated aldehydes are the only ones showing increased concentration after digestion. In this same line, the levels of the rest of aldehyde groups in samples DVSX and DRSX will be the result of the balance between their generation and their reaction with other compounds such as proteins present in the medium. Therefore, taking into account that reactions between aldehydes and amino acids residues can occur in a system containing proteins (Schaich, 2008; Uchida, 2203), the degree of oxidation can be easily underestimated if it is assessed through the measurement of aldehyde concentration.

Table 3. Concentration of the several kinds of oxidation compounds, expressed in mmol/mol acyl groups+fatty acids present in the oxidized virgin and refined soybean oil samples, before (VSX and RSX) and after *in vitro* digestion in the absence of ovalbumin (DVSX and DRSX), with a low proportion of ovalbumin added (D(VSX+LO) and D(RSX+LO)), and with a high proportion of this protein (D(VSX+HO) and D(RSX+HO)). Different letters within each row indicate a significant difference among the samples corresponding to the same type of oil ($p < 0.05$). 4-OOH: 4-hydroperoxy; 4-OH: 4-hydroxy.

	Virgin oil samples				**Refined oil samples**			
	VSX	**DVSX**	**D(VSX+LO)**	**D(VSX+HO)**	**RSX**	**DRSX**	**D(RSX+LO)**	**D(RSX+HO)**
Hydroperoxy-conjugated dienes								
(*Z*,*E*)-Hydroperoxy-dienes	**15.24 ± 0.46a**	8.83 ± 1.04b	8.32 ± 0.67b	6.90± 0.78a	**11.98 ± 1.99b**	9.07 ± 0.95a	8.88 ± 1.76a	7.46± 0.71a
(*E*,*E*)-Hydroperoxy-dienes	**23.84 ± 0.0.64b**	12.53 ± 1.32a	12.15 ± 2.66a	9.74 ± 0.87a	**24.44 ± 1.61b**	12.09 ± 0.93a	11.29 ± 1.12a	11.03 ± 1.31a
Total hydroperoxy-dienes	**39.08 ± 1.10c**	21.36 ± 2.36b	20.47 ± 3.33b	16.64 ± 1.66a	**36.42 ± 3.59b**	21.15 ± 1.89a	20.18 ± 2.87a	18.49 ± 2.02a
Hydroxy-conjugated dienes								
(*Z*,*E*)-Hydroxy-dienes	-	2.35 ± 0.38a	2.42 ± 0.34a	5.03 ± 1.09b	-	2.04 ± 0.40a	2.47 ± 0.31a	4.55 ± 0.66b
(*E*,*E*)-Hydroxy-dienes	-	nd	nd	nd	-	nd	nd	nd
Epoxides								
(*E*)-Epoxystearates	**0.85 ± 0.04**	nd	nd	nd	**1.05 ± 0.04**	nd	nd	nd
Giving signal "e"	**11.31 ± 1.09a**	10.60 ± 0.42a	10.69 ± 0.62a	11.01 ± 0.57a	**14.32 ± 0.52a**	12.59 ± 0.47a	12.70 ± 1.19a	13.02 ± 0.74a
Giving signal "f"	**25.79 ± 2.06b**	20.42 ± 2.29a	20.40 ± 1.68a	20.18 ± 1.65a	**32.40 ± 0.98b**	24.84 ± 3.01a	24.39 ± 0.47a	23.60 ± 2.06a
(*E*)-Epoxy-keto-enes	**1.58 ± 0.68a**	3.04 ± 0.20b	2.91 ± 0.34b	2.66 ± 0.13b	**1.65 ± 0.18a**	3.73 ± 0.44c	3.77 ± 0.27c	3.00 ± 0.09b
Keto-dienes								
(*Z*,*E*)-Keto-dienes	**2.02 ± 0.19a**	3.79 ± 0.57b	3.55 ± 0.46b	2.98 ± 0.55b	**1.63 ± 0.10a**	3.31 ± 0.40b	3.20 ± 0.25b	2.80 ± 0.54b
(*E*,*E*)-Keto-dienes	**3.97 ± 0.13a**	5.42 ± 0.72b	5.15 ± 0.45b	4.81 ± 0.24b	**4.31 ± 0.28a**	5.68 ± 1.02a	5.41 ± 0.86a	4.95 ± 0.66a
Potential hydroxy-derivatives								
Giving signal "j"	**2.65 ± 0.17b**	2.36± 0.32ab	2.27 ± 0.27ab	1.89 ± 0.27a	**3.25 ± 0.27b**	3.16 ± 0.59b	3.10 ± 0.14b	1.99 ± 0.39a
Giving signal "k"	**1.85 ± 0.12**	nd	nd	nd	**2.13 ± 0.19**	nd	nd	nd
Aldehydes								
n-Alkanals	**1.59 ± 0.16a**	4.27 ± 0.53c	3.75 ± 0.72bc	3.15 ± 0.29b	**2.25 ± 0.15a**	4.71 ± 0.87b	4.50 ± 1.10b	3.55 ± 0.51ab
4-OOH-+4-OH-(*E*)-2-alkenals	**7.48 ± 0.30c**	2.24 ± 0.34b	1.78 ± 0.16b	1.24 ± 0.18a	**9.01 ± 0.58b**	2.25 ± 0.14a	2.16 ± 0.64a	1.76 ± 0.30a
4,5-Epoxy-2-alkenals	**0.62 ± 0.06b**	0.42 ± 0.10a	0.34 ± 0.10a	-	**0.87 ± 0.23b**	0.38 ± 0.17a	0.34 ± 0.16a	-
(*E*,*E*)-2,4-Alkadienals	**0.91 ± 0.11b**	0.90 ± 0.10b	0.92 ± 0.02b	0.64 ± 0.11a	**0.99 ± 0.18 a**	0.91 ± 0.19a	0.89 ± 0.14a	0.70 ± 0.15a
(*E*)-2-Alkenals	**3.23 ± 0.21d**	2.63 ± 0.53c	1.79 ± 0.20b	0.80 ± 0.16a	**3.95 ± 0.19d**	2.36 ± 0.30c	1.77 ± 0.24b	1.11 ± 0.18a

-: not detected; nd: not determined

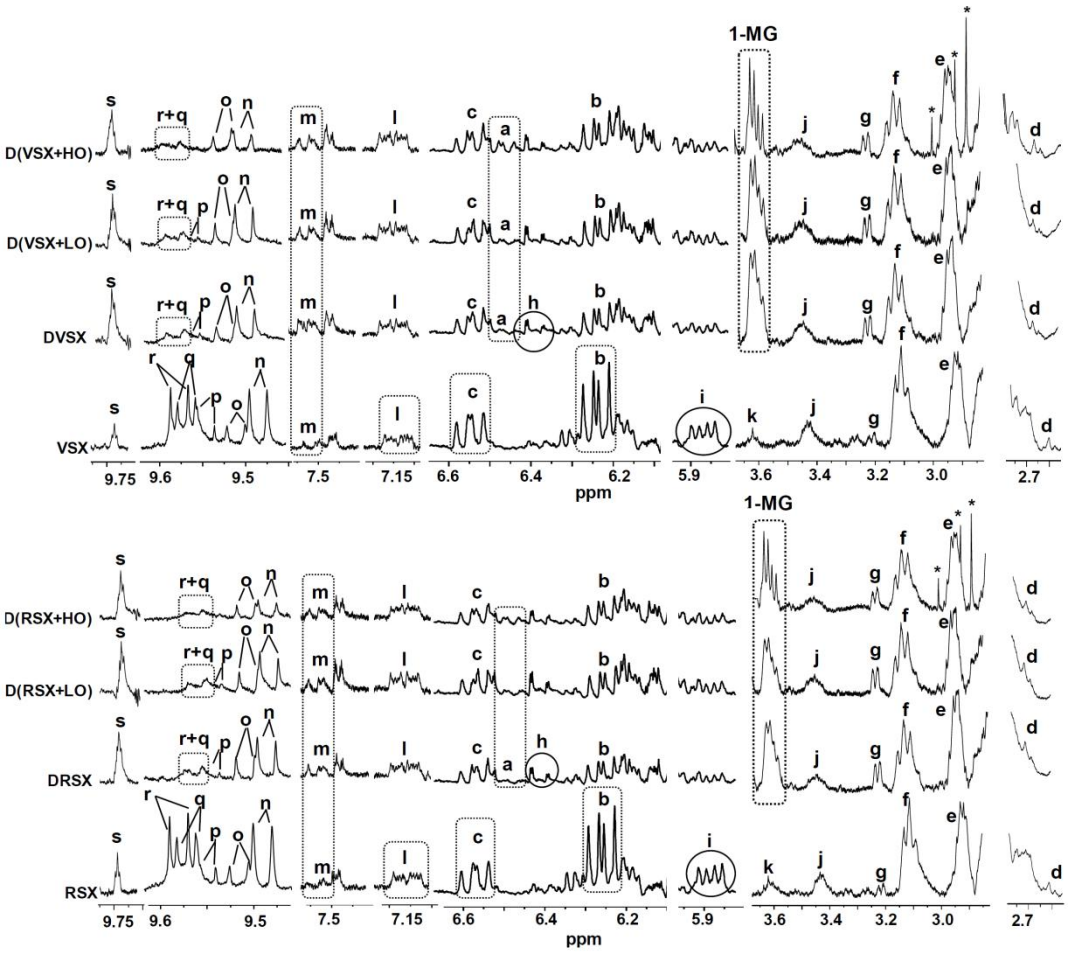

Figure 1. Enlargements of some regions of the ^{1}H NMR spectra of the highly oxidized virgin and refined oils (VSX and RSX), and of the lipid extracts obtained after their *in vitro* digestion process in the absence of ovalbumin (DVSX and DRSX), as well as in the presence of a low (D(VSX+LO) and D(RSX+LO)) and a high proportion of ovalbumin (D(VSX+HO) and D(RSX+HO)). The signal letters agree with those in Table S1, considering that "e" includes signals "e1-e6" and "f" signals "f1-f3". Signals marked with an asterisk are considered to come from the ovalbumin sample used. The plots corresponding to the same ^{1}H NMR spectral region are presented at a fixed value of absolute intensity, for them to be valid for comparative purposes.

3.2.3.2. In the samples digested with different proportions of ovalbumin

As shown in Table 3, the presence of a low ovalbumin proportion during digestion does not provoke significant changes in the concentration of most of the oxidation products present in the digestates when compared with the samples digested without ovalbumin, except for (*E*)-2-alkenals, which show a significant decrease.

Notwithstanding, slightly lower levels of the rest of aldehydes, as well as of hydroperoxy-dienes and keto-dienes, together with somewhat higher concentrations of (*Z*,*E*)-hydroxy-dienes, are perceived.

When ovalbumin is present in a high concentration in the samples subjected to digestion, a greater reduction in hydroperoxy-diene levels, along with a significant increase in the concentrations of (*Z*,*E*)-hydroxy-dienes, are noticed (see Table 3, samples D(VSX+HO) and D(RSX+HO)). This suggests that, as ovalbumin concentration increases, the formation of hydroxy-dienes is favoured over other types of reactions, thus reinforcing previous findings (Nieva-Echevarría et al., 2017a). Table 3 also reveals that while the levels of the epoxides giving signals "e" and "f" are not noticeably modified by the presence of a high proportion of ovalbumin, the concentration increments observed after digestion for (*E*)-epoxy-keto-enes, keto-dienes and n-alkanals are slightly smaller than in the rest of digested samples. Regarding the various groups of unsaturated aldehydes, their respective levels decrease to a greater extent than in the rest of digested samples, and even (*E*,*E*)-2,4-alkadienals show a concentration decline in samples D(VSX+HO) and D(RSX+HO). Finally, a drop is also noticed in the concentration of potential dihydroxy-derivatives in comparison with the samples digested without ovalbumin.

The lower concentrations of some oxidation products observed in the samples digested with a high ovalbumin proportion might be due to their reaction with this protein, but also to a smaller transformation of hydroperoxides into other types of oxidation products in favour of hydroxy-diene formation. In addition, considering that, on the basis of polyunsaturated group evolution (see section 3.2.2), a lower oxidation degree seems to have taken place in these samples, this could also contribute to the lower levels of some oxidation compounds.

3.5. Some remarks about the relevance of the presence of oxidation products in the gastrointestinal tract

In the light of the outcomes of this work, it is evident that a large proportion of the oxidation products present in the highly oxidized oils here studied, including hydroperoxides, remains after the *in vitro* digestion process. The bioaccessibility of all these compounds will depend on whether they are supported or not on absorbable molecules. Notwithstanding, irrespective of whether they are absorbed or not, their presence in the gastrointestinal tract could negatively affect human health, since damage

of the intestinal barrier due to reactions with oxidation compounds can trigger the development of various pathologies (Catalioto, Maggi & Giuliani, 2011). In this regard, hydroperoxides have been shown to induce oxidative damage and cell death in the colon, and this has been suggested to contribute to an enhanced risk of colon cancer (Udilova, Jurek, Marian, Gille, Schulte-Hermann & Nohl, 2003). In addition, the inhibition of gastrointestinal detoxifying enzymes by their reaction with aldehydes can favour a greater absorption of certain oxidation products (Márquez-Ruiz et al., 2008), with the consequent impact on the systemic oxidative stress level.

This work also evidences that, of all various classes of oxidation compounds studied, epoxides are in the highest proportion after digestion. This finding is in line with the results of Chalvardjian and coworkers (1962), who reported that the epoxy group survived the digestive system, and with those of Wilson and coworkers (2002), who observed that ^{13}C-labelled epoxides were absorbed intact. This can be considered a relevant issue, since among these epoxides, toxic monoepoxides of linoleic groups like leukotoxin and isoleukotoxin (Greene et al., 2000), as well as (*Z*)-epoxystearic acid, which has also been recently attributed toxic effects (Liu et al., 2018), could be present. Moreover, epoxides are considered in general as very reactive compounds (Schaich, 2008) that might affect the functionality of different types of biomolecules, and depending on their absorption extent, their deleterious effects could go beyond the intestine and reach other targets. Nevertheless, it must also be noticed that the reactivity of epoxides does not seem to be as high as that of oxygenated α,β-unsaturated aldehydes, since these latter exhibit, in general, a greater concentration decrease after digestion. These results offer a new perspective on the relevance of the various classes of oxidation products coming from dietary sources in biological damage and on the assessment of the health risks derived from them; thus, although some types of aldehydes are very reactive and toxic compounds (Guillén & Goicoechea, 2008), a great proportion of them seems to react with components of the digestive fluids, while in contrast, a considerable proportion of epoxides and also of other types of oxidation products are still present after the digestion process.

4. CONCLUSIONS

The extent of lipolysis, and in consequence lipid bioaccessibility, is negatively affected by the high oxidation level of the samples subject of study. In addition, the

degradation of polyunsaturated acyl groups during digestion due to oxidation also contributes to a reduction in the amount of bioaccessible lipid nutrients. Regarding the changes in the oxidation product profile of the starting oils after *in vitro* digestion, it has been shown that hydroperoxides exhibit a concentration decrease, either due to their evolution to give rise to other types of oxidation products, and/or to their reaction with components of the digestive juices. By contrast, other compounds like epoxides, keto-dienes and dihydroxyderivatives persist to a greater extent after digestion, and some of them even show an increase in concentration. When it comes to aldehydes, while the concentration of n-alkanals increases during digestion, those of the different groups of α,β-unsaturated aldehydes exhibit decreases of variable magnitude, these being especially marked for the very reactive and toxic oxygenated ones. This could be probably due to their reaction with enzymes and/or other proteins present in the digestive fluids; notwithstanding, part of them are still present in the digestates.

All this evidences that the reactions occurring through the *in vitro* digestion process are very complex, and entail generation, evolution and/or reaction of lipid primary and secondary oxidation products, thus showing the difficulty of adequately assessing the oxidation extent when proteins able to react with oxidation compounds are present in the system. This issue evidences the importance of selecting oxidation markers appropriate to the composition of the system subject of study.

The addition of a low proportion of ovalbumin has little effect on lipolysis and polyunsaturated acyl group degradation during digestion. However, when this protein is present at a high level, a slight increment in lipolysis and a lower oxidation degree are noticed during digestion, all this resulting in a small increase in the bioaccessibility of polyunsaturated fatty acids. Moreover, as the concentration of ovalbumin in the system increases, an enhancement in the reduction of hydroperoxides to their corresponding hydroxides, together with a diminution in the levels of (*E*)-epoxy-keto-enes, keto-dienes, dihydroxy-derivatives and especially of aldehydes, are observed. As a consequence, reduced concentration of some toxic compounds like oxygenated α,β-unsaturated aldehydes are noticed in the samples digested with a high ovalbumin proportion. This diminution in the concentration of oxidation compounds could be due to the lower oxidation extent observed in these samples and/or to their reaction with the ovalbumin present in the system. It is worth noticing that the reactions between aldehydes and proteins like ovalbumin, which can be considered of the Maillard-type,

could act as a detoxifying mechanism in the context of a food system. However, it must also be taken into account that the involvement of essential amino acid residues like for example lysine in these reactions, and the diminution in the susceptibility to be hydrolyzed of the so modified proteins, could lead to a decrease in their digestibility and in their nutritional value.

This knowledge could be useful in order to obtain a more global view of which type of oxidation compounds can remain or be generated during digestion and then be available for absorption or for reacting with components of the gastrointestinal tract; this would allow a better assessment of the impact on human health of dietary oxidation products.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abeyrathne, E. D. N. S., Lee, H. Y., Jo, C., Nam, K. C., & Ahn, D. U. (2014). Enzymatic hydrolysis of ovalbumin and the functional properties of the hydrolysates. *Poultry Science, 93*, 2678-2686.

Arroyo, R., Sánchez-Muniz, F. J., Cuesta, C., Sinisterra, J. V., & Sánchez-Montero, J. M. (1997). Thermoxidation of substrate models and their behavior during hydrolysis by porcine pancreatic lipase. *Journal of the American Oil Chemists' Society, 74*, 1509-1516.

Awada, M., Soulage, C. O., Meynier, A., Debard, C., Plaisancié, P., Benoit, B., Picard, G., Loizon, E., Chauvin, M. A., Estienne, M., Peretti, N., Guichardant, M., Lagarde, M., Genot, C., & Michalski, M. C. (2012). Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. *Journal of Lipid Research, 53*, 2069-2080.

Brühl, L., Weisshaar, R., & Matthäus, B. (2016). Epoxy fatty acids in used frying fats and oils, edible oils and chocolate and their formation in oils during heating. *European Journal of Lipid Science and Technology, 118*, 425-434.

Catalioto, R. M., Maggi, A. C., & Giuliani, S. (2011). Intestinal epithelial barrier dysfunction in disease and possible therapeutical interventions. *Current Medicinal Chemistry, 18*, 398-426.

Chalvardjian, A., Morris, L. J., & Holman, R. T. (1962). Effect of dietary epoxyoleic acid upon rats. *The Journal of Nutrition, 76*, 52-58.

Chen, C., Chi, Y. J., Zhao, M. Y., & Xu, W. (2012). Influence of degree of hydrolysis on functional properties, antioxidant and ACE inhibitory activities of egg white protein hydrolysate. *Food Science and Biotechnology, 21*, 27-34.

De Groot, W. T. M. (1972). Acyl migration solid phase isomerization of 1,2-diglycerides to 1,3-isomers. *Lipids, 7*, 626-628.

Esterbauer, H. (1993). Cytotoxicity and genotoxicity of lipid-oxidation products. *The American Journal of Clinical Nutrition, 57*, 779S-785S.

Gamage, P. T., & Matsushita, S. (1973). Interactions of the autoxidized products of linoleic acid with enzyme proteins. *Agricultural and Biological Chemistry, 37*, 1-8.

Gamage, P. T., Mori, T., & Matsushita, S. (1973). Mechanism of polymerization of proteins by autoxidized products of linoleic acid. *Journal of Nutritional Science and Vitaminology, 19*, 173-182.

Gardner, H. W. (1979). Lipid hydroperoxide reactivity with proteins and amino acids: a review. *Journal of Agricultural and Food Chemistry, 27*, 220-229.

Gardner, H. W., & Kleiman, R. (1981). Degradation of linoleic acid hydroperoxides by a cysteine FeCl3 catalyst as a model for similar biochemical reactions: II. Specificity in formation of fatty acid epoxides. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 665*, 113-125.

Gardner, H. W., Weisleder, D., & Kleiman, R. (1978). Formation of *trans*-12,13-epoxy-9-hydroperoxy-trans-10-octadecenoic acid from 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid catalyzed by either a soybean extract or cysteine-FeC13. *Lipids, 13*, 246-252.

Gardner, H. W., Weisleder, D., & Nelson, E. C. (1984). Acid catalysis of a linoleic acid hydroperoxide: formation of epoxides by an intramolecular cyclization of the hydroperoxide group. *The Journal of Organic Chemistry, 49*, 508-515.

Giuffrida, F., Destaillats, F., Robert, F., Skibsted, L. H., & Dionisi, F. (2004). Formation and hydrolysis of triacylglycerol and sterols epoxides: role of unsaturated triacylglycerol peroxyl radicals. *Free Radical Biology and Medicine, 37*, 104-114.

Goicoechea, E., Van Twillert, K., Duits, M., Brandon, E. D., Kootstra, P. R., Blokland, M. H., & Guillén, M. D. (2008). Use of an *in vitro* digestion model to study the bioaccessibility of 4-hydroxy-2-nonenal and related aldehydes present in oxidized oils rich in omega-6 acyl groups. *Journal of Agricultural and Food Chemistry, 56*, 8475-8483.

Goicoechea, E., Brandon, E. F., Blokland, M. H., & Guillén, M. D. (2011). Fate in digestion *in vitro* of several food components, including some toxic compounds coming from omega-3 and omega-6 lipids. *Food and Chemical Toxicology, 49*, 115-124.

Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau, C., & Hammock, B. D. (2000). Metabolism of monoepoxides of methyl linoleate: bioactivation and detoxification. *Archives of Biochemistry and Biophysics, 376*, 420-432.

Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated alpha, beta-unsaturated aldehydes and their study in foods: A review. *Critical Reviews in Food Science and Nutrition, 48*, 119-136.

Guillén, M. D., & Ruiz, A. (2003). Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups. *European Journal of Lipid Science and Technology, 105*, 688-696.

Guillén, M. D., & Uriarte, P. S. (2009). Contribution to further understanding of the evolution of sunflower oil submitted to frying temperature in a domestic fryer: study by ^{1}H nuclear magnetic resonance. *Journal of Agricultural and Food Chemistry, 57*, 7790-7799.

Kalogeropoulos, N., Salta, F. N., Chiou, A., & Andrikopoulos, N. K. (2007). Formation and distribution of oxidized fatty acids during deep-and pan-frying of potatoes. *European Journal of Lipid Science and Technology, 109*, 1111-1123.

Kanazawa, K., Ashida, H., Minamoto, S., Danno, G. I., & Natake, M. (1988). The effects of orally administered linoleic acid and its autoxidation products on intestinal mucosa in rat. *Journal of Nutritional Science and Vitaminology, 34*, 363-373.

Kanazawa, K., & Ashida, H. (1998). Catabolic fate of dietary trilinoleoylglycerol hydroperoxides in rat gastrointestines. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1393*, 336-348.

Li, Y., Hu, M., & McClements, D. J. (2011). Factors affecting lipase digestibility of emulsified lipids using an *in vitro* digestion model: proposal for a standardised pH-stat method. *Food Chemistry, 126*, 498-505.

Liu, Y., Cheng, Y., Li, J., Wang, Y., & Liu, Y. (2018). Epoxy stearic acid, an oxidative product derived from oleic acid, induces cytotoxicity, oxidative stress, and apoptosis in HepG2 cells. *Journal of Agricultural and Food Chemistry, 66*, 5237-5246.

Marmesat, S., Velasco, J., & Dobarganes, M. C. (2008). Quantitative determination of epoxy acids, keto acids and hydroxy acids formed in fats and oils at frying temperatures. *Journal of Chromatography A, 1211*, 129-134.

Márquez-Ruiz, G., García-Martínez, M. C., & Holgado, F. (2008). Changes and effects of dietary oxidized lipids in the gastrointestinal tract. *Lipid Insights, 2*, 11-19.

Martin-Rubio, A. S., Sopelana, P., & Guillén, M. D. (2019). Influence of minor components on lipid bioaccessibility and oxidation during *in vitro* digestion of soybean oil. *Journal of the Science of Food and Agriculture*. doi.org/10.1002/jsfa.9734

Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). A review of thermo-oxidative degradation of food lipids studied by ^{1}H NMR spectroscopy: influence of degradative conditions and food lipid nature. *Comprehensive Reviews in Food Science and Food Safety, 13*, 838-859.

Matsushita, S. (1975). Specific interactions of linoleic acid hydroperoxides and their secondary degraded products with enzyme proteins. *Journal of Agricultural and Food Chemistry, 23*, 150-154.

Matsushita, S., & Kobayashi, M. (1970). Effect of linoleic acid hydroperoxides on pepsin activity. *Agricultural and Biological Chemistry, 34*, 825-829.

Matsushita, S., Kobayashi, M., & Nitta, Y. (1970). Inactivation of enzymes by linoleic acid hydroperoxides and linoleic acid. *Agricultural and Biological Chemistry, 34*, 817-824.

Mattson, F. H., & Volpenhein, R. A. (1964). The digestion and absorption of triglycerides. *Journal of Biological Chemistry, 239*, 2772-2777.

Mattson, F. H., & Volpenhein, R. A. (1966). Enzymatic hydrolysis at an oil/water

interface. *Journal of the American Oil Chemists Society, 43*, 286-289.

Mine, Y., Noutomi, T., & Haga, N. (1991). Emulsifying and structural properties of ovalbumin. *Journal of Agricultural and Food Chemistry, 39*, 443-446.

Nakatsugawa, K. I., & Kaneda, T. (1983). Absorption and metabolism of methyl linoleate hydroperoxides in rats. *Journal of Japan Oil Chemists' Society*, 32, 361-366.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). A method based on ^{1}H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures. *Food Research International, 66*, 379-387.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2015). Usefulness of ^{1}H NMR in assessing the extent of lipid digestion. *Food Chemistry, 179*, 182-190.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016). A study by ^{1}H NMR on the influence of some factors affecting lipid *in vitro* digestion. *Food Chemistry, 211*, 17-26.

Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2017a). Effect of the presence of protein on lipolysis and lipid oxidation occurring during *in vitro* digestion of highly unsaturated oils. *Food Chemistry, 235*, 21-33.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2017b). ^{1}H NMR and SPME-GC/MS study of hydrolysis, oxidation and other reactions occurring during *in vitro* digestion of non-oxidized and oxidized sunflower oil. Formation of hydroxy-octadecadienoates. *Food Research International, 91*, 171-182.

Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2017c). Behaviour of non-oxidized and oxidized flaxseed oils, as models of omega-3 rich lipids, during *in vitro* digestion. Occurrence of epoxidation reactions. *Food Research International, 97*, 104-115.

Penumetcha, M., Khan, N., & Parthasarathy, S. (2000). Dietary oxidized fatty acids: an atherogenic risk?. *Journal of Lipid Research, 41*, 1473-1480.

Sánchez-Muniz, F. J., Benedí, J., Bastida, S., Olivero-David, R., & González-Muñoz, M. J. (2011). Enzymes and thermally oxidized oils and fats. In *Frying of foods. Oxidation, nutrient and non-nutrient antioxidants, biologically active compounds and high temperatures*, 2nd ed.; Boskou D., Elmadfa I., Eds.; Taylor and Francis Group: Boca Raton; Chapter 5, pp. 105-155.

Schaich, K. M. (2008). Co-oxidation of proteins by oxidizing lipids. *Lipid Oxidation Pathways, 2,* 181-272.

Schaich, K. M., Xie, J., & Bogusz, B. A. (2017). Thinking outside the classical chain reaction box of lipid oxidation: Evidence for alternate pathways and the importance of epoxides. *Lipid Technology, 29*, 91-96.

Steppeler, C., Haugen, J. E., Rødbotten, R., & Kirkhus, B. (2016). Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during *in vitro* digestion of cooked beef, pork, chicken, and salmon. *Journal of Agricultural and Food Chemistry, 64*, 487-496.

Uchida, K. (2003). Histidine and lysine as targets of oxidative modification. *Amino acids, 25*, 249-257.

Udilova, N., Jurek, D., Marian, B., Gille, L., Schulte-Hermann, R., & Nohl, H. (2003). Induction of lipid peroxidation in biomembranes by dietary oil components. *Food and Chemical Toxicology, 41*, 1481-1489.

Van Hecke, T., Vossen, E., Bussche, J. V., Raes, K., Vanhaecke, L., & De Smet, S. (2014). Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during *in vitro* digestion of meat. *PLOS One, 9*, e101122.

Versantvoort, C. H., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J., & Sips, A. J. (2005). Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food and Chemical Toxicology, 43*, 31-40.

Wilson, R., Fernie, C. E., Scrimgeour, C. M., Lyall, K., Smyth, L., & Riemersma, R. A. (2002). Dietary epoxy fatty acids are absorbed in healthy women. *European Journal of Clinical Investigation, 32*, 79-83.

SUPPLEMENTARY MATERIAL OF MANUSCRIPT 8

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^{1}H NMR STUDY OF THE *IN VITRO* DIGESTION OF HIGHLY OXIDIZED SOYBEAN OIL, FOCUSING ON THE BIOACCESSIBILITY OF MAJOR LIPID NUTRIENTS AND OF SOME OXIDATION COMPOUNDS. EFFECT OF THE PRESENCE OF OVALBUMIN.

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Table S1. Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study.

Components	**Saliva**	**Gastric juice**	**Duodenal juice**	**Bile juice**
KCl (mmol/L)	12.02	11.06	7.57	5.05
NaCl (mmol/L)	5.10	47.09	119.98	89.99
$NaHCO_3$ (mmol/L)	20.17	-	40.33	68.86
NaH_2PO_4 (mmol/L)	7.40	0.22	-	-
NH_4Cl (mmol/L)	-	5.72	-	-
KH_2PO_4 (mmol/L)	-	-	0.59	-
Na_2SO_4 (mmol/L)	4.79	-	-	-
KSCN (mmol/L)	2.06	-	-	-
$MgCl_2$ (mmol/L)	-	-	0.53	-
$CaCl_2*2H_2O$ (mmol/L)	-	2.72	1.36	1.51
HCl (37%) (mL/L)	-	6.50	0.18	0.15
Urea (mmol/L)	3.33	1.42	1.67	4.16
Glucose (mmol/L)	-	3.61	-	-
Glucuronic acid (mmol/L)	-	0.10	-	-
Uric acid (mmol/L)	0.09	-	-	-
Glucoseamine hydrochloride (mmol/L)	-	1.53	-	-
Bovine serum albumin (g/L)	-	1.00	1.00	1.80
Mucin (g/L)	0.025	3.00	-	-
Aspergillus oryzae α-amylase (g/L)	0.29	-	-	-
Aspergillus niger lipase (U/mL)	-	100	-	-
Pepsin (g/L)	-	2.50	-	-
Pancreatin (g/L)	-	-	9.00	-
Lipase type II from porcine pancreas (g/L)	-	-	1.50	-
Bovine bile extract (g/L)	-	-	-	18.75
pH	6.8±0.2	1.6±0.3	8.1±0.2	8.2±0.2

Some details of the in vitro digestion procedure used

The digestion experiment started by adding 6 mL of saliva to each of the oil samples. After 5 min of incubation, 12 mL of gastric juice were added and the mixture was rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. 1 hour after starting the gastric digestion, pH was set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring in vivo. After 2 h of gastric digestion, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice were added. Subsequently, pH was set between 6 and 7, and the mixture was rotated again at 40 rpm and incubated at 37±2ºC for 4 h.

Table S2. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of the main protons of glycerides, fatty acids and some oxidation compounds present in the samples before and after *in vitro* digestion. TG: triglycerides; DG: diglycerides; MG: monoglycerides. The signal letters agree with those given in Figures 1 and S1.

Signal	Chemical shift (ppm)	Multiplicity	Functional group	
			Type of protons	Compound
Main acyl groups (AG) and fatty acids (FA)[a,b]				
A	0.88	t	–C**H**$_3$	saturated and monounsaturated **ω-9 AG** and **FA**
	0.89	t	–C**H**$_3$	linoleic **AG** and **FA**
B	0.97	t	–C**H**$_3$	linolenic **AG** and **FA**
C	1.19–1.42	m*	–(C**H**$_2$)$_n$–	**AG** and **FA**
D	1.61	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **TG**
	1.62	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **1,2-DG**
	1.63	m	–OCO–CH$_2$–C**H**$_2$–, COOH–CH$_2$–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	1.64	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **2-MG**
E	1.92–2.15	m**	–C**H**$_2$–CH=CH–	**AG** and **FA**
F	2.26–2.36	dt	–OCO–C**H**$_2$–	**AG** in **TG**
	2.33	m	–OCO–C**H**$_2$–	**AG** in **1,2-DG**
	2.35	t	–OCO–C**H**$_2$–, COOH–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	2.38	t	–OCO–C**H**$_2$–	**AG** in **2-MG**
G	2.77	t	=HC–C**H**$_2$–CH=	Linoleic **AG** and **FA**
H	2.80	t	=HC–C**H**$_2$–CH=	Linolenic **AG** and **FA**
I	3.65	ddd	ROCH$_2$–CHOH–C**H**$_2$OH	glyceryl group in **1-MG**
J	3.73	m***	ROCH$_2$–CH(O*R*')–C**H**$_2$OH	glyceryl group in **1,2-DG**
K	3.84	m***	HOC**H**$_2$–CH(O*R*)–C**H**$_2$OH	glyceryl group in **2-MG**
L	3.94	m	ROCH$_2$–C**H**OH–CH$_2$OH	glyceryl group in **1-MG**
M	4.05–4.21	m	ROC**H**$_2$–C**H**OH–C**H**$_2$OR'	glyceryl group in **1,3-DG**
N	4.18	ddd	ROC**H**$_2$–CHOH–CH$_2$OH	glyceryl group in **1-MG**
O	4.22	dd,dd	ROC**H**$_2$–CH(O*R*')–C**H**$_2$OR''	glyceryl group in **TG**
P	4.28	ddd	ROC**H**$_2$–CH(O*R*')–CH$_2$OH	glyceryl group in **1,2-DG**
Q	4.93	m	HOCH$_2$–C**H**(O*R*)–CH$_2$OH	glyceryl group in **2-MG**
R	5.08	m	ROCH$_2$–C**H**(O*R*')–CH$_2$OH	glyceryl group in **1,2-DG**

S	5.27	m	$ROCH_2$–C**H**(O*R*')–CH_2OR''	glyceryl group in **TG**
T	5.28–5.46	m	–C**H**=C**H**–	**AG** and **FA**
		Signals related to oxidation compounds		
Conjugated dienic systems[c,d]				
–	5.44	ddd	–C**H**=C**H**–C**H**=C**H**–	(*Z*,*E*)-conjugated double bonds associated with hydroxy group (OH) in octadecadienoic **AG** and **FA**
-	5.66	dd		
-	5.97	t		
a	6.49	dd		
-	5.47	ddm	–C**H**=C**H**–C**H**=C**H**–	(*E*,*E*)-conjugated double bonds associated with hydroperoxy group (OOH) in octadecadienoic **AG** and **FA**
-	5.76	dtm		
-	6.06	ddtd		
b	6.27	ddm		
-	5.51	dtm	–C**H**=C**H**–C**H**=C**H**–	(*Z*,*E*)-conjugated double bonds associated with hydroperoxy group (OOH) in octadecadienoic **AG** and **FA**
-	5.56	ddm		
-	6.00	ddtd		
c	6.58	dddd		
Epoxides				
Epoxy-derivatives				
d	2.63[e]	m	-C**H**O**H**C-	(*E*)-9,10-epoxystearate
e1	2.88[e]	m	-C**H**O**H**C-	(*Z*)-9,10-epoxystearate
e2	2.9[f]	m	-C**H**O**H**C-	monoepoxy-octadecenoate groups
			-C**H**OHC-CH_2-CHO**H**C-	diepoxides
e3	2.94***	m	-C**H**O**H**C-	(*Z*)-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid
f1	3.10[f]	m	-CHO**H**C-CH_2-C**H**OHC-	diepoxides
Epoxy-keto-derivatives				
e4	2.89[g]/2.90[h]	td[g]/m[h]	-CO-CH=CH-CHO**H**C-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
e5	2.91[g]	td	-C**H**OHC-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
g	3.20[g,h]	dd	-CO-CH=CH-C**H**OHC-	(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
			-CHO**H**C-CH=CH-CO-	(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
h	6.38[g]	d		(*E*)-9,10-epoxy-13-keto-(*E*)-11-octadecenoate
				(*E*)-12,13-epoxy-9-keto-(*E*)-10-octadecenoate
Epoxy-hydroxy-derivatives				

e6	2.93[i]	dt	-C**H**OHC-CHOH-CH=CH-	*threo*-11-hydroxy-(*E*)-12,13-epoxy-(*Z*)-9-octadecenoate
f2	3.09[j]/3.097[k]	dd	-CHO**H**C-CH=CH-CHOH-	9-hydroxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate
Epoxy-hydroperoxy-derivatives				
f3	3.11[j]	dd	-CHO**H**C-CH=CH-CHOOH-	9-hydroperoxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate[l]
i	5.85[j]	dd	-CHO**H**C-CH=C**H**-CHOOH-	9-hydroperoxy-(*E*)-12,13-epoxy-(*E*)-10-octadecenoate
Hydroxy-derivatives				
j1	3.45[m,n]/3.48-3.41[o]	m[m,o]/bs[n]	-C**H**OH-C**H**OH-	9,10-dihydroxy-12-octadecenoate (leukotoxin diol, methyl ester)
j2	3.45[m]/3.42[n]	m[m]/bs[n]	-C**H**OH-C**H**OH-	12,13-dihydroxy-9-octadecenoate (isoleukotoxin diol, methyl ester)
k	3.62***	m	-C**H**OH-	12(R)-hydroxy-9(*Z*)-octadecenoate
Keto-dienes				
l	7.14***	dm	-CO−CH=C**H**−CH=CH-	9-keto-10(*E*),12(*E*)-octadecadienoic acid
m	7.50[p]/7.43[q]	dd[q]/ddd[r]	-CO−CH=C**H**−CH=CH-	(*Z*,*E*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
	7.49[p]/7.47[q]	ddd	-CO−CH=C**H**−CH=CH-	(*E*,*Z*)-conjugated double bonds associated with a keto group in octadecadienoic acyl groups
Aldehydes				
n	9.49[r]	d	−C**H**O	(*E*)-2-alkenals
o	9.52[r]	d	−C**H**O	(*E*,*E*)-2,4-alkadienals
p	9.55[r]	d	−C**H**O	4,5-epoxy-2-alkenals
q	9.57[r]	d	−C**H**O	4-hydroxy-(*E*)-2-alkenals
r	9.59[s]	d	−C**H**O	4-hydroperoxy-(*E*)-2-alkenals
s	9.75[r]	t	-C**H**O	n-alkanals

Abbreviations: t: triplet; m: multiplet; d: doublet. *Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group; **Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; ***This signal shows different

multiplicity if the spectrum is acquired from the pure compound or taking part in the mixture; ****Assignment made with the aid of standard compounds.

[a]Assignments of AG in TG taken from Guillén, M. D., & Ruiz, A. (2003). *Journal of the Science of Food and Agriculture, 83*, 338-346.

[b]Assignments of AG in partial glycerides (DG and MG) and of FA taken from Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). *Food Research International, 66*, 379-387.

[c]Data taken from E. Goicoechea and M. D. Guillén. *J. Agric. Food Chem.*, 2010, **58**, 6234-6245 (conjugated (*Z,E*)- and (*E,E*)-hydroperoxy-dienes).

[d]Data taken from Dong, M., Oda, Y., & Hirota, M. (2000). *Bioscience, Biotechnology and Biochemistry, 64*, 882-886 (conjugated (*Z,E*)-hydroxy-dienes).

[e]Data taken from Du, G., Tekin, A., Hammond, E. G., & Woo, L. K. (2004). *Journal of the American Oil Chemists' Society, 81*, 477-480.

[f]Data taken from Aerts, H. A. J., & Jacobs, P. A. (2004). *Journal of the American Oil Chemists' Society, 81*, 841-846 (monoepoxy-octadecenoates and diepoxides).

[g]Data taken from Lin, D., Zhang, J., & Sayre, L. M. (2007). *The Journal of Organic Chemistry, 72*, 9471-9480.

[h]Data taken from Gardner, H. W., Kleiman, R., & Weisleder, D. (1974). *Lipids, 9*, 696-706.

[i]Data taken from Garssen, G. J., Veldink, G. A., Vliegenthart, J. F., & Boldingh, J. (1976). *The FEBS Journal, 62*, 33-36.

[j]Data taken from Gardner, H. W., Weisleder, D., & Kleiman, R. (1978). *Lipids, 13*, 246-252.

[k]Data taken from Van Os Cornelis, P. A., Vliegenthart, J. F. G., Crawford, C. G., & Gardner, H. W. (1982). *Biochimica et Biophysica Acta, 713*, 173-176.

[l]δ-Ketols (hydroxy-keto-derivatives) could also contribute to this signal (Gardner et al., 1974).

[m]Data taken from Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau C., & Hammoc B. D. (2000). *Archives of Biochemistry and Biophysics, 376*, 420-43.

[n]Data taken from Yang, J., Morton, M. D., Hill, D. W., & Grant, D. F. (2006). *Chemistry and Physics of Lipids, 140*, 75-87.

[o]Data taken from Nilewski, C., Chapelain, C. L., Wolfrum, S., & Carreira, E. M. (2015). *Organic Letters, 17*, 5602-5605.

[p]Data taken from Dufour, C., & Loonis, M. (2005). *Chemistry and Physics of Lipids, 138*, 60-68.

[q]Data taken from Kuklev et al. (1997).

[r]Data taken from Guillén, M. D., & Ruiz, A. (2004). *European Journal of Lipid Science and Technology, 106*, 680-687.

[s]Data taken from Guillén, M. D., & Uriarte, P. S. (2009). *Journal of Agricultural and Food Chemistry, 57*, 7790-7799.

Standard compounds for the identification of some of the oxidation products present in the various samples studied

(*E*)-2-Hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E,E*)-2,4-hexadienal, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, 4,5-epoxy-(E)-2-decenal and 12,13-epoxy-9(Z)-octadecenoic acid methyl ester (isoleukotoxin methyl ester), acquired from Sigma-Aldrich, 4-hydroxy-(*E*)-2-nonenal, 4-hydroperoxy-(*E*)-2-nonenal, 9,10-dihydroxy-12(*Z*)-octadecenoic acid (leukotoxin diol), 12,13-dihydroxy-9(*Z*)-octadecenoic acid

(isoleukotoxin diol), trans-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, 9-keto-10(*E*),12(*E*)-octadecadienoic acid and 12R-hydroxy-9(*Z*)-octadecenoic acid methyl ester (ricinoleic acid methyl ester), purchased from Cayman Chemical (Ann Arbor, MI, USA), and cis-(12,13)-epoxy-9(*Z*),15(*Z*)-octadecadienoic acid and (±)-cis-9,10-epoxyoctadecanoic acid methyl ester, acquired from Cymit Quimica (Barcelona, Spain).

Quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and/or in the lipid extracts of the digestates and of Lipid Bioaccessibility

A. Lipolytic products and Lipid Bioaccessibility

The number of moles (N) of fatty acids and all the glycerides present in the lipid samples were expressed as follows:

$N_{2\text{-}MG}=Pc*A_K/4$ [eq. S1]
$N_{1\text{-}MG}=Pc*A_L$ [eq. S2]
$N_{1,2\text{-}DG}=Pc*(A_{I+J}-2A_L)/2$ [eq. S3]
$N_{TG}=Pc*(2A_{4.26\text{-}4.38}-A_{I+J}+2A_L)/4$ [eq. S4]
$N_{1,3\text{-}DG}=Pc*(A_{4.04\text{-}4.38}-2A_{4.26\text{-}4.38}-2A_L)/5$ [eq. S5]
$N_{FA}=(Pc*A_F-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2$ [eq. S6]
$N_{Gol}=(N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2N_{2\text{-}MG}-2N_{1\text{-}MG})/3$ [eq. S7]

where Pc is the proportionality existing between the area of the ^{1}H NMR signals and the number of protons that generate them, A_K, A_L, A_{I+J} and A_F are the areas of the corresponding signals indicated in Table S1, and $A_{4.26\text{-}4.38}$ and $A_{4.04\text{-}4.38}$ represent the areas of the signals between 4.26 and 4.38 ppm, and between 4.04 and 4.38 ppm, respectively (see Figure S1).

Using these equations, the molar percentages of the different kinds of glycerides in relation to the total number of moles of glyceryl structures present (NT_{GS}) were determined as follows:

$NT_{GS}=N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{Gol}$ [eq. S8]
$G\%=100N_G/NT_{GS}$ [eq. S9]

where G is each kind of glyceride (TG, 1,2-DG, 1,3-DG, 2-MG and 1-MG) and N_G the number of moles of each kind of glyceride.

$Gol\%=100N_{Gol}/NT_{GS}$ [eq. S10]

Likewise, the Lipid Bioaccessibility parameter was calculated as follows:

$L_{BA}\%=100(N_{1\text{-}MG}+N_{2\text{-}MG}+N_{FA})/NT_{AG+FA}$ [eq. S11]

$NT_{AG+FA}=Pc*A_F/2$ [eq. S12]

where NT_{AG+FA} is the total number of moles of AG plus FA present.

B Molar concentrations of polyunsaturated acyl groups and fatty acids

The concentrations of linolenic (Ln) and linoleic (L) acyl groups and fatty acids, expressed as millimoles per mole of the sum of AG+FA present in either the starting oils or the lipid extracts of the digested samples were estimated by using the following equations:

$[Ln] = [(A_H/4)/(A_F/2)]*1000$ [eq. S13]
$[L] = [(A_G/2)/(A_F/2)]*1000$ [eq. S14]

where A_H and A_G are the areas of signals H and G indicated in Table S1. It must be noted that due to partial overlapping of signals H and G, a previous correction of both areas must be undertaken to properly assess the area corresponding to each one of them. For this purpose, trilinolenin and trilinolein were used as references.

Finally, it should be pointed out that signal F is due to methylenic protons bonded to carbon atoms in *alpha* position in relation to carbonyl/carboxyl groups of AG and FA, modified or not, as well as to carbonyl groups of other compounds formed in the oxidation such as aldehydes, (*E*)-epoxy-keto-enes and keto-dienes. However, despite the high oxidation level of the studied samples, the inclusion in this signal of methylenic protons in *alpha* position in relation to carbonyl groups different from that of AG and FA does not affect the calculations before mentioned, in which A_F is included, because the concentration of oxidation products is negligible in relation with that of AG+FA.

C. Oxidation compounds

The concentration of the several kinds of oxidation compounds, expressed as millimoles per mol of the sum of AG+FA present, was estimated by using the following equation:
$[OP] = [(A_{OP}/n)/(A_F/2)]*1000$ [eq. S15]

where A_{OP} is the area of the signal selected for the quantification of each oxidation product (OP), shown in Table S1. In the case of epoxides (signals "e1-e6" in Table S1), the overlapped area due to the side band of *bis*-allylic protons signals G and H must be subtracted. Although the epoxy-compounds given in Table S1 can contribute to signal "e" either with one ("e4-e6") or with two protons ("e1-e3"), it has been assumed that all contribute with two protons.
4-Hydroperoxy- and 4-hydroxy-(*E*)-2-alkenals have been quantified together in order to accurately compare their concentrations before and after *in vitro* digestion, because in the digested samples it is very difficult to determine each one of these

kinds of aldehydes separately, due to the almost total overlap of their signals (see Figure 1, letters "r" and "q", respectively).

Particularities of the quantification of some compounds in the samples digested with ovalbumin

As shown in Figure S1 for D(RSX+HO), in the ^{1}H NMR spectra of the extracts obtained from the samples digested with a high proportion of ovalbumin, some signals attributed to this latter overlap with the signals used to estimate both the molar concentration of epoxides giving signal at 2.9 ppm (see signals "e1-e6" in Table S2) and the molar percentages of 1,3-DG and TG (see signals "M" and "O", respectively), so their contribution must be subtracted, especially in the case of epoxides. For this purpose, ovalbumin was added to the digestive juices after undergoing the digestion process and this mixture was extracted in the same way as the rest of digested samples; the relative areas of the different ovalbumin signals can be determined from the corresponding ^{1}H NMR spectrum, free of lipids. This enables one to subtract the area of the signals overlapping with those of lipid components in the spectra of the extracts obtained from the digested oil samples taking as a reference the signals that do not overlap with one another (see Figure S1). It is worth noticing that while some signals coming from ovalbumin sample also overlap with those of *bis*-allylic protons (signals "H+G"), their area is very small in relation to that of the latter, and so can be ignored.

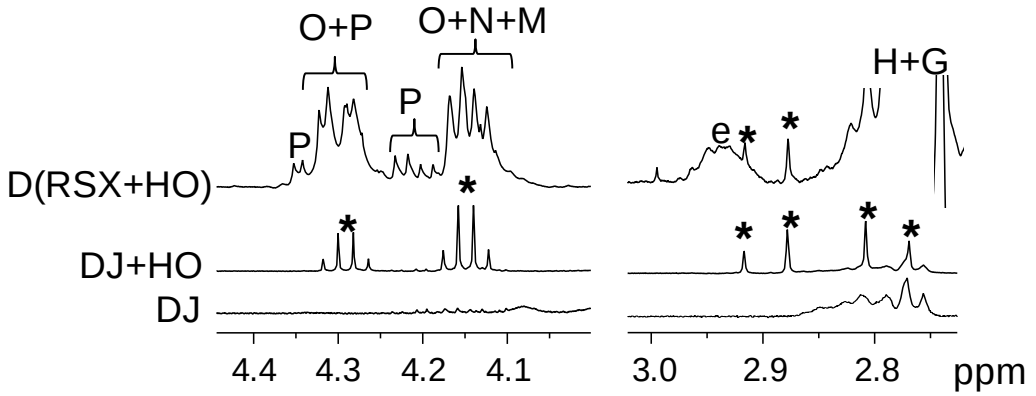

Figure S1. Enlargement of some spectral regions of the ^{1}H NMR spectra of the lipid extracts of: the digested fluids subjected to digestion conditions (DJ); the digestive fluids subjected to digestion conditions mixed with ovalbumin at the highest proportion tested (DJ+HO); the lipid extract of the digestate of sample D(RSX+HO). The signal letters agree with those in Table S2, considering that signal "e" includes signals "e1 to e6".

SUMMARY OF RESULTS

Aim 1 - STUDY OF THE INFLUENCE OF THE MINOR COMPOUNDS NATURALLY PRESENT IN COMMERCIAL SOYBEAN OIL ON ITS EVOLUTION UNDER ACCELERATED STORAGE CONDITIONS.

1. Analysis of the effect of the pool of minor components present in commercial virgin and refined soybean oils, determined by Direct Immersion SPME-GC/MS, on their evolution under accelerated storage conditions (70 ºC), monitored by ^{1}H NMR **(Manuscript 1)**

As before commented, the effect of minor soybean oil components on its oxidative stability is considered a subject of interest. To analyze this effect, two commercial soybean oils, one virgin and the other refined, were subjected to an accelerated storage process at 70 ºC. The composition of the oils was studied by means of ^{1}H NMR and DI SPME-GC/MS, and their evolution under oxidative conditions was monitored by ^{1}H NMR.

Composition in main and minor components of the studied soybean oils.

The analysis by ^{1}H NMR reveals that both the virgin and the refined soybean oils have very similar molar proportions of the different types of acyl groups, linoleic being the most abundant ones, followed by oleic, saturated and linolenic ones.

As determined by DI SPME-GC/MS, their content in tocopherols and sterols, considered as compounds with antioxidant ability, is higher in the refined oil than in the virgin type. By contrast, this latter is richer in free fatty acids, which are compounds especially prone to oxidation.

Study by ^{1}H NMR of the evolution of the oils under accelerated storage conditions

The evolution of the oils was analyzed through the degradation of unsaturated acyl groups and the generation of both primary and secondary oxidation products.

The molar percentages of all the types of unsaturated groups, especially those of the polyunsaturated ones (linolenic and linoleic), decrease with time in both oils, this diminution being slow during a first stage, but very quick afterwards. The first stage of acyl group degradation is slightly longer in the refined than in the virgin oil (8 and 6 days, respectively), what entails a slower degradation of the refined oil.

Hydroperoxides, which are the first oxidation compounds to be detected, appear one day later in the refined oil (day 2) than in the virgin one (day 1). Their evolution with

time follows the line of acyl groups, in such a way that their faster concentration increase begins earlier in the virgin oil and the maximum is also reached before in this latter (after 6 days *versus* 8 days in the refined oil), to decrease thereafter.

As a consequence of hydroperoxide decomposition, a wide variety of secondary oxidation products are generated, among which epoxides derived from polyunsaturated and from oleic groups, and aldehydes (n-alkanals, (*E*)-2-alkenals, 4-hydroperoxy-(*E*)-2-alkenals, 4-hydroxy-(*E*)-2-alkenals, 4,5-epoxy-2-alkenals and (*E*,*E*)-2,4-alkadienals) can be mentioned. In accordance with hydroperoxide evolution, all these types of compounds are detected later in the refined oil, their evolution throughout time being similar in both oils.

All these results evidence that the oxidative stability of the refined soybean oil is higher than that of the virgin one, and this can be attributed to both its greater content of tocopherols and sterols, and to its lower level of free fatty acids.

Aim 2 - ASSESSMENT OF THE EFFECT OF ADDING ALPHA-TOCOPHEROL, GAMMA-TOCOPHEROL OR L-LYSINE ON THE OXIDATIVE STABILITY AND THE OXIDATION PROCESS OF COMMERCIAL SOYBEAN OIL SUBMITTED TO ACCELERATED STORAGE CONDITIONS

2.1. Study of the effect of enriching a commercial soybean oil with different proportions of α-tocopherol (0.002, 0.02, 0.2, 2 and 5% in weight) on its evolution under accelerated storage conditions at 70 ºC by means of ^{1}H NMR, paying attention to both acyl group degradation and oxidation compound generation and evolution.

(Manuscript 2)

One of the strategies most commonly used to reduce the oxidation of edible oils is the addition of compounds with antioxidant ability. In this context, the current legislation allows the addition of α-tocopherol (α-T) to most refined oils under the *quantum satis* principle, this is without an established limit, despite prooxidant effects of α-T have been reported on different types of lipids under certain circumstances. In addition to this, it must be noticed that little is known about the effect of the α-T enrichment on the oxidation process of commercial edible oils, with all their original components. For

these reasons, the effect of adding α-T in proportions ranging from 0.002 to 5% in weight to a commercial soybean oil on its evolution under accelerated storage conditions at 70 ºC was studied by ^{1}H NMR, which allows one to obtain a wide vision of the oxidation process. To this aim, the oil acyl group degradation rate and the generation of both primary and secondary oxidation products were monitored, including hydroperoxides, epoxides, aldehydes, keto-dienes, compounds with conjugated hydroxy-dienes and other hydroxy-derivatives different from these latter.

Unsaturated oil acyl group degradation

In the reference oil, without α-T added, unsaturated acyl group degradation occurs slowly during a first stage of the accelerated storage process; this is followed by a second one, in which the acyl group degradation rate turns considerably faster.

While the lowest α-T proportion tested (0.002%) does not affect acyl group evolution, as the α-T concentration increases, the difference between the speeds at which acyl groups degrade during the first and the second stage of the oxidation course becomes smaller, to the point that in the sample with a 5% of α-T added, the acyl group degradation pace can be considered almost constant throughout the entire oxidation process. This results in an enlargement of the time necessary to reach oil total polymerization, especially noticeable in the oil with the highest α-T level (5%), where this process takes 20 days against 10 in the non-enriched oil.

Generation of hydroperoxides supporting conjugated diene systems (primary oxidation products)

In line with that commented on acyl groups, above the 0.002% proportion, increasing α-T enrichment levels progressively accelerate the hydroperoxide concentration increase and shorten the time to reach its maximum. Moreover, in the oils with the highest α-T levels (2 and 5%) hydroperoxides reach higher concentrations than in the rest of studied oils. However, the subsequent decrease of hydroperoxide concentration takes longer as the α-T amount rises.

Furthermore, the α-T enrichment modifies the oxidation pathway of soybean oil in relation to the non-enriched oil, promoting the generation of (*Z*,*E*)-hydroperoxides while delaying and reducing that of their (*E*,*E*)-counterparts in line with α-T concentration, to the point that, at the highest α-T level (5%), the former are basically the only ones detected during a large part of the oxidation process. This contrasts with

the evolution of (*Z,E*)-and (*E,E*)-hydroperoxides usually observed under the accelerated storage conditions here used, under which the proportions of both types of hydroperoxides are similar for an initial period of time, after which the concentration of (*E,E*)-hydroperoxides becomes higher than that of the (*Z,E*)-ones, both of them reaching their maximum at the same time.

In view of the findings relative to acyl group and hydroperoxide evolution, it can be said that, except in the lowest proportion tested, α-tocopherol reduces the oxidative stability of the oil and accelerates its oxidation process. However, the time needed to reach total polymerization is longer in the samples with the highest α-T concentrations.

Generation of secondary oxidation products

As it could be expected from the effect of the α-T enrichment on hydroperoxide evolution, the higher the α-T concentration, the earlier the appearance of secondary oxidation products. However, due to the different effect of α-T on (*Z,E*)- and (*E,E*)-hydroperoxides as the enrichment degree grows, not all oxidation compounds are affected in the same way by the different α-T addition levels. Thus, in agreement with that observed for (*Z,E*)-hydroperoxides, as the α-T concentration gets higher an earlier generation of (*Z,E*)-hydroxy-dienes is observed, together with an increase in their level in the samples with the highest α-T proportions (2 and 5%).

Regarding epoxides, although increasing levels of α-T provoke a faster generation of this type of compounds, as the α-T concentration rises, a progressive delay in the appearance of (*E*)-epoxystearates, derived from oleic groups, is observed when compared with the non-enriched oil.

Similarly to that commented for the rest of oxidation compounds, as the concentration of α-T increases, the formation of aldehydes begins earlier. However, in parallel to the changes provoked by the α-T enrichment in (*Z,E*)- and (*E,E*)-hydroperoxide evolutions, variations in the aldehyde generation pattern are also noticed. Thus, while in the non-enriched oil the toxic 4-hydroxy-(*E*)-2-alkenals are generated at a lower rate than other kinds of aldehydes such as n-alkanals, (*E*)-2-alkenals and 4-hydroperoxy-(*E*)-2-alkenals, as the α-T concentration increases, 4-hydroxy-(*E*)-2-alkenals appear earlier, together with 4-hydroperoxy-(*E*)-2-alkenals, while the appearance of the rest of aldehydes is postponed, more as higher is the α-T

concentration. Moreover, a group of aldehydes not usually detected under the conditions of this study also appear in the samples with 2 and 5% of α-T: (*Z,E*)-2,4-alkadienals.

The changes caused by the different α-T levels in the evolution of hydroperoxides can be considered to be well reflected in the evolution of (*Z,E*)- and (*E,E*)-keto-dienes. Thus, as in the case of hydroperoxides, as α-T concentration increases, an earlier and more profuse generation of (*Z,E*)-keto-dienes is noticed, together with a delay in the generation of (*E,E*)-keto-dienes in comparison with the (*Z,E*)-ones.

Similarly to comments on other types of oxidation products, the generation of hydroxy-derivatives different from those supporting conjugated hydroxy-dienes is accelerated as α-T level increases. Nonetheless, in the oils with the highest α-T levels (2 and 5%) a reduction in their concentration increasing rate is observed.

All the above mentioned reveals that, at levels between 0.02 and 5%, the α-T enrichment of soybean oil accelerates the appearance of secondary oxidation products, more as higher is the α-T concentration; among these, toxic or potentially toxic compounds are present, such as oxygenated α,β-unsaturated aldehydes and certain monoepoxides.

2.2. Assessment by means of ^{1}H NMR of the influence of different levels of γ-tocopherol (0.02, 0.2 and 2% in weight) added to a commercial refined soybean oil on acyl group evolution and oxidation compound generation throughout an accelerated storage process at 70 ºC, and comparison with that of α-tocopherol.

(Manuscript 3)

To go deeper into the effect of different types of tocopherols on the oxidative stability of edible oils and to analyze possible differences between their respective behaviours, the influence of the addition of γ-tocopherol (γ-T) in proportions between 0.02 and 2% in weight, on the oxidation process of a commercial refined soybean under accelerated storage conditions was studied by ^{1}H NMR and compared with that of α-T at levels of 0.2 and 2%.

Effect of the γ-T enrichment on the evolution of the soybean oil acyl groups and comparison with that of α-T

Unlike that observed previously for α-T, the addition of a 0.02% proportion of γ-T to soybean oil does not provoke changes detectable by ^{1}H NMR in the evolution of the oil acyl groups. However, similarly to that observed for α-T, although not so markedly, as the γ-T concentration increases over this level, the rate of acyl group degradation increases in comparison with that of the non-enriched refined soybean oil. In addition, the initial period in which acyl group degradation proceeds at a lower rate is lengthened with γ-T concentration, what leads to a considerable enlargement of the oil total polymerization process in the oil with the highest γ-T level (2%).

Effect of γ-T on the evolution of hydroperoxides and their associated conjugated dienes and comparison with that of α-T

As could be expected from acyl group evolution, and also similarly to that observed for α-T, the addition of γ-T to refined soybean oil in proportions higher than 0.02% provokes a faster generation of hydroperoxides and an acceleration in their concentration increase rate, greater as higher is the γ-T level. However, the effect is not so pronounced as in the case of α-T. Moreover, as described for α-T, γ-T modifies the oxidation pathway of soybean oil as it concentration increases, promoting the formation of (*Z*,*E*)-hydroperoxides over that of the (*E*,*E*)-ones, although less noticeably than α-T.

Despite the above mentioned similarities between the effects of α- and γ-T on hydroperoxides, some differences can also be found between the actions of both tocopherols. Thus, contrary to α-T, in the samples with the highest γ-T enrichment levels (0.2 and 2%) the maximum hydroperoxide concentration is reached later than in the reference oil. Furthermore, hydroperoxide decomposition also takes longer in the oil with the highest γ-T amount (2%) than in the rest of the samples, the magnitude of this effect being greater than in the case of α-T.

Effect of the γ-T enrichment on the generation of secondary oxidation products and comparison with that of α-T

The effect of both γ- and α-T on the nature, relative proportions and generation rate of secondary oxidation products is determined by the influence of the enrichment levels tested on the evolution of hydroperoxides. Thus, when added at refined soybean oil, and under accelerated storage conditions, increasing levels of both tocopherols cause an earlier appearance and an increase in the concentration of conjugated (*Z*,*E*)-hydroxy-

dienes but, in line with the differences observed between the effects of both tocopherols on hydroperoxide evolution, at the 2% enrichment level, the increase in hydroxy-diene concentration is more marked and their existence period shorter in the α-T than in the γ-T enriched sample.

Within the broad group of oxidation products designated as epoxides, a distinction was made between major epoxides and other minor epoxides, found in lower concentrations than the previous ones. Major epoxides encompass those epoxides giving ^{1}H NMR signals at 2.9 and 3.1 ppm, which could derive from all the types of unsaturated acyl groups, while the minor ones include (*E*)-epoxystearates, derived from oleic groups, and epoxy-keto-enes, which presumably come exclusively from polyunsaturated groups. Not all the various classes of compounds included in this group are affected similarly by the γ-T enrichment. Thus, while the so-called major epoxides are detected earlier in the oil with the highest γ-T level than in the non-enriched oil, a delay in the appearance of epoxy-keto-enes and (*E*)-epoxystearates is observed as the γ-T proportion is increased over 0.02%. In contrast, an acceleration in the appearance of all the various types of epoxides is observed when refined soybean oil is enriched with increasing amounts of α-T.

Regarding aldehydes, the γ-T enrichment above the 0.02% proportion provokes a delay in aldehyde formation, in line with γ-T concentration. By contrast, the addition of α-T accelerates the appearance of aldehydes in relation to the non-enriched oil, the higher the α-T concentration gets. Nonetheless, despite these differences between the effects of both tocopherols on aldehyde generation rate, there are also some features common to the action of both. Thus, unlike that observed in the rest of the oils, in the samples with the highest level of either α- or γ-T, 4-hydroxy-(*E*)-2-alkenals are detected at the same time than 4-hydroperoxy-(*E*)-2-alkenals, and the generation of (*Z*,*E*)-2,4-alkadienals is noticed.

The effect of the γ-T enrichment on the evolutions of (*Z*,*E*)- and (*E*,*E*)-keto-dienes follows the line of that on their respective hydroperoxide precursors. Thus, in the sample with the highest γ-T concentration (*Z*,*E*)-keto-dienes reach higher levels than in the rest of the samples and they are detectable for a longer time, while the formation of the (*E*,*E*)-ones is markedly retarded compared to the non-enriched oil. As for the samples enriched with α-T, the higher the α-T concentration, the earlier the generation

of (*Z,E*)-keto-dienes and the later the generation of the (*E,E*)-ones in relation to the former.

Finally, in the oils with γ-T proportions higher than 0.02% a delay is observed in the formation of compounds tentatively identified as mono- and di-hydroxy-derivatives different from those supporting conjugated hydroxy-dienes, more marked the higher the γ-T amount added. However, the opposite effect is observed in the case of the α-T enrichment.

All the aforementioned reveals that both similarities and differences exist between the actions of α- and γ-T. Thus, although both tocopherols reduce the oxidative stability and accelerate the degradation of refined soybean oil, the effect is less marked in the case of γ-T. However, important variations in the effect of both tocopherols are observed concerning the generation of most secondary oxidation products, which, in general, is accelerated and retarded by the α- and the γ-T enrichment, respectively. Furthermore, in the oil with the highest proportion of added γ-T (2%), the total oil polymerization process is considerably extended when compared with the rest of the samples. These findings evidence the complex effect of tocopherols on the generation of oxidation products, and the difficulty to define their action on the oil oxidation process as either antioxidant or prooxidant.

2.3. Investigation of the effect of different proportions of L-lysine (1 and 2% in weight) on the evolution of a commercial refined soybean oil throughout a thermal treatment at 70 ºC by means of ^{1}H NMR, paying attention to the evolution of oil acyl groups and γ-tocopherol, and to the generation of a wide range of oxidation products.

(Manuscript 4)

Taking into account that the antioxidant ability of amino acids on edible oils has been described, and that they could constitute an alternative to other types of compounds considered to be able to act as antioxidants, the effect of 1 and 2% proportions of L-lysine on the oxidation process of a refined soybean oil, performed at 70 ºC under stirring conditions, was studied by ^{1}H NMR. Attention was focused on oil acyl group degradation and oxidation compound generation. In addition, the evolution of γ-T throughout the oxidation process was also monitored by ^{1}H NMR.

Effect of lysine on the evolution of oil acyl groups and γ-T

The addition of lysine to the oil reduces the degradation rate of unsaturated acyl groups, extending the duration of the first stage of the acyl group degradation process from 4 days in the reference oil to 11 and 14 days in the samples with 1 and 2% of lysine, respectively. This causes an enlargement of the time required to reach the oil total polymerization from 7 days in the oil without lysine to 15 and 17 days in those enriched with 1 and 2% of lysine, respectively.

The oil enrichment with lysine also decreases the diminution rate of γ-T concentration, more in the oil with a 2% of lysine added that in that with a 1%.

Influence of the lysine enrichment on the generation and further evolution of oxidation products

With regard to ***primary oxidation products***, in the lysine-enriched samples hydroperoxides and their associated (*Z*,*E*)- and (*E*,*E*)-conjugated dienes are detected at the same time as in the reference oil (after 2 days under oxidative conditions), although in concentrations that decrease in line with lysine enrichment. In addition, as it could be expected from acyl group evolution in the different samples, hydroperoxide concentration rises much more slowly in the oils with lysine added than in the non-enriched oil.

Unlike that observed in the case of α- and γ-T, the addition of lysine at any of the levels tested does not modify either the relative proportions of (*Z*,*E*)- and (*E*,*E*)-hydroperoxides or their respective evolutions in comparison with the reference oil. However, small ^{1}H NMR signals that could be assigned to conjugated (*Z*,*E*)-hydroxy-dienes were found in the spectra of the oils enriched with lysine during part of the oxidation process, in general before hydroperoxides reached their maximum concentration, but not in those of the original oil. This type of compounds were detected for a longer period and in a slightly higher concentration in the oil with a 2% of lysine.

Regarding ***secondary oxidation products***, the addition of lysine to the oil causes a remarkable delay in the appearance of all the different types of epoxides (major epoxides, epoxy-keto-enes and (*E*)-epoxystearates) from day 5 in the reference oil to days 13 and 15-16 in those with 1 and 2% proportions of lysine, respectively. In addition, a reduction in the maximum concentration reached by most of them throughout the oxidation process is observed, especially at the 2% level.

Similarly to that observed for epoxides, the appearance of aldehydes is also delayed in the lysine-enriched samples, from day 5 in the non-enriched oil to days 12 and 14 in those containing a 1 and 2% of lysine, respectively. However, unlike in the case of epoxides, differences in the appearance rate and in the relative concentrations of the various types of aldehydes are observed in the lysine-enriched samples in comparison with the reference oil. Thus, in the former the concentrations of all the different kinds of toxic oxygenated α,β-unsaturated aldehydes (4-hydroperoxy-(*E*)-2-alkenals, 4-hydroxy-(*E*)-2-alkenals and 4,5-epoxy-alkenals) are notably lower than in the original oil, especially in presence of the highest lysine proportion, probably due to their reaction with lysine.

The lysine enrichment at the two concentration levels tested also delays the appearance of both (*Z*,*E*)- and (*E*,*E*)-keto-dienes, to a similar extent than in the case of epoxides and aldehdyes.

In agreement with all the aforementioned, the presence of lysine also postpones considerably the emergence of compounds tentatively identified as mono- and dihydroxy-derivatives, particularly in the oil with a 2% of lysine added.

Therefore, in view of all the above mentioned, it could be said that the addition of lysine at the levels tested delays considerably the degradation of refined soybean oil, preserving its content in major and some minor nutrients for longer, and extending its shelf-life. In addition, the presence of lysine reduces the concentration of some toxic oxidation compounds in the oil, especially of oxygenated α,β-unsaturated aldehdyes.

2.4. Study of the effect of the cooxidation of soybean oil and L-lysine, added in a proportion of a 2% in weight, on their respective evolutions throughout a thermal treatment at 70 ºC by combining ^{1}H NMR and LC/MS analyses.

(Manuscript 5)

In an attempt to go further into the cooxidation process of lipids and amino acids, and into the mechanism through which lysine could delay the oxidation process of soybean oil, another refined soybean oil was enriched with lysine in a proportion of 2% in weight and subjected to an oxidation process at 70 ºC under stirring conditions. Its evolution was compared to that of the same oil without lysine. Although the evolution of the oil was analyzed, interest was especially focused on studying the effect of oxidation on the amino acid with the aim of establishing relationships between both

types of processes. The progress of the oil throughout the oxidation process was monitored by ^{1}H NMR, and that of the amino acid by both ^{1}H NMR and LC/MS, in this case after a previous extraction process. The evolutions of lysine and their derivatives were monitored by LC/MS only up to the 22nd day of the oxidation process since, from this point onwards, the oil polymerization degree impaired the extraction of lysine and lysine derivatives in the aqueous phase.

Effect of the lysine enrichment on the oxidation process of the oil, analyzed by ^{1}H NMR

The outcomes of the analysis of the evolution of the oil acyl groups and of the generation and further evolution of hydroperoxides, hydroxy-dienes, epoxides, aldehydes and keto-dienes agree with that observed previously with another type of refined soybean oil enriched with 1 and 2% proportions of lysine, thus reinforcing previous results. An enlargement of the oil total polymerization process from 13 days in the non-enriched oil to 25 days in presence of lysine was observed.

Analysis of the changes that lysine might undergo and the reactions in which it could be involved during its cooxidation process with soybean oil

- ***Decrease in lysine concentration*.** The abundance of lysine, monitored by LC/MS, decreases slightly during most of the oil oxidation process until day 22, when it exhibits a sharp decrease. This coincides with the moment when lipid hydroperoxides are close to their maximum concentration and the levels of all the secondary oxidation products monitored begin to increase.

- ***Reactions with lipid oxidation products*.** According to the scientific literature, lysine could react with several types of oxidation products such as hydroperoxides, epoxides and aldehydes. With regard to a potential reaction of lysine with hydroperoxides, the generation of hydroxy-dienes detected by ^{1}H NMR throughout the oxidation process of the oil containing lysine, but not in the non-enriched oil, could be indicating the paticipation of hydroperoxides in covalent modifications of lysine by saturated aldehydes. This reaction would give rise to N-alkanoyl (amide type) lysine adducts, while hydroperoxides would be reduced to alkyl-hydroxides.

The reaction of lysine with epoxides might also have taken place considerig the slightly lower level of this type of compounds found by ^{1}H NMR at the end of the oxidation process in the lysine-enriched oil.

Notwithstanding, the clearest evidence of the reaction of lysine with lipid oxidation products is the detection of several adducts of lysine with different aldehydes. Thus, lysine adducts with saturated aldehydes were tentatively identified by means of LC/MS in the aqueous extracts obtained throughout the oxidation process from the oil containing lysine, in concentrations that, in general, increase with time. Although both adducts at the Nε and Nα positions were detected, the former were more numerous than the latter, what evidences the higher reactivity of the Nε position. Among this type of adducts, the one due to the reaction of lysine with formaldehyde (*Nε-formyl-lysin*e) was the most abundant, followed by lysine adducts with acetaldehyde (*Nε-acetyl-lysine*), propanal (*Nε-propanoyl-lysine*) and hexanal (*Nε-hexanoyl-lysine*). Furthermore, Nε-lysine adducts with butanal, pentanal and octanal were also detected, although in lower abundances than those of the previously mentioned compounds. Of all these lysine-alkanal adducts detected by LC/MS, only the most abundant one, *Nε-formyl-lysine*, could be identified by ^{1}H NMR, from day 21 onwards, probably due to the lower sensitivity of this technique in comparison with the previous one.

In addition to the adducts with n-alkanals, 7 adducts of lysine with other types of aldehydes considered more reactive than the former were tentatively identified from days 14 to 22. Two of them were considered to come from malondialdehyde (*Nε-(2-propenal)lysine* and *N,N'-disubstituted 1-amino-3-iminopropene lysine derivative*), 2 from (*E*)-2-alkenals (*Michael adduct of lysine with (E)-2-heptenal* and *FDP-lysine*) and 3 from oxygenated α,β-unsaturated aldehydes (*HNA-lysine*, *Nε-4- hydroxy-(2Z)-nonenoyl-lysine* and *4-HNE-LYS Michael adduct stabilized as hemiacetal*). Of all the kinds of aldehydes involved in the mentioned adducts, (*E*)-2-alkenals and the oxygenated α,β-unsaturated type exhibit markedly reduced concentrations in the oil containing lysine when compared to the reference oil, this being especially noticeable in the latter case.

- *Pyrrolization reactions*. Also by means of LC/MS, another compound with a pyrrolic structure (*Nε-2-hexyl-pyrrole*) was tentatively identified at the end of the oxidation process. It was supposed to come from the reaction of lysine with an unidentified aldehyde of the oil, but other possibilities should not be discarded either.

- *Polymerization reactions*. Given that a change in colour from yellow to dark orange took place during the oxidation process of the oil containing lysine, and that coloured

polymers could be generated from the reaction between lipid oxidation compounds and amino acids, polymerization reactions might also have taken place, despite the resulting products not being detected in this study. In fact, the oil with lysine added totally polymerized with a lower degradation degree than the original oil, evidenced by a higher molar percentage of linoleic acyl groups.

In summary, although lysine could take part in various types of reactions under oxidative conditions in presence of lipids, only its reaction with aldehydes has been evidenced in this work, which reinforces observations made from the oil evolution relative to the reduction in the concentration of certain types of aldehydes. It must be noticed that, although some products coming from the reaction of lysine with α,β-unsaturated aldehydes have been reported to exhibit antioxidant activity in soybean oil, the compounds identified in this work do not match with those for which antioxidant ability is claimed. In addition, a retardation in the oil oxidation process is already evident before these adducts are detected. Therefore, other mechanisms seem to be involved in the effect observed.

Aim 3 - STUDY OF THE *IN VITRO* DIGESTION PROCESS OF COMMERCIAL SOYBEAN OIL AND OF THE INFLUENCE OF THE OIL COMPOSITION IN MINOR COMPONENTS, OF ITS INITIAL OXIDATIVE STATUS AND OF THE PRESENCE OF DIFFERENT PROPORTIONS OF OVALBUMIN ON LIPOLYSIS AND OXIDATION REACTIONS.

3.1. Study of the influence of minor components present in commercial virgin and refined soybean oils, determined by Direct Immersion SPME-GC/MS, on lipid bioaccessibility and oxidation during gastrointestinal *in vitro* digestion, studied by ^{1}H NMR and SPME-GC/MS.

(Manuscript 6)

Another process that could lead to the oxidation of food lipids is digestion. In fact, the occurrence of oxidation and the extent of lipolysis achieved during this process are determining factors for the bioaccessibility of dietary lipids, and the knowledge of the factors affecting these types of reactions a crucial issue to advance in the field of lipid nutrient bioaccessibility. In this context, it was considered of interest to assess whether the composition in minor components of commercial edible oils can affect the course of

these processes. To this aim, the evolution of virgin and refined soybean oils throughout a gastrointestinal *in vitro* digestion process was studied by ^{1}H NMR and SPME-GC/MS, paying attention to both lipolysis and oxidation reactions. In addition, the fate during digestion of γ-tocopherol, the main tocopherol present in soybean oil, was also analyzed by ^{1}H NMR.

Characterization of the samples

The ^{1}H NMR analysis of the oils reveals that the two studied soybean oils have very similar molar proportions of the different types of acyl groups.

Regarding minor components, determined by DI-SPME-GC/MS, higher concentrations of all tocopherols and of squalene, all of them compounds attributed with antioxidant ability, are found in the refined oil compared to the virgin type. In contrast, free fatty acids, which show a high tendence to oxidize, are only detected in the latter.

Extent of lipolysis reached throughout *in vitro* digestion, monitored by ^{1}H NMR

Despite the differences in minor components between the virgin and the refined soybean oils, significant differences between the lipolysis extent of the two oils were not observed. Thus, in both cases molar proportions of triglycerides, 2-monoglycerides, 1,2-diglycerides, 1-monoglycerides, 1,3-diglycerides and glycerol of around 22%, 24%, 15%, 5%, 3.5% and 30%, respectively, were found after digestion.

Assessment of lipid oxidation throughout *in vitro* digestion

The occurrence of oxidation during digestion was assessed, on the one hand, ***by means of ^{1}H NMR***, through the study both of unsaturated acyl group degradation and of the generation of oxidation compounds.

The analysis of the molar percentages of the different kinds of oil acyl groups+fatty acids did not show significant changes either in the virgin or in the refined oil after being submitted to *in vitro* digestion. However, the study of the oxidation product profile reveals that some oxidation takes place during digestion, especially in the virgin oil, evidenced by the appearance of (*Z,E*)-conjugated hydroperoxy- and hydroxy-dienes. In contrast, this type of compounds are not detected in the case of the refined oil.

The ***study by SPME-GC/MS*** of the volatile compounds present in the digestates, focused on aldehydes and 2-pentyl-furan, shows that in agreement with the results obtained by ^{1}H NMR, some lipid oxidation occurs during the digestion process of the virgin oil, since the abundances of all the aldehydes present in the headspace of the non-digested reference sample (n-alkanals, (*E*)-2-alkenals and 2,4-alkadienals), and also of

2-pentyl-furan, increase after digestion. In the case of the refined oil, aldehyde and 2-pentyl-furan generation is also noticed after digestion, but to a lesser extent than in the virgin oil.

Evolution of γ-T during the *in vitro* digestion process, monitored by ^{1}H NMR

A reduction in the concentration of γ-T, and consequently in its bioaccessibility, was observed after digestion in the two studied oils. However, this was somewhat more pronounced in the case of the virgin oil, what might be related to the slightly higher oxidation extent found after digestion in this oil when compared to the refined one.

In view of all the above, it could be said that the lower tocopherol and squalene concentrations of the virgin oil, together with its considerable abundance of free fatty acids in comparison with the refined oil, result in a slightly lower oxidative stability during *in vitro* in the former.

3.2. Analysis of the lipolysis extent, of γ-tocopherol fate and of oxidation compound evolution during the *in vitro* digestion of slightly oxidized virgin and refined soybean oils, and of the influence of two proportions of ovalbumin on these processes by combining ^{1}H NMR and SPME-GC/MS analyses.

(Manuscript 7)

Continuing with the study of the factors that could exert an influence on the digestion process of soybean oil and on the bioaccessibility of some of their components, the behaviour of slightly oxidized virgin and refined soybean oils, coming from a thermal treatment at 70 ºC, during *in vitro* digestion was studied by ^{1}H NMR and SPME-GC/MS. The main objectives were to analyze lipolysis extent and oxidation during digestion, and to assess the impact of two different proportions of ovalbumin on both processes. At the same time γ-tocopherol fate was monitored, when possible, by ^{1}H NMR.

Starting oil samples composition

The ^{1}H NMR analysis of the starting oils reveals that they contain noticeable concentrations of linolenic groups and much higher levels of linoleic ones. These oils exhibit low levels of hydroperoxides supporting (*Z*,*E*)- and (*E*,*E*)-conjugated dienes, together with some epoxides. Moreover, a small amount of (*Z*,*E*)-conjugated hydroxy dienes is also noticed in the virgin oil

Extent of lipolysis throughout the *in vitro* digestion

- ***In the samples digested without ovalbumin.*** The lipolysis extent achieved after digestion in the slightly oxidized oils here studied was found to be lower than that observed in the digestates of the same fresh oils. This is evidenced by a higher proportion of triglycerides (around 31% *versus* 22% in the case of the unoxidized oils) and also by a lower proportion of glycerol (around 22% *versus* near 30% in the fresh oils). The lower hydrolysis extent in these oxidized oils could be due to the reaction of some oxidation products with amino acid residues of lipases, this leading to a decrease in their functionality and to a reduction in the bioaccessibility of the major oil components.
- ***In the samples digested with different proportions of ovalbumin*.** While the addition of a low proportion of ovalbumin to the slightly oxidized oils studied does not cause significant variations in the lipolytic process occurred during *in vitro* digestion, the presence of a high level of this protein greatly enhances lipolysis degree, provoking a marked decrease in the triglyceride molar percentage (from around 31% to 5-7%) and a notable surge in that of 2-monoglycerides. This, in turn, improves the bioaccessibility of lipophilic nutrients.

Assessment of lipid oxidation during *in vitro* digestion by ^{1}H NMR through polyunsaturated acyl group evolution and oxidation compound profile analysis

The ***molar concentrations of polyunsaturated acyl groups*** are not significantly affected by the *in vitro* digestion process, either in the absence or in presence of the two ovalbumin proportions tested, so the occurrence of oxidation cannot be inferred from acyl group degradation.

Regarding the ***evolution and/or generation of oxidation compounds***, a diminution in the concentration of hydroperoxides, monitored through the evolution of their associated (*Z*,*E*)- and (*E*,*E*)-hydroperoxy-dienes, is observed after digestion, being this more pronounced for the (*E*,*E*)-isomers. While statistically significant differences were not found between the samples digested in the absence and in presence of a low proportion of ovalbumin, a more pronounced decline in the concentration of hydroperoxy-dienes occurs in the samples digested with a high ovalbumin proportion.

Both in the oils digested in the absence and in presence of a low proportion of ovalbumin, and to a similar extent, the generation of conjugated (*Z*,*E*)- and (*E*,*E*)-hydroxy-dienes is observed after *in vitro* digestion, probably coming from the reduction

of hydroperoxides. This increase in hydroxy-diene concentration is greater in the samples digested with a high ovalbumin amount.

In addition to conjugated hydroxy-dienes, the formation of epoxides and of n-alkanals also occur after the *in vitro* digestion of all the studied samples. However, while no significant differences were found between the oils digested without ovalbumin and with a low proportion of this protein, in general smaller increments in the concentration of both classes of compounds were found in the samples digested with a high ovalbumin proportion.

In view of all the above mentioned, it appears that when ovalbumin is present in a high concentration during digestion, the reduction of hydroperoxides to hydroxy-dienes seems to be favoured over other reactions leading to the generation of epoxides and saturated aldehydes. However, a potential reaction of all these compounds with the amino acid residues of ovalbumin might also contribute to their lower concentration increase in comparison with the rest of digested samples.

Assessment of lipid oxidation throughout *in vitro* digestion by SPME-GC/MS

Although not detectable by ^{1}H NMR, the study of the starting oils by SPME-GC/MS shows that both the virgin and the refined oils contain several classes of aldehydes (n-alkanals, (*E*)-2-alkenals and 2,4-alkadienals), as well as 2-pentyl-furan.

Both in the samples digested without ovalbumin and with a low proportion of this protein, concentration of most n-alkanals increases significantly after *in vitro* digestion. However, when a high ovalbumin proportion is present in the system, most saturated aldehydes either remain unchanged or show a decrease in relation to the non-digested samples.

With regard to unsaturated aldehydes, the concentration of most (*E*)-2-alkenals and of all 2,4-alkadienals also increases after digestion in the samples digested without ovalbumin, although to a lesser extent than in the case of the saturated ones. The same is true for the samples digested with a low proportion of ovalbumin, where the overall increases of both types of unsaturated aldehydes, in general, are not so marked as in the samples digested without ovalbumin. By contrast, in the samples digested with a high ovalbumin proportion, the concentrations of (*E*)-2-alkenals and 2,4-alkadienals generally diminishes after digestion, this reinforcing the idea that reactions between aldehydes and ovalbumin take place during digestion.

The concentration of 2-pentyl-furan also increases after digestion, to a similar extent in the samples without ovalbumin and with a low proportion of this protein. Nevertheless, the increase observed in the concentration of this compound is considerably lower in the samples digested with a high proportion of ovalbumin than in the rest of digested samples, which might be interpreted as a certain antioxidant effect of this high ovalbumin level during digestion.

Analysis of the volution of γ-tocopherol during the *in vitro* digestion process by ^{1}H NMR

The presence of γ-T could only be detected by ^{1}H NMR in the refined oil and, after digestion, only a small concentration of this compound was found in the sample digested with a high proportion of ovalbumin, possibly due to a decrease in oxidative reactions.

All these results evidence that a high proportion of ovalbumin could play a key and beneficial role during the digestion of slightly oxidized oils by increasing their absorbable fraction and γ-T bioaccessibility, and also by contributing to reducing the concentration in the gastrointestinal tract of certain types of oxidation products, such as certain aldehydes and epoxides, either by reducing their generation and/or by reacting with them. These types of compounds could negatively affect the lipolytic process and even human health.

3.3. Study by ^{1}H NMR of the *in vitro* digestion of highly oxidized soybean oil, focusing on the bioaccessibility of major lipid nutrients and of some oxidation compounds, as well as of the influence of the presence of two different proportions of ovalbumin on this process.

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The study of the evolution during digestion of different types of oxidation products present in oxidized oils, and of the parameters influencing this evolution, as well as the analysis of the effect that the presence of such compounds can have on lipid hydrolysis, are considered relevant issues due to their potential health implications. For this reason, the bioaccessibility after *in vitro* digestion of lipid nutrients and oxidation compounds present in highly oxidized virgin and refined soybean oils, obtained after a prolonged accelerated storage process at 70 ºC, was assessed by ^{1}H NMR. Moreover, the effect of

two ovalbumin proportions on lipolysis, oxidation occurrence and fate of oxidation compounds was also addressed.

Starting oil samples composition

The oils subject of study contain polyunsaturated acyl groups (linoleic and linolenic), supported basically on triglycerides, and exhibit a wide variety of oxidation products: hydroperoxides with conjugated (*Z*,*E*)- and (*E*,*E*)-dienes, tentatively identified (*E*)-epoxystearates, other epoxides giving ^{1}H NMR signals either at 2.9 or/and at 3.1 ppm including (*E*)-epoxy-keto-enes, (*Z*,*E*)- and (*E*,*E*)-keto-dienes, tentatively identified mono- and dihydroxy-derivatives and several classes of aldehydes (n-alkanals, (*E*)-2-alkenals, (*E*,*E*)-2,4-alkadienals, 4-hydroperoxy-(*E*)-2-alkenals, 4-hydroxy-(*E*)-2-alkenals and 4,5-epoxy-alkenals).

***In vitro* bioaccessibility of some compounds initially present in the samples after being digested**

The *in vitro* bioaccessibility of the compounds present in the starting oil samples will be influenced, among other factors, by the extent of lipolysis reached after the *in vitro* digestion process, by the occurrence of oxidation, which can lead to the degradation of unsaturated acyl groups, and by the transformations undergone by the oxidation compounds either initially present in the samples or newly formed during digestion.

- ***Lipolysis extent.*** The lipolysis extent of the highly oxidized virgin and refined soybean oils here studied is lower than that observed after the *in vitro* digestion of these same oils when they are unoxidized and when they exhibit a lower oxidation degree. However, the differences between the slightly and the highly oxidized oils are not very marked. This could be due to several factors such as the ability of the various proteins present in the digestive juices, including enzymes, to react with oxidation products, the susceptibility of lipases to be inactivated by different classes of oxidation compounds, or the polarity of the system, among others.

  Although the presence of a low ovalbumin level during digestion hardly affects lipolysis, the presence of a high proportion of this protein slightly enhances lipolysis, since a decrease is observed in the amount of remaining triglycerides from approximately 35% in the samples digested without ovalbumin to 31% in those digested with a high ovalbumin level. This is accompanied by an increment of the molar proportions of 1,2-diglycerides, and especially of 2-monoglycerides. The small effect of this high concentration of ovalbumin on lipid bioaccessibility, which only

increases from around 54% in the samples digested without ovalbumin to 55-57% in those containing a high ovalbumin level, contrasts with that observed in less oxidized soybean oil samples, where this parameter rises from approximately 57% to around 80%. This suggests that the high oxidation degree of the oils causes a loss in the efficiency of ovalbumin in improving lipolysis.

- ***Assessment of oxidation occurrence during digestion through polyunsaturated group degradation.*** Clear and important diminutions in the concentrations of linolenic and linoleic acyl groups+fatty acids occur after digestion, more pronounced for linolenic than for linoleic groups, which evidence the occurrence of oxidation reactions during digestion.

  While the presence of a low ovalbumin proportion in the system does not have a noticeable impact on the evolution of polyunsaturated acyl groups+fatty acids during digestion, the addition of ovalbumin at a high level leads to a less pronounced decrease in the concentrations of polyunsaturated acyl groups+fatty acids when compared to the rest of digested samples. This suggests that a high concentration of ovalbumin during digestion could exert an antioxidant effect.

- ***Changes in the oxidation compound profile after digestion*****.** Similarly to that observed in the less oxidized oils studied previously, the concentrations of (*Z*,*E*)- and especially of (*E*,*E*)-hydroperoxy-dienes decrease after digestion and the generation of (*Z*,*E*)-hydroxy-dienes is noticed.

  Regarding epoxides, while the concentration of those giving signal at 2.9 ppm drops only slightly after digestion, the level of those with signal at 3.1 ppm decreases to a greater extent. In contrast, the concentration of (*E*)-epoxy-keto-enes rises significantly after digestion.

  An increase after digestion is also observed for keto-diene levels, possibly due to their generation from the initially present hydroperoxides. In contrast, the concentration of the tentatively identified dihydroxy-derivatives decreases very slightly after digestion.

  When it comes to aldehydes, significant increments in the concentration of n-alkanals take place after digestion. Conversely, the concentrations of α,β-unsaturated aldehydes decrease, this diminution being very sharp for the most reactive and toxic aldehydes among those detected, which are the oxygenated ones (4-hydroperoxy-(*E*)-

2-alkenals, 4-hydroxy-(*E*)-2-alkenals and 4,5-epoxy-2-alkenals), less pronounced for (*E*)-2-alkenals and practically null for (*E,E*)-2,4-alkadienals.

The presence of a low ovalbumin proportion during digestion does not provoke significant changes in the concentration of most of the oxidation products present in the digestates. Notwithstanding, slightly lower levels of aldehydes, as well as of hydroperoxy-dienes and keto-dienes, together with somewhat higher concentrations of (*Z,E*)-hydroxy-dienes, are perceived.

When ovalbumin is present in a high concentration in the samples subjected to digestion, a greater reduction in hydroperoxy-diene levels, along with a significant increase in the concentrations of (*Z,E*)-hydroxy-dienes, are noticed. This reinforces previous outcomes obtained with less oxidized oils. Moreover, while the levels of the epoxides giving signals at 2.9 and 3.1 ppm are not noticeably modified by the presence of a high proportion of ovalbumin, slightly smaller concentration increments are observed after digestion for (*E*)-epoxy-keto-enes, keto-dienes and n-alkanals than in the rest of digested samples. Regarding the various groups of unsaturated aldehydes, their respective levels decrease to a greater extent than in the rest of samples, and even (*E,E*)-2,4-alkadienals show a concentration decline after digestion. A drop is also noticed in the concentration of potential dihydroxy-derivatives in comparison with the samples digested without ovalbumin.

Similarly to that commented in the case of the digestion of the slightly oxidized oils studied previously, the lower concentrations of some oxidation products observed in the samples digested with a high ovalbumin proportion, like for example α,β-unsaturated aldehdyes, might be due to their reaction with this protein. However, the lower concentrations observed for some types of oxidation products could also be due to an antioxidant effect of ovalbumin, evidenced by a smaller decrease in the concentrations of polyunsaturated acyl groups in these samples.

It is worth noticing that, among all the oxidation products identified by ^{1}H NMR after the *in vitro* digestion of all the highly oxidized soybean oil samples studied, epoxides are those present in higher concentrations. Therefore, the potential effect of the presence of this type of compounds in the digestates should be further investigated.

CONCLUSIONS

Aim 1 - STUDY OF THE INFLUENCE OF THE MINOR COMPOUNDS NATURALLY PRESENT IN COMMERCIAL SOYBEAN OIL ON ITS EVOLUTION UNDER ACCELERATED STORAGE CONDITIONS

1.1. Contrary to what the denomination of the two studied soybean oils might suggest, the virgin oil has a lower content of tocols and sterols than the refined one. Therefore, it should not be generally assumed that virgin oils are better sources of minor components considered to be beneficial for human health than refined ones.

1.2. The presence of a higher concentration of tocopherols and of a lower amount of free fatty acids is associated to a higher oxidative stability under accelerated storage conditions in soybean oils having similar proportions of the different kinds of acyl groups.

1.3. A simple analysis of the composition in minor components of commercial oils by means of DI SPME followed by GC/MS would make it possible to establish different categories or quality levels in relation to oxidative stability and content in some bioactive compounds considered beneficial for human health within oils of the same botanical origin and similar unsaturation level.

1.4. Despite all edible oils of the same botanical origin must meet certain requirements established in the food legislation, it is evident that differences exist among them that can be associated with higher or lower quality levels. Therefore, the establishment of parameters indicative of this quality, not considered until now, could be valuable for consumers and also for oil producers and food industry.

Aim 2 - ASSESSMENT OF THE EFFECT OF ADDING ALPHA-TOCOPHEROL, GAMMA-TOCOPHEROL OR L-LYSINE ON THE OXIDATIVE STABILITY AND THE OXIDATION PROCESS OF COMMERCIAL SOYBEAN OIL SUBMITTED TO ACCELERATED STORAGE CONDITIONS

2.1. To the best of our knowledge, the methodology used, based on ^{1}H NMR spectroscopy and partially developed in this work, is the only one that allows studying the oxidation process of edible oils through the degradation of acyl groups and the formation of primary and secondary oxidation compounds simultaneously and in a simple way. It does not require either chemical changes of the sample or

the use of reagents. In addition, this methodology has been proved to be extremely useful to assess the real effect of the addition of compounds up to now described as either antioxidants or prooxidants.

2.2. <u>In relation to the effect of α-T</u>

2.2.1. The addition of α-T to soybean oil at a very low level (0.002% in weight) does not affect either its oxidative stability or its oxidation process under accelerated storage conditions monitored by ^{1}H NMR.

2.2.2. The addition of α-T proportions ranging from 0.02 to 5% to soybean oil submitted to accelerated storage conditions reduces its oxidative stability, more the higher α-T concentration. This effect has been proved through a higher rate of degradation of acyl groups, a more elevated pace of hydroperoxide concentration increase and an earlier appearance of secondary oxidation products, including some toxic compounds such as oxygenated α,β-unsaturated aldehydes. In spite of this, as the α-T concentration gets higher, an enlargement in the time needed to reach oil total polymerization is observed.

2.2.3. The α-T enrichment modifies the oxidation pathway of soybean oil forcing the almost exclusive generation of (*Z,E*)-hydroperoxides as the α-T level rises, while postponing that of their (*E,E*)-counterparts to the most advanced stages of the oxidation process and reducing their levels. This has direct consequences in the nature and relative proportions of secondary oxidation products, in such a way that oxidation compounds with (*Z,E*)-isomerism such as conjugated hydroxy-dienes and (*Z,E*)-keto-dienes are also generated earlier and in higher concentrations that go in line with the α-T level. Even (*Z,E*)-2,4-alkadienals appear, not usually detected under the accelerated storage conditions used.

2.2.4. The parallelism observed between the effect of increasing α-T levels on (*Z,E*)- and (*E,E*)-hydroperoxides and on the generation sequence of the various types of aldehydes suggest a potential relationship between each type of hydroperoxides and specific groups of aldehydes, not described previously.

2.3. <u>In relation to the effect of γ-T</u>

2.3.1. Unlike that observed for α-T, the addition of a low level of γ-T (0.02%) does not provoke an effect detectable by ^{1}H NMR, either on the oxidative stability or on the oxidation process of refined soybean oil.

2.3.2. The enrichment of refined soybean oil with γ-T at higher levels reduces its oxidative stability under the accelerated conditions here tested, and modifies the oxidation process in relation to the non-enriched oil, more the higher the γ-T concentration.

2.3.3. Similarly to α-T, increasing levels of γ-T speed up the degradation of acyl groups and the surge of (*Z,E*)-hydroperoxide concentration, while the generation of the (*E,E*)-isomers is delayed, but to a lower extent than α-T.

2.3.4. As the γ-T level rises, an extension of the time necessary to reach oil total polymerization is observed, this effect being considerably more pronounced than in the case of α-T. This leads to important differences in the generation rate of secondary oxidation products in the oils enriched with each tocopherol since, contrary to α-T, γ-T delays the appearance of most secondary oxidation products more the higher the enrichment degree, with the exception of some epoxides, which are detected earlier than in the non-enriched oil.

2.3.5. The same effect of the various tocopherols on the oxidative stability of edible oils should not be assumed, since small structural variations can lead to significant differences in their respective actions on the oxidation process under accelerated storage conditions.

2.3.6. The results obtained evidence the difficulty to define the performance of α- and γ-T on soybean oil oxidation under accelerated storage conditions, either as antioxidant or prooxidant since, on the one hand, they reduce the oxidative stability of the oil, and on the other they extend the time needed to reach the oil total polymerization.

2.3.7. Given the complexity of the effect of tocopherols on soybean oil oxidation process under accelerated storage conditions, the behaviour of these compounds towards edible oil oxidation should be addressed on the basis of a broad range of lipid oxidation markers, monitored throughout the whole oxidation process, or on the global study of this latter. Otherwise, the monitoring of only one or two compounds or group of compounds at certain times throughout the process could lead to erroneous conclusions.

2.3.8. The results obtained suggest that it would be necessary to revise the European regulations to limit the addition of α- and γ-T to products intended for human consumption since, as in the vegetable oil here studied, the supplementation with

these tocopherols could have a negative effect not only on the oxidative stability of the oil, but also potentially on human health due to the acceleration in the appearance of some toxic oxidation products.

2.4. <u>In relation to the effect of lysine</u>

2.4.1. The enrichment of refined soybean oil with L-lysine increases its oxidative stability, although no large differences were observed between the effects of the 1 and 2% enrichment levels.

2.4.2. The addition of lysine not only delays considerably the degradation of the oil acyl groups and the generation rate of hydroperoxides, but also postpones the appearance of secondary oxidation compounds, while preserving the γ-T oil content. All this extends considerably the oil shelf life.

2.4.3. Contrary to α- and γ-tocopherols, lysine does not modify the oxidation pathway of soybean oil although, unlike in the non-enriched oil, the presence of conjugated hydroxy-dienes is detected during part of the oxidation process, in general before hydroperoxides reach their maximum concentration.

2.4.4. It could be said that the presence of lysine exerts a detoxifying effect on soybean oil submitted to oxidative conditions, since it considerably reduces the concentrations of some toxic secondary oxidation products such as the very reactive oxygenated α,β-unsaturated aldehydes, possibly due to their reaction with the amino acid.

2.4.5. Lysine has been shown to be much more effective than α- and γ-tocopherols to reduce the oxidation rate of soybean oil, so it could constitute a worthy alternative to consider in order to increase the oxidative stability of edible oils and diminish their oxidation.

2.4.6. The selection of appropriate markers to assess lipid oxidation is also a crucial issue when amino compounds able to react with oxidation products are present in the system since, as in the case of lysine, reduced concentrations of aldehydes in relation to other types of oxidation compounds can be found at very advanced stages of the oxidation process.

2.4.7. The cooxidation of soybean oil and lysine provokes changes in the amino acid that, under the studied conditions, seem to be mainly due to its reaction with different types of aldehydes, thus confirming the results suggested by the study

of the oil. However, the involvement of lysine in other types of reactions like polymerization ones could also be plausible.

2.4.8. In the soybean oil oxidation process in presence of lysine, the most abundant lysine-aldehyde adducts and the first ones to be detected by LC/MS are those formed at the reactive N-ε position with low molecular weight n-alkanals and malondialdehdye, followed by the ones with α,β-unsaturated aldehydes and (*E*)-2-alkenals. To the best of our knowledge, this is the first time that several lysine adducts with aldehydes of varying nature have simultaneously been detected in a complex food model system.

2.4.9. Despite the great decrease observed in the concentration of oxygenated α,β-unsaturated aldehydes in presence of lysine, the adducts of lysine with this kind of aldehydes are not the most abundant among those tentatively identified. Therefore, it could be thought that this class of compounds might also be taking part in other types of reactions with lysine.

2.4.10. Although some antioxidant ability has been attributed to certain products of the reaction of lysine with unsaturated aldehydes, they do not match with those identified in this study, and in addition, a retardation in the oil oxidation process is already evident before these adducts are detected. This suggests that much more complex mechanisms seem to be involved in the observed effect.

Aim 3 - STUDY OF THE *IN VITRO* DIGESTION PROCESS OF COMMERCIAL SOYBEAN OIL AND OF THE INFLUENCE OF THE OIL COMPOSITION IN MINOR COMPONENTS, OF ITS INITIAL OXIDATIVE STATUS AND OF THE PRESENCE OF DIFFERENT PROPORTIONS OF OVALBUMIN ON LIPOLYSIS AND OXIDATION REACTIONS

In relation to the effect of the minor components present in unoxidized soybean oil

3.1. In line with that observed under accelerated storage conditions, higher concentrations of tocopherols and squalene and a lower content of free fatty acids in soybean oils with similar unsaturation degrees, result in a higher oxidative stability during *in vitro* digestion and also in a smaller reduction in the bioaccessibility of γ-T, the main tocopherol present in soybean oil.

3.2. In relation to the influence of the initial oxidative status of soybean oil

3.2.1. An initial oxidation degree in soybean oil negatively influences the lipolysis extent during *in vitro* digestion, reducing the bioaccessibility of the major oil components, which include some essential fatty acids like the ω-3 ones. This could be due to the reaction of certain oxidation products present in the oxidized oils with the amino acid residues of lipolytic enzymes, which in turn could lead to a decrease in their activity. However, great differences between the lipolysis degree achieved in oils with two different initial levels of oxidation are not observed. This evidences the complexity of oxidized lipid digestion and of the factors that can affect this process.

3.2.2. The initial oxidative status of the oil also affects the occurrence of oxidation during *in vitro* digestion, this being greater and deductible from polyunsaturated acyl group degradation when the initial oxidation degree is high.

3.2.3. The *in vitro* digestion process of soybean oil causes changes in its oxidation compound profile that entail generation, transformation and/or reaction with components of the digestive fluids. The balance among all these processes will determine which kinds of compounds appear to a lesser or greater extent in the digestates.

3.2.4. Not all the types of oxidation products present in oxidized soybean oil evolve in the same way during *in vitro* digestion, epoxides being among those remaining to a greater extent and the most abundant oxidation compounds present in the digestates. This raises the need to go deeper into the effect that the presence of epoxides in the gastrointestinal tract can have on human health.

3.2.5. It is considered of special interest the case of those samples having notable levels of toxic oxygenated α,β-unsaturated aldehydes because, although a pronounced decrease in their concentration occurs after digestion, a certain amount is present in the digestates. This suggests that this type of compounds react with enzymes and/or other proteins present in the digestive fluids, being able to affect their functionality; however, part of them remain bioaccessible for absorption.

3.3. <u>In relation to the effect of the addition of different proportions of ovalbumin to oxidized soybean oil</u>

3.3.1. The presence of a low proportion of ovalbumin during the *in vitro* digestion of oxidized soybean oil does not significantly affect either lipolysis or oxidation or

oxidation compound evolution. By contrast, a high proportion of this protein can affect all these processes.

3.3.2. A high level of ovalbumin during the *in vitro* digestion of oxidized soybean oil greatly enhances lipolysis when the oxidation degree of the oil is low, improving lipid bioaccessibility. This might be due to the emulsifying ability of the ovalbumin added. However, this effect is much smaller when the initial oxidation degree of the oil is high, this revealing a negative effect on ovalbumin performance during digestion.

3.3.3. The presence of a high level of ovalbumin during the *in vitro* digestion of oxidized soybean oil, irrespectively of the sample oxidation degree, modifies to some extent the oxidation product profile of the digestates, favouring the reduction of hydroperoxides to their corresponding hydroxides and diminishing the levels of some oxidation products such as hydroperoxides, certain epoxides and above all aldehydes.

3.3.4. A high proportion of ovalbumin, and maybe of other proteins of similar nature, could play a beneficial role in the digestion of lipids, to a greater or lesser extent depending on the initial oxidation degree of the oil, by increasing the bioaccessibility of major lipid nutrients, of γ-T and presumably of other compounds of the same nature. This could be due to an enhancement of lipolysis and/or to a reduction in oxidation reactions consequence of a likely antioxidant effect. Moreover, the presence of ovalbumin in high enough proportion during digestion can contribute to reduce the concentration in the gastrointestinal tract of oxidation products that could exert negative effects on human health, such as certain aldehydes and epoxides, either by diminishing their generation and/or by reacting with them.

3.3.5. Taking into account the different evolution of the various kinds of oxidation compounds during the *in vitro* digestion of soybean oil depending on its initial oxidative status, a battery of markers should be used to assess oxidation during digestion of oils. In addition to this, another important issue that should be borne in mind is that the presence of proteins able to react with certain oxidation products can lead to an underestimation of the oxidation extent depending on the oxidation compounds monitored.

REFERENCES

Ahmad, M. M., Al-Hakim, S., Adel, A., & Shehata, Y. (1983). The antioxidant activity of amino acids in two vegetable oils. *Journal of the American Oil Chemists' Society*, 60, 837-840.

Alaiz, M., Zamora, R., & Hidalgo, F. J. (1995a). Antioxidative activity of (E)-2-octenal/amino acids reaction products. *Journal of Agricultural and Food Chemistry*, 43, 795-800.

Alaiz, M., Zamora, R., & Hidalgo, F. J. (1995b). Natural antioxidants produced in oxidized lipid/amino acid browning reactions. *Journal of the American Oil Chemists' Society*, 72, 1571-1575.

Alberdi-Cedeño, J., Ibargoitia, M. L., Cristillo, G., Sopelana, P., & Guillén, M. D. (2017). A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components. *Food Chemistry, 221*, 1135-1144.

Al-Shamsi, K. A., Mudgil, P., Hassan, H. M., & Maqsood, S. (2018). Camel milk protein hydrolysates with improved technofunctional properties and enhanced antioxidant potential in vitro and in food model systems. *Journal of Dairy Science, 101*, 47-60.

Awada, M., Soulage, C. O., Meynier, A., Debard, C., Plaisancié, P., Benoit, B., Picard, G., Loizon, E., Chauvin, M. A., Estienne, M., Peretti, N., Guichardant, M., Lagarde, M., Genot, C., & Michalski, M. C. (2012). Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. *Journal of Lipid Research, 53*, 2069-2080.

Azizkhani, M., Kamkar, A., & Nejad, A. S. M. (2011). Effects of tocopherols on oxidative stability of margarine. *Journal of the Chemical Society of Pakistan, 33*, 134-137.

Baker, J. K., & Myers, C. W. (1991). One-dimensional and two-dimensional ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) analysis of vitamin E raw materials or analytical reference standards. *Pharmaceutical Research, 8*, 763-770.

Bakota, E. L., & Winkler-Moser, J. K. (2017). Differences in antioxidant activity between two rice protein concentrates in an oil-in-water emulsion. *European Journal of Lipid Science and Technology, 119*, 1600421.

Banni, S., Contini, M. S., Angioni, E., Deiana, M., Dessì, M. A., Melis, M. P., ... & Corongiu, F. P. (1996). A novel approach to study linoleic acid autoxidation:

importance of simultaneous detection of the substrate and its derivative oxidation products. *Free radical research, 25*, 43-53.

Birringer, M., Pfluger, P., Kluth, D., Landes, N., & Brigelius-Flohe, R. (2002). Identities and differences in the metabolism of tocotrienols and tocopherols in HepG2 cells. *The Journal of nutrition, 132*, 3113-3118.

Blekas, G., Tsimidou, M., & Boskou, D. (1995). Contribution of α-tocopherol to olive oil stability. *Food Chemistry*, *52*, 289-294.

Brühl, L., Weisshaar, R., & Matthäus, B. (2016). Epoxy fatty acids in used frying fats and oils, edible oils and chocolate and their formation in oils during heating. *European journal of lipid science and technology*, *118*, 425-434.

Carocho, M., & Ferreira, I. C. (2013). A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and chemical toxicology*, *51*, 15-25.

Chalvardjian, A., Morris, L. J., & Holman, R. T. (1962). Effect of dietary epoxyoleic acid upon rats. *The Journal of Nutrition, 76*, 52-58.

Cheetangdee, N., & Benjakul, S. (2015). Antioxidant activities of rice bran protein hydrolysates in bulk oil and oil-in-water emulsion. *Journal of the Science of Food and Agriculture, 95*, 1461-1468.

Chen, Z. Y., & Nawar, W. W. (1991). The role of amino acids in the autoxidation of milk fat. *Journal of the American Oil Chemists' Society, 68*, 47–50.

Choe, E., & Min, D. B. (2006). Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety, 5*, 169-186.

Cillard, J., Cillard, P., Cormier, M., & Girre, L. (1980). α-Tocopherol prooxidant effect in aqueous media: increased autoxidation rate of linoleic acid. *Journal of the American Oil Chemists' Society*, *57*, 252.

Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union*, 12.11.2011, L 295/1-177.

Davies, M.J. (2005). The Oxidative Environment and Protein Damage. *Biochim. Biophys, 1703*, 93-109

Dolde, D., & Wang, T. (2011). Oxidation of corn oils with spiked tocols. *Journal of the American Oil Chemists' Society*, *88*, 1759-1765.

Dwyer, S. P. O., O'Beirne, D., Ní Eidhin, D., & O'Kennedy, B. T. (2012). Effects of green tea extract and α-tocopherol on the lipid oxidation rate of omega-3 oils, incorporated into table spreads, prepared using multiple emulsion technology. *Journal of food science*, *77*, N58-N65.

Eghbaliferiz, S., & Iranshahi, M. (2016). Prooxidant activity of polyphenols, flavonoids, anthocyanins and carotenoids: updated review of mechanisms and catalyzing metals. *Phytotherapy Research*, *30*, 1379-1391.

Elez-Martínez, P., Soliva-Fortuny, R., & Martín-Belloso, O. (2007). Oxidative rancidity in avocado purée as affected by α-tocopherol, sorbic acid and storage atmosphere. *European Food Research and Technology*, *226*, 295.

Elias, R. J., Bridgewater, J. D., Vachet, R. W., Waraho, T., McClements, D. J., & Decker, E. A. (2006). Antioxidant mechanisms of enzymatic hydrolysates of β-lactoglobulin in food lipid dispersions. *Journal of Agricultural and Food Chemistry*, *54*, 9565-9572.

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical reviews in food science and nutrition*, *48*, 430-441.

Esterbauer, H. (1993). Cytotoxicity and genotoxicity of lipid-oxidation products. *The American Journal of Clinical Nutrition*, *57*, 779S-785S.

Evans, J. C., Kodali, D. R., & Addis, P. B. (2002). Optimal tocopherol concentrations to inhibit soybean oil oxidation. *Journal of the American Oil Chemists' Society*, *79*, 47-51.

Farag, R. S., Osman, S. A., Hallabo, S. A. S., & Nasr, A. A. (1978). Cottonseed oil oxidation catalyzed by amino acids and albumin in aqueous and nonaqueous media. *Journal of the American Oil Chemists' Society*, *55*, 613–615.

Filippenko, T. A., & Gribova, N. Y. (2011). Antioxidant activity of amino acids during oxidation of sunflower oil in an emulsion. *Pharmaceutical Chemistry Journal*, *45*, 296–298

Frankel E.N., Neff W.E., Miyashita K. (1990). Autoxidation of polyunsaturatedtriacylglycerols. II. Trilinolenoylglycerol. *Lipids*, *25*, 40–7

Frankel E.N., Neff W.E., Selke E., Brooks D.D. (1988). Analysis of autoxidizedfats by gas chromatography-mass spectrometry: X. Volatile thermaldecomposition products of methyl linolenate dimers. *Lipids*, *23*, 295–8.

Frankel E.N., Neff W.E., Selke E., Weisleder D. (1982). Photosensitizedoxidation of methyl linoleate: secondary and volatile thermal decompositionproducts. *Lipids, 17*, 11–8.

Frankel, E. N. (1993). In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends in Food Science & Technology, 4*, 220-225.

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture*, *80*, 1925-1941.

Frankel, E. N., Evans, C. D., & Cooney, P. M. (1959). Autoxidation of Fats, Tocopherol Oxidation in Natural Fats. *Journal of Agricultural and Food Chemistry*, *7*, 438-441.

Fuster, M. D., Lampi, A. M., Hopia, A., & Kamal-Eldin, A. (1998). Effects of α-and γ-tocopherols on the autooxidation of purified sunflower triacylglycerols. *Lipids*, *33*, 715-722.

Gardner, H. W. (1979). Lipid hydroperoxide reactivity with proteins and amino acids: a review. *Journal of Agricultural and Food Chemistry*, *27*, 220-229.

Gardner, H. W., Plattner, R. D., & Weisleder, D. (1985). The epoxyallylic radical from homolysis and rearrangement of methyl linoleate hydroperoxide combines with the thiyl radical of N-acetylcysteine. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *834*, 65-74.

Gliszczynska-Swiglo, A., Sikorska, E., Khmelinskii, I., & Sikorski, M. (2007). Tocopherol content in edible plant oils. *Polish Journal of Food and Nutrition Sciences*, *57*, 157-161.

Gobert, M., Rémond, D., Loonis, M., Buffière, C., Santé-Lhoutellier, V., & Dufour, C. (2014). Fruits, vegetables and their polyphenols protect dietary lipids from oxidation during gastric digestion. *Food & function*, *5*, 2166-2174.

Goicoechea, E., & Guillen, M. D. (2010). Analysis of hydroperoxides, aldehydes and epoxides by 1H nuclear magnetic resonance in sunflower oil oxidized at 70 and 100 C. *Journal of Agricultural and Food Chemistry, 58*, 6234-6245.

Goicoechea, E., Brandon, E. F., Blokland, M. H., & Guillén, M. D. (2011). Fate in digestion in vitro of several food components, including some toxic compounds coming from omega-3 and omega-6 lipids. *Food and Chemical Toxicology, 49*, 115-124.

Goicoechea, E., Van Twillert, K., Duits, M., Brandon, E. D., Kootstra, P. R., Blokland, M. H., & Guillén, M. D. (2008). Use of an *in vitro* digestion model to study the bioaccessibility of 4-hydroxy-2-nonenal and related aldehydes present in oxidized oils rich in omega-6 acyl groups. *Journal of agricultural and food chemistry*, *56*, 8475-8483.

Golding, M., & Wooster, T. J. (2010). The influence of emulsion structure and stability on lipid digestion. *Current Opinion in Colloid & Interface Science*, *15*, 90-101.

Gopala Krishna, A. G., & Prabhakar, J. V. (1994). Antioxidant efficacy of amino acids in methyl linoleate at different relative humidities. *Journal of the American Oil Chemists' Society, 71,* 645–647.

Gottstein, T., & Grosch, W. (1990). Model study of different antioxidant properties of α- and γ-tocopherol in fats. *European Journal of Lipid Science and Technology*, 92, 139-144.

Greene, J. F., Williamson, K. C., Newman, J. W., Morisseau, C., & Hammock, B. D. (2000). Metabolism of monoepoxides of methyl linoleate: bioactivation and detoxification. *Archives of biochemistry and biophysics*, *376*, 420-432.

Grüneis, V., & Pignitter, M. (2018). Epoxide Value-A Novel Marker for the Quality Assessment of Food Lipids. *Journal of Agricultural and Food Chemistry, 66*, 5039-5040.

Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α, β-unsaturated aldehydes and their study in foods: a review. *Critical reviews in food science and nutrition*, *48*, 119-136.

Guillén, M. D., & Ruiz, A. (2005). Oxidation process of oils with high content of linoleic acyl groups and formation of toxic hydroperoxy- and hydroxyalkenals. A study by ^{1}H nuclear magnetic resonance. *Journal of the Science of Food and Agriculture*, *85*, 2413–2420.

Guillén, M. D., & Ruiz, A. (2008). Monitoring of heat-induced degradation of edible oils by proton NMR. *European Journal of Lipid Science and Technology, 110*, 52-60.

Guillén, M. D., & Uriarte, P. S. (2009). Contribution to further understanding of the evolution of sunflower oil submitted to frying temperature in a domestic fryer: study

by 1H Nuclear Magnetic Resonance. *Journal of Agricultural and Food Chemistry*, 57, 7790–7799.

Gutteridge, J. M., Paterson, S. K., Segal, A. W., & Halliwell, B. (1981). Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. *Biochemical Journal*, *199*, 259-261.

Haila, K. M., Lievonen, S. M., & Heinonen, M. I. (1996). Effects of lutein, lycopene, annatto, and γ-tocopherol on autoxidation of triglycerides. *Journal of Agricultural and Food Chemistry*, *44*, 2096-2100.

Heinonen, M., Haila, K., Lampi, A. M., & Piironen, V. (1997). Inhibition of oxidation in 10% oil-in-water emulsions by β-carotene with α-and γ-tocopherols. *Journal of the American Oil Chemists' Society*, *74*, 1047.

Hidalgo, F. J., & Zamora, R. (2003). Edible oil analysis by high-resolution nuclear magnetic resonance spectroscopy: recent advances and future perspectives. *Trends in Food Science & Technology*, *14*, 499-506.

Hidalgo, F. J., & Zamora, R. (2016). Amino acid degradations produced by lipid oxidation products. *Critical Reviews in Food Science and Nutrition, 56*, 1242-1252.

Hidalgo, F. J., León, M. M., & Zamora, R. (2006). Antioxidative activity of amino phospholipids and phospholipid/amino acid mixtures in edible oils as determined by the Rancimat method. *Journal of Agricultural and Food Chemistry, 54*, 5461-5467.

Hu, M., McClements, D. J., & Decker, E. A. (2003). Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *51*, 1435-1439.

Huang, S. W., Frankel, E. N., & German, J. B. (1994). Antioxidant activity of α- and γ-tocopherols in bulk oils and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *42*, 2108-2114.

Huang, S. W., Frankel, E. N., & German, J. B. (1995). Effects of individual tocopherols and tocopherol mixtures on the oxidative stability of corn oil triglycerides. *Journal of Agricultural and Food Chemistry*, *43*, 2345-2350.

Hwang, H. S., & Winkler-Moser, J. K. (2017). Antioxidant activity of amino acids in soybean oil at frying temperature: Structural effects and synergism with tocopherols. *Food Chemistry*, *221*, 1168-1177.

Ishino, K., Shibata, T., Ishii, T., Liu, Y. T., Toyokuni, S., Zhu, X., Sayre. L. M. & Uchida, K. (2008). Protein N-acylation: H2O2-mediated covalent modification of protein by lipid peroxidation-derived saturated aldehydes. *Chemical research in toxicology*, *21*, 1261-1270.

Isnardy, B., Wagner K.-H., & Elmadfa, I. (2003). Effects of α-, γ-, and δ- tocopherols on the autoxidation of purified rapeseed oil triacylglycerols in a system containing low oxygen. *Journal of Agricultural and Food Chemistry*, *51*, 7775–7780.

Jayasinghe, C., Gotoh, N., & Wada, S. (2013). Pro-oxidant/antioxidant behaviours of ascorbic acid, tocopherol, and plant extracts in n-3 highly unsaturated fatty acid rich oil-in-water emulsions. *Food chemistry*, *141*, 3077-3084.

Johnson, L. A., White, P. J., & Galloway, R. (Eds.). (2015). Soybeans: chemistry, production, processing, and utilization. Elsevier.

Jung, M. Y., Yoon, S. H., & Min, D. B. (1989). Effects of processing steps on the contents of minor compounds and oxidation of soybean oil. *Journal of the American Oil Chemists' Society*, *66*, 118-120.

Jung, M.Y., & Min, D.B. (1990). Effects of α-, γ-, and δ-tocopherols on oxidative stability of soybean oil. *Journal of Food Science*, *55*, 1464-1465.

Kalogeropoulos, N., Salta, F. N., Chiou, A., & Andrikopoulos, N. K. (2007). Formation and distribution of oxidized fatty acids during deep-and pan-frying of potatoes. European *Journal of Lipid Science and Technology*, *109*, 1111-1123.

Kamal-Eldin, A., & Appelqvist, L. Å. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids*, *31*, 671-701.

Kamal-Eldin, A., & Budilarto, E. (2014). Antioxidant activities and interactions of α-and γ-tocopherols within canola and soybean emulsions. *European journal of lipid science and technology*, *116*, 781-782.

Kamal-Eldin, A., & Pickova, J. (2008). Balance between polyunsaturated fatty acids and antioxidants in nutrition. *Lipid technology*, *20*, 80-83.

Kanazawa, K., & Ashida, H. (1998a). Catabolic fate of dietary trilinoleoylglycerol hydroperoxides in rat gastrointestines. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1393*, 336-348.

Kanazawa, K., & Ashida, H. (1998b). Dietary hydroperoxides of linoleic acid decompose to aldehydes in stomach before being absorbed into the body. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1393*, 349-361.

Kanazawa, K., Ashida, H., Minamoto, S., Danno, G. I., & Natake, M. (1988). The effects of orally administered linoleic acid and its autoxidation products on intestinal mucosa in rat. *Journal of Nutritional Science and Vitaminology, 34*, 363-373.

Karel, M., Schaich, K., & Roy, R. B. (1975). Interaction of peroxidizing methyl linoleate with some proteins and amino acids. *Journal of Agricultural and Food Chemistry, 23*, 159-163.

Karel, M., Tannenbaum, S. R., Wallace, D. H., & Maloney, H. (1966). Autoxidation of methyl linoleate in freeze- dried model systems. III. Effects of added amino acids. *Journal of Food Science, 31*, 892–896.

Kenmogne-Domguia, H. B., Moisan, S., Viau, M., Genot, C., & Meynier, A. (2014). The initial characteristics of marine oil emulsions and the composition of the media inflect lipid oxidation during *in vitro* gastrointestinal digestion. *Food Chemistry, 152*, 146-154.

King, R. E., Min, D. B., & Min, S. C. (2011). Study of α-, γ-, and δ-tocopherols in the oxidative stability of lard. *Food Science and Biotechnology, 20*, 817-822.

Koskas, J. P., Cillard, J., & Cillard, P. (1984). Autoxidation of linoleic acid and behavior of its hydroperoxides with and without tocopherols. *Journal of the American Oil Chemists' Society, 61*, 1466-1469.

Kostewicz, E. S., Abrahamsson, B., Brewster, M., Brouwers, J., Butler, J., Carlert, S., Dickson, P. A., Dressman, J., Holm, R., Klein, S. & Mann, J. (2014). *In vitro* models for the prediction of in vivo performance of oral dosage forms. *European Journal of Pharmaceutical Sciences, 57*, 342-366.

Kulås, E., & Ackman, R. G. (2001). Properties of α-, γ-, and δ-tocopherol in purified fish oil triacylglycerols. *Journal of the American Oil Chemists' Society, 78*, 361-367.

Lampi, A. M., Kataja, L., Kamal-Eldin, A., & Vieno, P. (1999). Antioxidant activities of α-and γ-tocopherols in the oxidation of rapeseed oil triacylglycerols. *Journal of the American Oil Chemists' Society, 76*, 749-755.

Larsson, K., Cavonius, L., Alminger, M., & Undeland, I. (2012). Oxidation of cod liver oil during gastrointestinal *in vitro* digestion. *Journal of agricultural and food chemistry*, *60*, 7556-7564.

Larsson, K., Tullberg, C., Alminger, M., Havenaar, R., & Undeland, I. (2016). Malondialdehyde and 4-hydroxy-2-hexenal are formed during dynamic gastrointestinal *in vitro* digestion of cod liver oils. *Food & function*, *7*, 3458-3467.

Let, M. B., Jacobsen, C., & Meyer, A. S. (2007). Lipid oxidation in milk, yoghurt, and salad dressing enriched with neat fish oil or pre-emulsified fish oil. *Journal of agricultural and food chemistry*, *55*, 7802-7809.

Li, Y., Hu, M., & McClements, D. J. (2011). Factors affecting lipase digestibility of emulsified lipids using an *in vitro* digestion model: Proposal for a standardised pH-stat method. *Food Chemistry*, *126*, 498-505.

Liu, Y., Cheng, Y., Li, J., Wang, Y., & Liu, Y. (2018). Epoxy stearic acid, an oxidative product derived from oleic acid, induces cytotoxicity, oxidative stress, and apoptosis in hepg2 cells. *Journal of agricultural and food chemistry*, *66*, 5237-5246.

Lorrain, B., Dangles, O., Loonis, M., Armand, M., & Dufour, C. (2012). Dietary iron-initiated lipid oxidation and its inhibition by polyphenols in gastric conditions. *Journal of agricultural and food chemistry*, *60*, 9074-9081.

Maestre, R., Douglass, J. D., Kodukula, S., Medina, I., & Storch, J. (2013). Alterations in the intestinal assimilation of oxidized PUFAs are ameliorated by a polyphenol-rich grape seed extract in an *in vitro* model and Caco-2 cells. *The Journal of nutrition*, *143*, 295-301.

Mäkinen, M. M., Kamal-Eldin, A., Lampi, A. M., & Hopia, A. (2000). Effects of α-and γ-tocopherols on formation of hydroperoxides and two decomposition products from methyl linoleate. *Journal of the American Oil Chemists' Society*, *77*, 801-806.

Marcuse, R. (1962). The effect of some amino acids on the oxidation of linoleic acid and its methyl ester. *Journal of the American Oil Chemists' Society, 39*, 97-103.

Marmesat, S., Velasco, J., & Dobarganes, M. C. (2008). Quantitative determination of epoxy acids, keto acids and hydroxy acids formed in fats and oils at frying temperatures. *Journal of Chromatography A, 1211*, 129-134.

Márquez-Ruiz, G., Garcia-Martinez, M. C., & Holgado, F. (2008). Changes and effects of dietary oxidized lipids in the gastrointestinal tract. *Lipid Insights*, *2*, LPI-S904.

Martínez-Yusta, A., & Guillén, M. D. (2019). Enrichment of sunflower oil with γ-tocopherol. Study by ^{1}H NMR of its effect under accelerated storage conditions. *European Journal of Lipid Science and Technology*, *121*, 1800457. DOI: 10.1002/ejlt.201800457.

Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). A review of thermo-oxidative degradation of food lipids studied by 1H NMR spectroscopy: influence of degradative conditions and food lipid nature. *Comprehensive Reviews in Food Science and Food Safety*, *13*, 838-859.

Morales, P., Ramírez-Moreno, E., de Cortes Sanchez-Mata, M., Carvalho, A. M., & Ferreira, I. C. (2012). Nutritional and antioxidant properties of pulp and seeds of two xoconostle cultivars (Opuntia joconostle FAC Weber ex Diguet and Opuntia matudae Scheinvar) of high consumption in Mexico. *Food Research International*, *46*, 279-285.

Neff, W. E., Warner, K., & Eller, F. (2003). Effect of γ-tocopherol on formation of nonvolatile lipid degradation products during frying of potato chips in triolein. *Journal of the American Oil Chemists' Society*, *80*, 801-806.

Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2017a). Behaviour of non-oxidized and oxidized flaxseed oils, as models of omega-3 rich lipids, during *in vitro* digestion. Occurrence of epoxidation reactions. *Food Research International*, *97*, 104-115.

Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2017c). Effect of the presence of protein on lipolysis and lipid oxidation occurring during *in vitro* digestion of highly unsaturated oils. *Food chemistry*, *235*, 21-33.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2015). Usefulness of 1H NMR in assessing the extent of lipid digestion. *Food chemistry*, *179*, 182-190.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). A method based on ^{1}H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures. *Food Research International*, *66*, 379-387.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016). A study by ^{1}H NMR on the influence of some factors affecting lipid *in vitro* digestion. *Food Chemistry*, *211*, 17-26.

Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2017b). ^{1}H NMR and SPME-GC/MS study of hydrolysis, oxidation and other reactions occurring during *in vitro* digestion of non-oxidized and oxidized sunflower oil. Formation of hydroxy-octadecadienoates. *Food Research International, 91*, 171-182.

Nieva-Echevarría, B; Goicoechea, E.; Guillén, M. D. (2018). The challenge of monitoring the hydrolysis of foods lipids during gastrointestinal digestion. *SDRP Journal of Food Science & Technology, 3,* 247-254.

Nik, A. M., Wright, A. J., & Corredig, M. (2010). Interfacial design of protein-stabilized emulsions for optimal delivery of nutrients. *Food & Function, 1*, 141-148.

Nogala-Kalucka, M., Korczak, J., Elmadfa, I., & Wagner, K. H. (2005). Effect of α-and δ-tocopherol on the oxidative stability of a mixed hydrogenated fat under frying conditions. *European Food Research and Technology*, *221*, 291-297.

Ohm, V. A., Stöckmann, H., & Schwarz, K. (2005). The more–The better? Estimating the inhibitory activity of alpha-tocopherol towards lipid oxidation. *Journal of plant physiology, 162,* 785-789.

Osborn-Barnes, H. T., & Akoh, C. C. (2003). Effects of α-tocopherol, β-carotene, and soy isoflavones on lipid oxidation of structured lipid-based emulsions. *Journal of agricultural and food chemistry, 51,* 6856-6860.

Park, E. Y., Murakami, H., & Matsumura, Y. (2005). Effects of the addition of amino acids and peptides on lipid oxidation in a powdery model system. *Journal of Agricultural and Food Chemistry, 53*, 8334–8341.

Phillips, K. M., Ruggio, D. M., Toivo, J. I., Swank, M. A., & Simpkins, A. H. (2002). Free and esterified sterol composition of edible oils and fats. *Journal of Food Composition and Analysis, 15,* 123–142.

Riisom, T., Sims, R. J., & Fioriti, J. A. (1980). Effect of amino acids on the autoxidation of safflower oil in emulsions. *Journal of the American Oil Chemists' Society, 57*, 354-359.

Rogalski, M., & Szterk, A. (2015). Oxidative stability of α-linolenic acid in corn chips enriched with linseed oil pro/antioxidative activity of tocopherol. *Journal of the American Oil Chemists' Society*, *92*, 1461-1471.

Sakihama, Y., Cohen, M. F., Grace, S. C., & Yamasaki, H. (2002). Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicology*, *177*, 67-80.

Sánchez-Muniz, F. J., Arroyo, R., Sánchez-Montero, J. M., & Cuesta, C. (2000). *In vitro* digestibility study of thermal oxidized palm oleins. *Food science and technology international*, *6*, 449-456.

Schaich, K. M. (2008). Co-oxidation of proteins by oxidizing lipids. *Lipid oxidation pathways*, *2*, 181-272.

Schaich, K. M. (2014). Lipid co-oxidation of proteins: One size does not fit all. *Inform*, *25*, 134-139.

Schaich, K. M., & Karel, M. (1975). Free radicals in lysozyme reacted with peroxidizing methyl linoleate. *Journal of Food Science*, *40*, 456-459.

Schaich, K. M., & Karel, M. (1976). Free radical reactions of peroxidizing lipids with amino acids and proteins: an ESR study. *Lipids*, *11*, 392-400.

Schaich, K. M., & Pryor, W. A. (1980). Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation—Part III: Free radical transfer from oxidizing lipids. *Critical Reviews in Food Science & Nutrition*, *13*, 189-244.

Schaich, K. M., Xie, J., & Bogusz, B. A. (2017). Thinking outside the classical chain reaction box of lipid oxidation: Evidence for alternate pathways and the importance of epoxides. *Lipid Technology*, *29*, 91-96.

Schneider, C. (2005). Chemistry and biology of vitamin E. *Molecular Nutrition & Food Research*, *49*, 7-30.

Seppanen, C. M., Song, Q., & Csallany, A. S. (2010). The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *Journal of the American Oil Chemists' Society*, *87*, 469-481.

Shimozu, Y., Hirano, K., Shibata, T., Shibata, N., & Uchida, K. (2011). 4-Hydroperoxy-2- nonenal is not just an intermediate but a reactive molecule that covalently modifies proteins to generate unique intramolecular oxidation products. *Journal of Biological Chemistry, 286*, 29313–29324.

Singh, P., Singh, T. P., & Gandhi, N. (2018). Prevention of lipid oxidation in muscle foods by milk proteins and peptides: A review. *Food Reviews International*, 34, 226-247.

Stadtman, E. R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annual review of biochemistry*, *62*, 797-821.

Steppeler, C., Haugen, J. E., Rødbotten, R., & Kirkhus, B. (2016). Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during *in vitro* digestion of cooked beef, pork, chicken, and salmon. *Journal of agricultural and food chemistry*, *64*, 487-496.

Tabee, E., Azadmard-Damirchi, S., Jägerstad, M., & Dutta, P. C. (2008). Effects of α-tocopherol on oxidative stability and phytosterol oxidation during heating in some regular and high-oleic vegetable oils. *Journal of the American Oil Chemists' Society*, *85*, 857-867.

Terao, J., & Matsushita, S. (1986). The peroxidizing effect of α-tocopherol on autoxidation of methyl linoleate in bulk phase. *Lipids*, *21*, 255-260.

Tirosh, O., Shpaizer, A., & Kanner, J. (2015). Lipid peroxidation in a stomach medium is affected by dietary oils (olive/fish) and antioxidants: the Mediterranean versus western diet. *Journal of agricultural and food chemistry*, 63, 7016-7023.

Uchida, K. (2015). Aldehyde adducts generated during lipid peroxidation modification of proteins. *Free radical research*, *49*, 896-904.

Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J. & De Smet, S. (2014). Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)- specific DNA adduct formation during *in vitro* digestion. *Journal of Agricultural and Food Chemistry* 62, 1980-1988.

Van Hecke, T., Vossen, E., Bussche, J. V., Raes, K., Vanhaecke, L., & De Smet, S. (2014). Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during *in vitro* digestion of meat. *PLoS One*, *9*, e101122.

Van Hecke, T., Wouters, A., Rombouts, C., Izzati, T., Berardo, A., Vossen, E., Claeys, E., Van Camp, J., Raes, K., Vanhaecke, L., Peeters, M., De Vos, W. H. & De Smet, S. (2016). Reducing compounds equivocally influence oxidation during digestion of a high-fat beef product, which promotes cytotoxicity in colorectal carcinoma cell lines. *Journal of agricultural and food chemistry*, *64*, 1600-1609.

Velasco, J., Marmesat, S., Bordeaux, O., Márquez-Ruiz, G., & Dobarganes, C. (2004). Formation and evolution of monoepoxy fatty acids in thermoxidized olive and sunflower oils and quantitation in used frying oils from restaurants and fried-food outlets. *Journal of agricultural and food chemistry*, *52*, 4438-4443.

Versantvoort, C. H., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J., & Sips, A. J. (2005). Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food and Chemical Toxicology*, *43*, 31-40.

Viljanen, K., Kylli, P., Hubbermann, E. M., Schwarz, K., & Heinonen, M. (2005). Anthocyanin antioxidant activity and partition behavior in whey protein emulsion. *Journal of agricultural and food chemistry*, *53*, 2022-2027.

Wagner, K. H., & Elmadfa, I. (2000). Effects of tocopherols and their mixtures on the oxidative stability of olive oil and linseed oil under heating. *European Journal of Lipid Science and Technology, 102*, 624-629.

Wagner, K. H., Isnardy, B., & Elmadfa, I. (2004). γ- and δ-tocopherols are more effective than α-tocopherol on the autoxidation of a 10% rapeseed oil triacylglycerol-in-water emulsion with and without a radical initiator. *European Journal of Lipid Science and Technology, 106*, 44-51.

Waraho, T., McClements, D. J., & Decker, E. A. (2011). Impact of free fatty acid concentration and structure on lipid oxidation in oil-in-water emulsions. *Food Chemistry, 129*, 854−859.

Warner, K., & Frankel, E. N. (1987). Effects of β-carotene on light stability of soybean oil. *Journal of the American Oil Chemists' Society*, *64*, 213-218.

Wilson, R., Fernie, C. E., Scrimgeour, C. M., Lyall, K., Smyth, L., & Riemersma, R. A. (2002). Dietary epoxy fatty acids are absorbed in healthy women. *European journal of clinical investigation*, *32*, 79-83.

Winkler-Moser, J. K., Logan, A., & Bakota, E. L. (2014). Antioxidant activities and interactions of α-and γ-tocopherols within canola and soybean oil emulsions. *European journal of lipid science and technology*, *116*, 606-617.

Wolf, G. (2006). How an increased intake of alpha-tocopherol can suppress the bioavailability of gamma-tocopherol. *Nutrition reviews*, *64*, 295-299.

Yee, J. J., & Shipe, W. F. (1982). Effects of sulfhydryl compounds on lipid oxidations catalyzed by copper and heme.*Journal of Dairy Science*, *65*, 1414-1420)

Yoshida, Y., & Niki, E. (2003). Antioxidant effects of phytosterol and its components. *Journal of nutritional science and vitaminology*, *49*, 277-280.

Zamora, R., & Hidalgo, F. J. (1994). Modification of lysine amino groups by the lipid peroxidation product 4,5(E)-Epoxy-2(E)-heptenal. *Lipids, 29*, 243–249.

Zamora, R., & Hidalgo, F. J. (2005). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition*, *45*, 49-59.

Zhu, X., Ye, A., Verrier, T., & Singh, H. (2013). Free fatty acid profiles of emulsified lipids during *in vitro* digestion with pancreatic lipase. *Food chemistry*, *139*, 398-404.

Zuta, P. C., Simpson, B. K., Zhao, X., & Leclerc, L. (2007). The effect of α-tocopherol on the oxidation of mackerel oil. *Food Chemistry*, *100*, 800-807.

OTHER CONTRIBUTIONS

Some of the results derived from the pre-doctoral research period gave rise to other contributions exposed in several national and international scientific congresses.

XIII Euro Feed Lipid Congress. (Florence, Italy) 27/09/2015 - 30/09/2015

Poster contribution

Contribution I: María L. Ibargoitia, Giovanna Cristillo, Ana San Martin, Patricia Sopelana and María D. Guillén

Simultaneous monitoring of the degradation of minor oil components able to exhibit antioxidant ability and of the formation of secondary oxidation products during accelerated storage of refined soybean oil

XVIII Euro Food Chem. (Madrid, Spain) 13/10/2015 - 16/10/2015

Poster contribution

Contribution II: A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén

Influence of High Concentrations of Phytosterols on ohe Evolution of Refined Soybean Oil under Accelerated Storage Conditions

VIII Congreso Español de Ingeniería de Alimentos (CESIA). (badajoz, Spain) 7/04/2015 - 10/04/2015

Poster contribution

Contribution III: A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén

La Adición de α -Tocoferol a Aceite de Soja Refinado Puede Acelerar su Oxidación.

VI Congreso Internacional de Autocontrol y Seguridad Alimentaria (KAUSAL). (Vitoria, Spain) 25/05/2016 - 27/05/2016

Three Poster contributions

Contribution IV: A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén

Controversia en relación al contenido de aceite de soja virgen y refinado en componentes beneficiosos para la salud.

Contribution V: A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén

Efecto de α-tocoferol en alta concentración en la evolución de aceite de soja durante la digestión *in vitro*

Contribution VI: M.D. Guillén, P. Sopelana, A. S Martín, M.L. Ibargoitia.

Los ésteres de esteroles incorporados a ciertos alimentos como compuestos saludables se oxidan bajo condiciones oxidativas igual que el resto de lípidos dando lugar a sustancias tóxicas.

V Infogest. (Rennes, France) 04/04/2017 – 06/04/2017

Two poster contributions

Contribution VII: A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén.

Advance of oxidation during *in vitro* digestion of edible oils of different unsaturation degree containing hydroperoxides. A study by SPME-GC/MS and by ^{1}H NMR.

Contribution VIII: A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén.

Study by 1H Nuclear Magnetic Resonance of the evolution of refined soybean oil with different oxidation status under *in vitro* digestion conditions.

IX CESIA. (Madrid, Spain) 16/05/2017 – 18/05/2017

Poster contribution

Contribution IX: A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén

Efecto del enriquecimiento de aceite de soja con γ-tocoferol sobre su proceso de oxidación bajo condiciones de almacenaje acelerado. Comparación con α-tocoferol.

Second International Symposium on Lipid Oxidation and Antioxidants (Euro Fed Lipid). (Graz, Austria) 04/06/2018 - 06/06/2018

Poster contribution

Contribution X: A. S Martín, P. Sopelana, M.D. Guillén

Antioxidant effect of lysine on soybean oil evidenced by 1H Nuclear Magnetic Resonance

X CESIA. (Leon, España) 15/05/2018 - 17/05/2019

Poster contribution

Contribution XI: A. S Martín, P. Sopelana, M.D. Guillén

La adición de L-cisteina permite alargar la vida útil del aceite de soja

CONTRIBUTION I

Simultaneous monitoring of the degradation of minor oil components able to exhibit antioxidant ability and of the formation of secondary oxidation products during accelerated storage of refined soybean oil

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Vegetable oils undergo oxidation when they are submitted to degradative conditions. The oxidation of edible oils has received much attention due to its influence on oil nutritional and sensory quality, and its economical and technological repercussions on the food industry. A great variety of methodologies have been developed and implemented to evaluate the extent of the oxidative deterioration in vegetable oils, and in foods, in general. Some of these are directed to the determination of certain parameters, requiring a different method for each parameter, involving the use of reagents and the chemical modification of the sample. In the last time, it has been described a methodology, based on ^{1}H NMR spectroscopy, able to provide simultaneously information on the degradation of main oil components, and on the formation of some new components; this methodology require the use of a solvent although the chemical modification of the sample is not required (1). In this communication the capability of a new methodology to provide simultaneously information, on the degradation rate of those oil components able to exhibit antioxidant ability and on the formation of new compounds derived from these and from oil triglycerides is shown. This methodology uses Solid Phase Microextraction and Gas Chromatography-Mass Spectrometry and does not require solvents. In this communication the oxidation of refined soybean oil submitted to accelerated storage is analyzed by using the above mentioned method.

REFERENCES

1. A. Martinez-Yusta, E. Goicoechea, M.D. Guillén. A Review of thermo-oxidative degradation of food lipids studied by ^{1}H NMR spectroscopy: influence of degradative conditions and food lipid nature. *Compr Rev Food Sci F*, 13, 838-859, 2015.

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Simultaneous monitoring of the degradation of minor oil components able to exhibit antioxidant ability and of the formation of secondary oxidation products during accelerated storage of refined soybean oil

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INTRODUCTION

Due to the great influence of the oxidation of edible oils on their nutritional and sensory quality, a great variety of methodologies have been developed to evaluate the extent of their oxidative deterioration. Some of these are aimed at the determination of certain parameters, which requires different methods for each one of them; these usually involve the chemical modification of the sample and the use of reagents. This has motivated the search for simpler methods, with minimum reagents consumption, without sample modification, and able to provide a more global view of the oxidation process. Although some approaches have already been developed based on ^{1}H Nuclear Magnetic Resonance spectroscopy [1], methods based on Gas Chromatography/Mass Spectrometry (GC/MS) would also be very valuable due to the specificity and sensitivity of this technique. For this reason, in this work, a methodology that does not require either the modification of the sample or the use of solvents, based on Solid Phase Microextraction and GC/MS (SPME-GC/MS), has been used.

OBJECTIVE

To assess the capability of a methodology based on Direct Immersion SPME-GC/MS to provide simultaneously information on the degradation rate of those oil components able to exhibit antioxidant ability and on the formation of new compounds derived from these and from oil triglycerides, throughout the accelerated storage of refined soybean oil.

SAMPLES AND METHODOLOGY

SAMPLES: Refined soybean oil (RSO), rich in polyunsaturated acyl groups.

ACCELERATED STORAGE CONDITIONS: Heating at 70 °C in an oven with aeration for 11 days.

STUDY OF THE SAMPLES: Both the samples taken before the heating process and those taken daily throughout the accelerated storage process were subjected to SPME by Direct Immersion (DI-SPME) of a fiber of 65 µm PDMS/DVB into the oil, followed by GC/MS analysis.

RESULTS AND DISCUSSION

Evolution of RSO throughout the accelerated storage process

Figure 1 shows the chromatograms of RSO recorded before heating (day 0) and at different stages of the accelerated storage process. It can be clearly observed that the methodology based on DI-SPME-GC/MS enables one to detect simultaneously different types of oil minor components, such as squalene, tocopherols and vegetable sterols. It is also possible to follow their evolution throughout the oxidative process; this reveals that the degradation rate throughout heating differs from some components to others. At the same time, the formation of triglycerides oxidation products can also be monitored and related to the evolution of those oil minor components able to exhibit antioxidant ability.

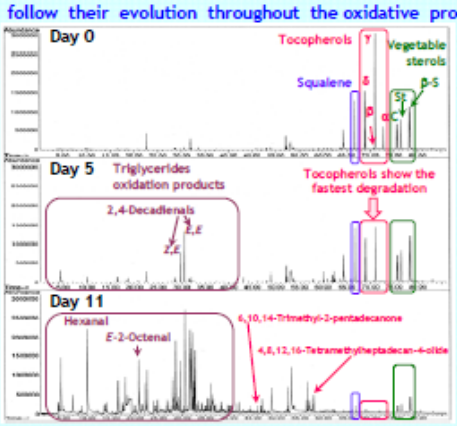

Figure 1: Chromatograms of RSO at different stages of the accelerated storage process. β-S: β-sitosterol; C: campesterol; St: stigmasterol.

Evolution of the abundance of tocopherols and some of their oxidation products

Figure 2 shows the evolution of tocopherols, considered essential to stabilize unsaturated acyl groups against oxidative deterioration [2], throughout the heating time. As this figure reveals, the most abundant one in the studied soybean oil is the γ-isomer, followed by δ-, α- and β-isomers. All tocopherols show similar degradative evolutions, starting to decrease between the 2nd and the 3rd day, when oil triglycerides oxidation starts, and disappearing almost completely after 8 days of heating. The γ-isomer exhibits the fastest degradation rate.

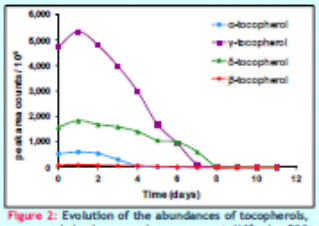

Figure 2: Evolution of the abundances of tocopherols, expressed in base peak area counts/10^6, in RSO throughout the accelerated storage process.

As Figure 3 shows, this methodology also enables to monitor the formation of some α-tocopherol oxidation products [3]. Their kinetic of formation agrees with that of α-tocopherol degradation.

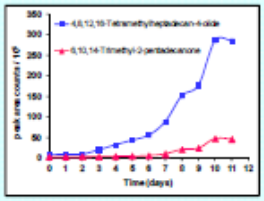

Figure 3: Formation and evolution of some α-tocopherol oxidation products in RSO throughout the accelerated storage process.

CONCLUSION

The use of SPME fibers in combination with GC/MS constitutes a powerful technique that provides a great deal of information regarding the degradation of minor oil components and the formation of oxidation products. All this is possible in a single analysis and without either the use of solvents or sample modification.

REFERENCES
[1] Martínez-Yusta, A.; Goicoechea, E.; Guillén, M.D. (2015). Comprehensive Reviews in Food Science and Food Safety 13: 838-859.
[2] Kamal-Eldin, A.; Appelqvist , L.-A. (1996). Lipids 31: 671-701.
[3] Rontani, J.F.; Nassiry, M.; Mouzdahir, A. (2007). Organic Geochemistry 38: 37-47.

ACKNOWLEDGEMENTS
This work has been supported by the Spanish Ministry of Economy and Competitiveness (MINECO, AGL2012-36466), the Basque Government (EJ-GV, GIC10/85-IT-463-10) and the UPV/EHU (UFI-11/21). S Martin, A. thanks the MINECO the granting of a predoctoral grant-recruit.

CONTRIBUTION II

INFLUENCE OF HIGH CONCENTRATIONS OF PHYTOSTEROLS ON THE EVOLUTION OF REFINED SOYBEAN OIL UNDER ACCELERATED STORAGE CONDITIONS

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Keywords: refined soybean oil, phytosterols, antioxidant ability, ^{1}H NMR

Different types of phytosterols, either in mixtures or individually, have been added to various vegetable oils in order to increase their thermal stability and reduce polymerization at high temperatures, even though results vary among phytosterols (1). However, very little has been studied in relation to the effect of phytosterols on the oxidative stability of oils at lower temperatures. Concerning this latter issue, some authors have taken for granted a lack of antioxidant activity of phytosterols at low temperatures, apparently without any explanation for it (2). In contrast, an antioxidant effect of some of these compounds at 37 ºC has been reported by other researchers (3).

The objective of this research was to study whether phytosterols added to refined soybean oil in higher amounts than those usually present in this type of oil could exert an effect on its evolution under accelerated storage conditions. For this purpose, refined soybean oil (RSO) and the same oil enriched with phytosterols (5% in weight) were subjected to heating at 70 ºC in an oven with aeration for a long period of time. The evolution of the oil was monitored by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and both acyl groups degradation and formation of different kinds of oxidation products were monitored throughout time.

The results obtained reveal that the presence of a high concentration of phytosterols in RSO modifies slightly the oxidation process of the RSO oil. On the one hand, a small reduction in the degradation rate of linoleic groups, the most abundant in RSO, after 6 days under accelerated storage conditions, was observed. On the other hand, a slight delay in the formation of secondary oxidation products was also noticed. Although primary oxidation products were detected at the same time in the RSO with and without added phytosterols, their evolution as well as the formation of aldehydes was found to be slower in the enriched RSO. Therefore, it can be said that the presence of high amounts of phytosterols slows down slightly the oxidative degradation of RSO once this has begun.

References

(1) Winkler JK, Warner K. (2008) Effect of phytosterol structure on thermal polymerization of heated soybean oil. Eur J Lipid Sci Technol 110, 1068–1077.

(2) Gertz C, Klostermann S, Kochhar P. (2000) Testing and comparing oxidative stability of vegetable oils and fats at frying temperature. Eur J Lipid Sci Technol 102, 543–551.

(3) Yoshida Y, Niki, E. (2003) Antioxidant effects of phytosterol and its components. J Nutr Sci Vitaminol, 49, 277-280.

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INFLUENCE OF HIGH CONCENTRATIONS OF PHYTOSTEROLS ON THE EVOLUTION OF REFINED SOYBEAN OIL UNDER ACCELERATED STORAGE CONDITIONS

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INTRODUCTION

Different types of phytosterols, either in mixtures or individually, have been added to various vegetable oils in order to increase their thermal stability and reduce polymerization at frying temperatures, even though results vary among phytosterols [1]. However, very little has been studied in relation to the effect of phytosterols on the oxidative stability of oils at lower temperatures. Concerning this latter issue, some authors have taken for granted a lack of antioxidant activity of phytosterols at low temperatures, apparently without any explanation for it [2]. In contrast, an antioxidant effect of some of these compounds at 37 ºC has been reported by other researchers [3].

OBJECTIVE

Studying whether phytosterols added to refined soybean oil in higher amounts than those usually present in this type of oil could exert an effect on its evolution under accelerated storage period (70 ºC).

MATERIALS AND METHOD

SAMPLES: Refined soybean oil (RSO), and the same oil enriched in phytosterols (5% in weight) (PE-RSO).

ACCELERATED STORAGE CONDITIONS: Heating at 70 ºC in an oven with aeration for a period of 12 days. The accelerated storage process was performed in duplicate for each sample.

STUDY OF THE SAMPLES: Aliquots taken from the original oils and periodically throughout the accelerated storage period were studied by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR). Both acyl groups evolution and formation of different kinds of oxidation products were monitored throughout time, as in previous studies [4].

RESULTS AND DISCUSSION

Figure 1 shows the MOLAR PERCENTAGE EVOLUTION OF LINOLEIC AND LINOLENIC ACYL GROUPS, both of them polyunsaturated groups, in RSO and in PE-RSO. It can be observed that, from the 6th day onwards, the degradation rate of linoleic groups, the most abundant ones in this type of oil, is slightly lower in PE-RSO, the phytosterols enriched sample, than in RSO. A similar tendency can be noticed in the evolution of linolenic groups.

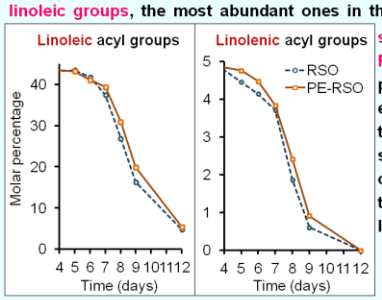

Figure 1: Molar percentage evolution of linoleic and linolenic acyl groups in RSO and PE-RSO throughout the accelerated conditions storage period.

Concerning the FORMATION AND EVOLUTION OF OXIDATION PRODUCTS, Figure 2 shows the generation and evolution of hydroperoxides, primary oxidation products, throughout the accelerated conditions storage period.

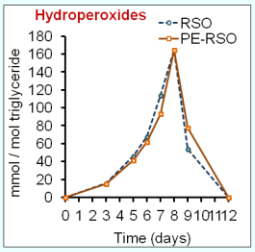

Figure 2: Generation and evolution of hydroperoxides in RSO and PE-RSO throughout the accelerated conditions storage period.

Hydroperoxides were detected at the same time in RSO and in PE-RSO. However, from 6th day onwards, their evolution was somewhat slower in PE-RSO. This means that both the increase of their concentration and their decomposition to give rise to other oxidation products were found to be slightly slower in the phytosterols enriched oil.

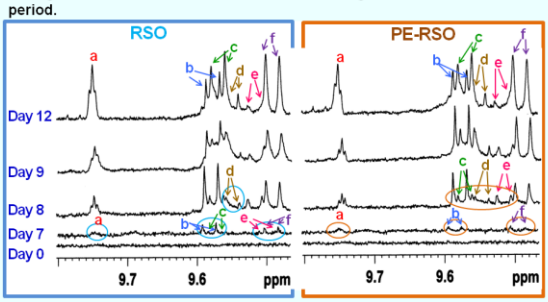

Figure 3: Evolution of the aldehydic proton signals throughout the accelerated conditions storage period in RSO and PE-RSO. The aldehydes identified were: n-alkanals (a), 4-hydroperoxy-(*E*)-2-alkenals (b), 4-hydroxy-(*E*)-2-alkenals (c), 4,5-epoxy-alkenals (d), (*E,E*)-2,4-alkadienals (e) and (*E*)-2-alkenals (f).

Primary oxidation products evolve to form secondary oxidation products. Among these latter, aldehydes deserve special attention since some of them can have toxic effects [5]. The evolution throughout heating of the ^{1}H NMR spectral signals of aldehydes in RSO and in PE-RSO can be observed in Figure 3. This figure reveals the presence of aldehydes after 7 days of heating in both oils. However, whereas in RSO most of the types of aldehydes were already detectable at this stage of the accelerated storage period, in PE-RSO only n-alkanals (a), 4-hydroperoxy-(*E*)-2-alkenals (b) and (*E*)-2-alkenals (f) were detected. Moreover, the concentrations of these latter were somewhat lower in PE-RSO than in RSO. These findings seem to be in agreement with the evolution of both acyl groups and hydroperoxides.

CONCLUSIONS

- The enrichment of RSO with phytosterols at 5% level gives rise to a small reduction in the degradation rate of polyunsaturated groups throughout the oxidation process underwent at 70 ºC with aeration.
- Although primary oxidation products were detected at the same time in both oils, their evolution, as well as the formation of secondary oxidation products like aldehydes, was found to be slightly slower in the phytosterols enriched oil.
- The results obtained reveal that the addition of a 5% of phytosterols to RSO seems to retard slightly the oxidation process of RSO once this has begun.

REFERENCES
[1] Winkler JK, Warner K. (2008) Eur J Lipid Sci Technol 110, 1068–1077.
[2] Gertz C, Klostermann S, Kochhar P. (2000) Eur J Lipid Sci Technol 102, 543–551.
[3] Yoshida Y, Niki E. (2003) J Nutr Sci Vitaminol 49, 277-280.
[4] Guillén MD, Uriarte PS. (2012) Food Chem 134,162-172.
[5] Guillén MD, Goicoechea E. (2008) Crit Rev Food Sci 48, 119-136.

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CONTRIBUTION III

LA ADICIÓN DE α-TOCOFEROL A ACEITE DE SOJA REFINADO PUEDE ACELERAR SU OXIDACION

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Resumen – En este trabajo se estudia el efecto de la adición de diferentes concentraciones de α-tocoferol (α-T) sobre la estabilidad oxidativa de aceite de soja refinado mantenido en condiciones de almacenaje acelerado. El objetivo es analizar si el α-T actúa como anti- o como pro-oxidante en las concentraciones utilizadas, y contribuir al conocimiento del mecanismo de acción de este compuesto fenólico. El seguimiento de la evolución del aceite se llevó a cabo mediante Resonancia Magnética Nuclear de Protón (RMN de ^{1}H). Los resultados obtenidos ponen de manifiesto que el α-T actúa como pro-oxidante en las concentraciones ensayadas.

Palabras clave – aceite de soja refinado, actividad prooxidante, alfa-tocoferol, estabilidad oxidativa, RMN

I. INTRODUCCIÓN

El α-tocoferol (α-T) puede actuar como antioxidante; de hecho su adición a aceites comestibles y otros alimentos está permitida en la Unión Europea sin indicación de límite máximo (principio *quantum satis*). Sin embargo, algunos autores han señalado que el α-T, dependiendo de distintos factores entre los que se ha citado la concentracion, puede comportarse también como pro-oxidante [1,2]. En un intento de contribuir al conocimiento de la actuación de este compuesto bajo condiciones oxidativas, en este trabajo se ha estudiado el efecto de la adición de altas concentraciones de α-T sobre la estabilidad oxidativa de aceite de soja refinado mantenido bajo condiciones de almacenaje acelerado. El estudio se ha llevado a cabo mediante RMN de ^{1}H ya que proporciona información difícil, si no imposible, de obtener por otros métodos.

II. MATERIALES Y MÉTODOS

Las muestras objeto de estudio fueron aceite de soja refinado y el mismo aceite de soja enriquecido con dos concentraciones diferentes de α-T: 0,2 y 2% en peso.

Todas las muestras se mantuvieron bajo condiciones de almacenaje acelerado a 70 ºC en una estufa con aireación durante 13 días. Con el fin de seguir su evolución, se tomaron alícuotas a lo largo del almacenaje a intervalos regulares de tiempo y su evolución fue estudiada mediante RMN de ^{1}H, del mismo modo que en estudios previos [3,4].

III. RESULTADOS Y DISCUSIÓN

La adición de α-T en las concentraciones estudiadas acelera la degradación de los grupos acilo del aceite, así como la formación de compuestos primarios de oxidación, siendo esta aceleración mayor cuanto mayor es la cantidad de α-T añadido. Es de destacar que la naturaleza de los compuestos primarios de oxidación derivados del aceite está claramente afectada por la adición de α-T. También se acelera la formación de compuestos secundarios de oxidación. Los resultados obtenidos sugieren que el proceso de oxidación transcurre por un mecanismo diferente en el aceite con y sin α-T añadido.

IV. CONCLUSIÓN

La adición de α-T en concentraciones del 0,2 y del 2% a aceite de soja refinado reduce la estabilidad oxidativa de éste poniendo de manifiesto que el α-T actúa como pro-oxidante.

Es evidente que aunque la legislación no limita la cantidad a la que el α-T se puede añadir a los alimentos, la adición al aceite de soja en las cantidades ensayadas es perjudicial, ya que reduce su vida útil, acelerando la formación de compuestos tóxicos.

AGRADECIMIENTOS

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REFERENCES

[1] Kamal-Eldin, A. & L.-A. Appelqvist (1996). Lipids 31: 671-701.
[2] Fuster, M. D. et al. (1998). Lipids 33: 715-722.
[3] Guillén, M. D. & A. Ruiz (2003). Eur. J. Lipid Sci. Technol. 106: 680-687.
[4] Guillén, M. D. & P.S. Uriarte (2012). Food Chem. 134:162-172

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LA ADICIÓN DE α-TOCOFEROL A ACEITE DE SOJA REFINADO PUEDE ACELERAR SU OXIDACIÓN

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INTRODUCCIÓN

El α-tocoferol (α-T) puede actuar como antioxidante; de hecho, su adición a aceites comestibles y otros alimentos está permitida en la Unión Europea sin indicación de límite máximo (principio *quantum satis*). Sin embargo, algunos autores han señalado que el α-T, dependiendo de distintos factores entre los que se ha citado la concentracion, puede comportarse también como pro-oxidante [1,2].

OBJETIVO

Estudio mediante RMN de ^{1}H del efecto de la adición de altas concentraciones de α-T sobre la estabilidad oxidativa de aceite de soja refinado mantenido bajo condiciones de almacenamiento acelerado (70 ºC).

MATERIALES Y MÉTODOS

MUESTRAS: Aceite de soja refinado (ASR), rico en grupos acilo poliinsaturados y el mismo aceite de soja enriquecido con dos concentraciones diferentes de α-T: 0,2 y 2% en peso.

CONDICIONES DE ALMACENAMIENTO ACELERADO: Calentamiento a 70 °C en una estufa con aireación durante un periodo de 13 días.

ESTUDIO DE LAS MUESTRAS: Toma de alícuotas a lo largo del almacenamiento acelerado a intervalos regulares de tiempo para su estudio mediante RMN de ^{1}H, del mismo modo que en estudios previos [3,4].

RESULTADOS Y DISCUSIÓN

En la Figura 1 se muestra la *evolución de los porcentajes molares* de los *grupos acilo poliinsaturados*, tanto en el ASR sin α-T añadido como en los aceites enriquecidos, a lo largo del período de almacenamiento acelerado. En ella se puede observar una degradación de los grupos linolénico y linoleico con el tiempo de calentamiento, la cual es más rápida cuanto mayor es la cantidad de α-T añadido.

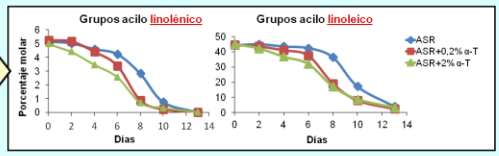

Figura 1: Evolución de los porcentajes molares de los grupos acilo poliinsaturados a lo largo del período de almacenaje acelerado en los 3 aceites estudiados.

En la Figura 2 se muestra la *evolución* a lo largo del calentamiento de la señales en espectro de ^{1}H RMN de los protones de *dienos conjugados* asociados a hidroperóxidos (*compuestos primarios de la oxidación*).

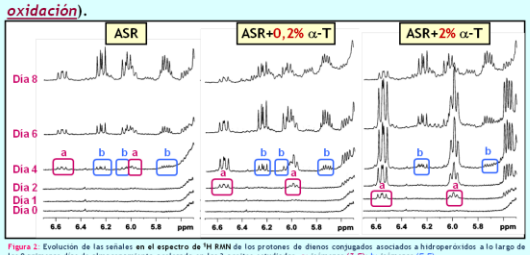

Figura 2: Evolución de las señales en el espectro de ^{1}H RMN de los protones de dienos conjugados asociados a hidroperóxidos a lo largo de los 8 primeros días de almacenamiento acelerado en los 3 aceites estudiados. a: isómeros (*Z,E*); b: isómeros (*E,E*).

Esta figura pone de manifiesto que:

1º) En concordancia con lo observado en la evolución de los grupos acilo, la adición de α-T acelera la formación de compuestos primarios de la oxidación, siendo éstos detectables al cabo de 1 y 2 días de calentamiento en los aceites enriquecidos con un 2% y un 0,2% de α-T, respectivamente, y al cabo de 4 días en el aceite sin enriquecer.

2º) La naturaleza de los compuestos primarios de oxidación está claramente afectada por la adición de α-T. Mientras que en el aceite sin enriquecer (ASR) los isómeros (*Z,E*) (a) y (*E,E*) (b) se detectan al mismo tiempo y en proporciones similares, en los aceites enriquecidos con α-T, los (*Z,E*) se generan en primer lugar y en más concentración que los (*E,E*). Este efecto es más pronunciado cuando la concentración de α-T es más alta.

En la Figura 3 se muestra la *evolución* de las señales de protones *aldehídicos* (*compuestos secundarios de la oxidación*) a lo largo del calentamiento.

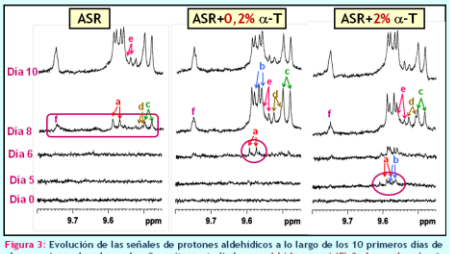

Figura 3: Evolución de las señales de protones aldehídicos a lo largo de los 10 primeros días de almacenaje acelerado en los 3 aceites estudiados. a: 4-hidroperoxi-(*E*)-2-alquenales; b: 4-hidroxi-(*E*)-2-alquenales; c: (*E*)-2-alquenales; d: (*E,E*)-2,4-alcadienales; e: 4,5-epoxi-(*E*)-2-alquenales; f: n-alcanales.

Se puede observar que, de acuerdo con todo lo señalado anteriormente, también la generación de aldehídos se acelera en los aceites enriquecidos con α-T, sobre todo la de algunos aldehídos oxigenados α,β-insaturados (a, b). Esto reviste especial importancia ya que, entre estos últimos, se encuentran algunos potencialmente tóxicos como los 4-hidroperoxi- (a) y 4-hidroxi-(*E*)-2-alquenales (b). Esta aceleración es mayor cuanto más alta es la cantidad de α-T añadida.

CONCLUSIONES

- La adición de α-T en las concentraciones estudiadas acelera la degradación de los grupos acilo del aceite, así como la formación de compuestos primarios y secundarios de oxidación, siendo esta aceleración mayor cuanto mayor es la cantidad de α-T añadido.
- La adición de α-T en concentraciones del 0,2 y del 2% a aceite de soja refinado reduce la estabilidad oxidativa de éste poniendo de manifiesto que el α-T actúa como pro-oxidante.
- Los resultados obtenidos sugieren que el proceso de oxidación transcurre por un mecanismo algo diferente en el aceite con y sin α-T añadido.
- Aunque la legislación no limita la cantidad a la que el α-T se puede añadir a los alimentos, la adición al aceite de soja en las cantidades ensayadas es perjudicial, ya que reduce su vida útil, acelerando la formación de compuestos tóxicos.

REFERENCIAS
[1] Kamal-Eldin, A. & L.-A. Appelqvist (1996). Lipids 31: 671-701.
[2] Fuster, M. D. et al. (1998). Lipids 33: 715-722.
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CONTRIBUTION IV

CONTROVERSIA EN RELACIÓN AL CONTENIDO DE ACEITE DE SOJA VIRGEN Y REFINADO EN COMPONENTES BENEFICIOSOS PARA LA SALUD

A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén

INTRODUCCION

Los aceites vegetales de semillas normalmente se someten a un proceso de refinado antes de ser destinados a consumo humano. Sin embargo, algunos de ellos como el de soja, también pueden ser consumidos sin refinar. Diversos estudios han puesto de manifiesto que el proceso de refinado provoca la pérdida de componentes minoritarios, algunos de los cuales tienen capacidad antioxidante, como tocoferoles y esteroles vegetales, a los que se atribuye efectos saludables en el organismo humano. Sin embargo, en los últimos tiempos se están buscando alternativas para que el impacto del refinado en los componentes beneficiosos del aceite sea el mínimo posible.

OBJETIVO

Comparar el contenido de algunos componentes minoritarios de interés en aceite de soja virgen y aceite de soja refinado.

MATERIALES Y MÉTODOS

Las muestras estudiadas fueron aceite de soja virgen y refinado. El estudio se llevó a cabo mediante Microextracción en Fase Sólida seguida de Cromatografía de Gases/Espectrometría de Masas.

RESULTADOS

Contrariamente a lo que cabría esperar teniendo en cuenta la denominación de los aceites estudiados y las características que normalmente se atribuyen a los aceites vírgenes y refinados, el aceite virgen presenta un contenido menor de tocoles, mayoritariamente tocoferoles, así como de esteroles vegetales, que el refinado. Sin embargo, en el aceite virgen las concentraciones de ácidos grasos libres son más altas que en el refinado. En ambos tipos de aceite se han detectado compuestos derivados de reacciones de oxidación, si bien éstos se encuentran en concentraciones más altas en el virgen.

CONCLUSIONES

*En el caso de los aceites de soja estudiados, el aceite virgen es más pobre que el refinado en ciertos componentes considerados beneficiosos.

*La composición observada podría sugerir que tal vez el etiquetado de los aceites estudiados no fuera correcto. Sin embargo, la presencia de una concentración más elevada de ácidos grasos libres en el aceite virgen es una característica de este tipo de aceites.

*La calidad de algunos aceites refinados de soja presentes en el mercado puede ser comparable o incluso superior a la de algunos vírgenes.

AGRADECIMIENTOS

Este trabajo ha sido financiado por el Ministerio de Economía y Competitividad (MINECO AGL2015-65450-R) y por la UPV/EHU (UFI-11/21). Ana San Martín agradece al MINECO el contrato predoctoral.

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CONTROVERSIA EN RELACIÓN AL CONTENIDO DE ACEITE DE SOJA VIRGEN Y REFINADO EN COMPONENTES BENEFICIOSOS PARA LA SALUD

S Martín, A., Ibargoitia, M.L., Sopelana, P., Guillén, M.D.*
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INTRODUCCIÓN

Los aceites vegetales de semillas normalmente se someten a un proceso de refinado antes de ser destinados a consumo humano. Sin embargo, algunos de ellos como el de soja, también pueden ser consumidos sin refinar. Diversos estudios han puesto de manifiesto que el proceso de refinado provoca la pérdida de componentes minoritarios [1], algunos de los cuales tienen capacidad antioxidante, como tocoferoles y esteroles vegetales, a los que se atribuye efectos saludables en el organismo humano. Sin embargo, en los últimos tiempos se están buscando alternativas para que el impacto del refinado en los componentes beneficiosos del aceite sea el mínimo posible.

OBJETIVO

Comparar el contenido de algunos componentes minoritarios de interés en aceite de soja virgen y aceite de soja refinado.

MATERIALES Y MÉTODOS

MUESTRAS: Aceite de soja refinado (ASR) y dos aceites de soja virgen (ASV1 y ASV2).
ESTUDIO DE LAS MUESTRAS: Mediante Microextracción en Fase Sólida seguida de Cromatografía de Gases/Espectrometría de Masas.

RESULTADOS Y DISCUSIÓN

La Figura 1 muestra el contenido de TOCOLES en los dos tipos de aceite de soja. Contrariamente a lo que cabría esperar teniendo en cuenta la denominación de los aceites estudiados y las características que normalmente se atribuyen a los aceites vírgenes y refinados, el aceite virgen presenta concentraciones más bajas de este grupo de componentes, constituido mayoritariamente por tocoferoles. No obstante, el perfil es el mismo en ambos casos, siendo el γ-tocoferol el tocol más abundante.

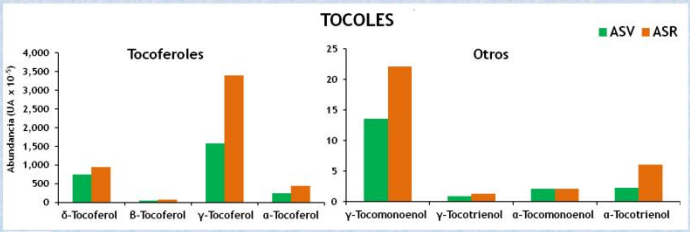

Figura 1: Contenido en tocoles de ASR y ASV (medias de ASV1 y ASV2), expresado en unidades arbitrarias de área (UA) x 10^{-5}.

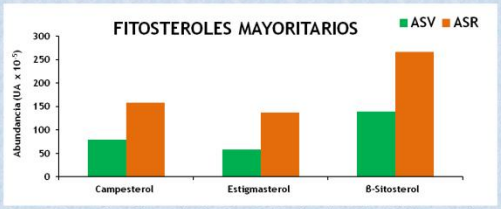

Figura 2: Contenido en fitosteroles de ASR y ASV (medias de ASV1 y ASV2), expresado en unidades arbitrarias de área (UA) x 10^{-5}.

En la Figura 2 se representa el contenido de FITOSTEROLES MAYORITARIOS de estos dos aceites. Aun cuando algunos autores indican que el proceso de refinado provoca una disminución en la concentración de fitosteroles [1], en el caso de los aceites de soja estudiados, el aceite refinado es más rico que el virgen en este tipo de componentes considerados bioactivos.

La Figura 3 muestra el contenido de los aceites estudiados en ÁCIDOS GRASOS LIBRES, concretamente ácido palmítico y ácidos grasos insaturados (oleico, linoleico y linolénico). En el aceite virgen las concentraciones de ácidos grasos libres son más altas que en el refinado, como era de esperar.

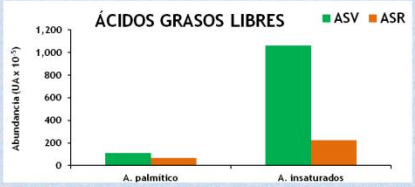

Figura 3: Contenido en ácidos grasos libres de ASR y ASV (medias de ASV1 y ASV2) expresado en unidades arbitrarias de área (UA) x 10^{-5}.

CONCLUSIONES

- En el caso de los aceites de soja estudiados, el aceite virgen es más pobre que el refinado en ciertos componentes considerados beneficiosos.
- La composición observada podría sugerir que tal vez el etiquetado de los aceites estudiados no fuera correcto. Sin embargo, la presencia de una concentración más elevada de ácidos grasos libres en el aceite virgen es una característica de este tipo de aceites.
- La calidad de algunos aceites refinados de soja presentes en el mercado puede ser comparable o incluso superior a la de algunos vírgenes.

REFERENCIAS
[1] Chen, B., McClements, D. J., Decker, E.A. (2011). Crit . Rev. Food Sci. Nutr., 51, 901-916.

AGRADECIMIENTOS
Este trabajo ha sido financiado por el Ministerio de Economía y Competitividad (MINECO AGL2015-65450-R), por el Departamento de Agricultura, Pesca y Alimentación del EJ-GV (PA 16/02) y por la UPV/EHU (UFI-11/21). S Martín A. agradece al MINECO la concesión de una beca-contrato predoctoral.

CONTRIBUTION V

EFECTO DE α-TOCOFEROL EN ALTA CONCENTRACIÓN EN LA EVOLUCIÓN DE ACEITE DE SOJA DURANTE LA DIGESTIÓN *IN VITRO*

A. S Martín, M.L. Ibargoitia, P. Sopelana, M.D. Guillén

INTRODUCCIÓN

Actualmente existe en la industria alimentaria una tendencia a enriquecer alimentos con compuestos con capacidad antioxidante con el fin de aumentar su estabilidad oxidativa, y a su vez, también buscando un efecto beneficioso para la salud. En este contexto, cabe destacar el α-tocoferol, bien conocido por su capacidad antioxidante y por su actividad como vitamina E. Sin embargo, existen evidencias científicas de que, en contra de lo esperado, este compuesto puede ejercer un efecto prooxidante en altas concentraciones. Por otra parte, también existen estudios que muestran que la digestión *in vitro* puede promover la oxidación de lípidos alimentarios. Teniendo esto en cuenta, cabe pensar que ese mismo efecto prooxidante podría observarse también durante el proceso de digestión.

OBJETIVO

Estudiar el efecto que la presencia de α-tocoferol en una concentración del 2% en peso tiene en la evolución de aceite de soja refinado durante el proceso de digestión *in vitro.*

MATERIALES Y MÉTODOS

Las muestras estudiadas fueron un aceite de soja refinado y ese mismo aceite enriquecido con un 2% en peso de α-tocoferol. Ambas fueron sometidas a digestión *in vitro*, tomándose alícuotas antes y después del proceso. El estudio se llevó a cabo mediante Microextracción en Fase Sólida seguida de Cromatografía de Gases/Espectrometría de Masas.

RESULTADOS

Tanto en el aceite de soja sin enriquecer en α-tocoferol como en el enriquecido se producen reacciones de oxidación durante el proceso de digestión *in vitro*. Esto se deduce de un aumento en la concentración de productos de oxidación, tales como aldehídos, en las muestras digeridas en comparación con las mismas muestras sin digerir. Sin embargo, la concentración de estos compuestos es más alta entre los productos de digestión del aceite enriquecido en α-tocoferol.

CONCLUSIONES

La adición de α-tocoferol en un 2% al aceite de soja refinado durante la digestión *in vitro*, no solo no frena la oxidación del aceite generada en este proceso, sino que aumenta la extensión de ésta ejerciendo un efecto prooxidante.

AGRADECIMIENTOS

Este trabajo ha sido financiado por el Ministerio de Economía y Competitividad (MINECO AGL2015-65450-R) y por la UPV/EHU (UFI-11/21). Ana San Martín agradece al MINECO el contrato predoctoral.

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S Martín, A., Ibargoitia, M.L., Sopelana, P., Guillén, M.D.*

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INTRODUCCIÓN

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MATERIALES Y MÉTODOS

MUESTRAS: Aceite de soja refinado (ASR) y el mismo aceite de soja enriquecido con α-tocoferol un 2% en peso (ASR+2%α-T).

DIGESTIÓN *IN VITRO*: Siguiendo el modelo descrito por Versantwoort et al. [3] con alguna modificación. Como blanco se emplearon los reactivos utilizados para la digestión ("jugos"), sin mezclar con aceite, sometidos a este mismo proceso.

ESTUDIO DE LAS MUESTRAS: Mediante Microextracción en Fase Sólida seguida de Cromatografía de Gases/Espectrometría de Masas.

OBJETIVO: Estudiar el efecto que la adición de α-tocoferol en una concentración del 2% en peso tiene en la evolución de aceite de soja refinado durante el proceso de digestión *in vitro*.

RESULTADOS Y DISCUSIÓN

Tanto en el aceite de soja sin enriquecer en α-tocoferol como en el enriquecido se producen reacciones de oxidación durante el proceso de digestión *in vitro*. Esto se deduce de un aumento en la concentración de productos de oxidación, tal como se puede observar en las Figuras 1 y 2.

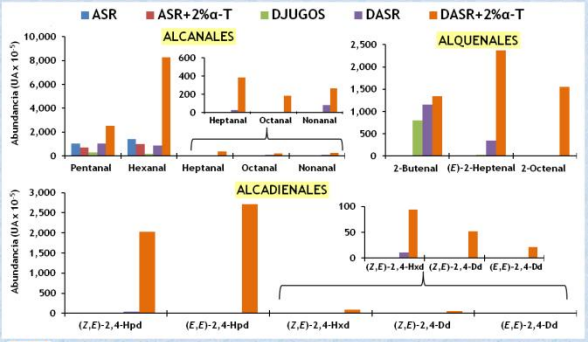

Figura 1: Abundancias de aldehídos en los aceites de partida (ASR y ASR+2%α-T), en los jugos sometidos al proceso de digestión (DJUGOS) y en los aceites digeridos (DASR y DASR+2%α-T), expresadas en unidades de área arbitrarias (UA) x 10^{-5}. Hpd: heptadienal; Hxd: hexadienal; Dd: decadienal.

En la Figura 1 se presentan los distintos tipos de ALDEHÍDOS detectados en las diferentes muestras y sus abundancias. Puede comprobarse que los aceites de partida (ASR y ASR+2%α-T) contienen ya algunos alcanales, como pentanal y hexanal.

Tras el proceso de digestión, se observa:

- Un aumento de la abundancia de los aldehídos presentes inicialmente, en el caso del aceite enriquecido en α-T (DASR+2%α-T).
- La generación de nuevos aldehídos tales como heptanal, (*E*)-2-heptenal o (*Z*,*E*)-2,4-heptadienal en ambos tipos de aceite, si bien en concentraciones más altas en el enriquecido en α-T (DASR+2%α-T) que en el aceite sin enriquecer (DASR).
- La generación de octanal, 2-octenal, (*E*,*E*)-2,4-heptadienal, y (*Z*,*E*)- y (*E*,*E*)-2,4-decadienales únicamente en el aceite con α-T añadido (DASR+2% α-T).

En la Figura 2 se presentan las abundancias de OTROS MARCADORES DE OXIDACIÓN, antes y después del proceso de digestión *in vitro*. Entre ellos, el 2-pentilfurano se encontraba ya presente en los aceites de partida.

Tras el proceso de digestión, se observa:

- Un aumento en la abundancia de 2-pentilfurano y la generación de 2-butilfurano, tanto en el aceite enriquecido como sin enriquecer en α-T.
- La generación de alcoholes y cetonas no detectadas en los aceites de partida, también en ambos tipos de aceite.
- Una mayor concentración de todos estos compuestos de oxidación en el aceite con α-T añadido en comparación con el aceite sin enriquecer.

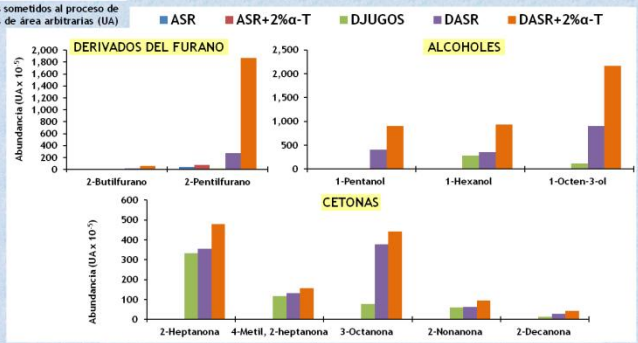

Figura 2: Abundancias de diversos marcadores de oxidación en los aceites originales (ASR y ASR+2%a-T), en los jugos sometidos al proceso de digestión (DJUGOS) y en los aceites digeridos (DASR y DASR+2%a-T), expresadas en unidades de área arbitrarias (UA) x 10^{-5}.

CONCLUSIONES

- Los componentes del aceite de soja refinado enriquecido con un 2% de α-T sufren mayor nivel de oxidación durante la digestión *in vitro* que los del aceite sin enriquecer.
- Se evidencia que esta adición no solo no frena la oxidación del aceite sino que aumenta la extensión de ésta ejerciendo un efecto prooxidante.

REFERENCIAS

[1] Evans, J.C., Kodali, D.R., Addis, P.B. (2002) J. Am. Oil Chem. Soc., 79, 47-51.
[2] Larsson, K., Cavonius, L., Alminger, M., Undeland, I. (2012) J. Agric. Food Chem., 60, 7556-7564.
[3] Versantwoort, C.H.M., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J.M., Sips, A.J.A.M. (2005) Food Chem. Toxicol., 43, 31-40.

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CONTRIBUTION VI

LOS ÉSTERES DE ESTEROLES INCORPORADOS A CIERTOS ALIMENTOS COMO COMPUESTOS SALUDABLES SE OXIDAN BAJO CONDICIONES OXIDATIVAS IGUAL QUE EL RESTO DE LÍPIDOS DANDO LUGAR A SUSTANCIAS TÓXICAS

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INTRODUCCIÓN

Los alimentos enriquecidos en esteroles vegetales gozan de gran popularidad en la sociedad actual debido a la capacidad de este tipo de compuestos para reducir los niveles de colesterol. Sin embargo, los esteroles se oxidan cuando se someten a calentamiento, dando lugar a la formación de derivados tóxicos. En la manufactura de ciertos alimentos se emplean ésteres de esteroles en lugar de esteroles, ya que son más solubles en lípidos. En este contexto se centra esta comunicación, referida a la oxidación de mezclas de ésteres de esteroles bajo condiciones oxidativas y su posible repercusión en la seguridad de tales alimentos cuando se calientan.

OBJETIVO

Estudiar el comportamiento de una mezcla de ésteres de esteroles vegetales cuando es sometida a condiciones de termooxidación, atendiendo tanto a los grupos esterilo como a los grupos acilo.

MATERIALES Y MÉTODOS

La muestra objeto de estudio fue una mezcla comercial de ésteres de esteroles vegetales. Ésta fue sometida a un proceso de termooxidación a 180 ºC en una estufa con aireación durante un período de 6 h. Las alícuotas tomadas antes y a lo largo del calentamiento fueron estudiadas por Resonancia Magnética Nuclear de Protón.

RESULTADOS

A lo largo del proceso de termooxidación se observa que los grupos esterilo se degradan, dando lugar a la formación de derivados oxidados. Asimismo, los grupos acilo, entre los cuales predominan las cadenas insaturadas, también sufren un proceso de termodegradación, dando lugar a diferentes compuestos de oxidación. Entre ellos cabe destacar la presencia de aldehídos, algunos de ellos tóxicos.

CONCLUSIÓN

Los ésteres de esteroles incorporados a alimentos se pueden oxidar en las dos partes que conforman su molécula por efecto del calentamiento a alta temperatura, lo que puede repercutir en la seguridad de estos alimentos.

AGRADECIMIENTOS

Este trabajo ha sido financiado por el Ministerio de Economía y Competitividad (MINECO AGL2015-65450-R) y por la UPV/EHU (UFI-11/21). Ana San Martín agradece al MINECO el contrato predoctoral.

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LOS ÉSTERES DE ESTEROLES INCORPORADOS A CIERTOS ALIMENTOS COMO COMPUESTOS SALUDABLES SE OXIDAN BAJO CONDICIONES OXIDATIVAS IGUAL QUE EL RESTO DE LÍPIDOS DANDO LUGAR A SUSTANCIAS TÓXICAS

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INTRODUCCIÓN

Los alimentos enriquecidos en esteroles vegetales gozan de gran popularidad en la sociedad actual debido a la capacidad de este tipo de compuestos para reducir los niveles de colesterol. Sin embargo, los esteroles se oxidan cuando se someten a calentamiento, dando lugar a la formación de derivados tóxicos [1]. En la manufactura de ciertos alimentos se emplean ésteres de esteroles en lugar de esteroles, ya que son más solubles en lípidos. En este contexto se centra esta comunicación, referida a la oxidación de mezclas de ésteres de esteroles bajo condiciones termooxidativas y su posible repercusión en la seguridad de tales alimentos cuando se calientan.

OBJETIVO

Estudiar el comportamiento de una mezcla de ésteres de esteroles vegetales cuando es sometida a condiciones de termooxidación, atendiendo tanto a los grupos esterilo como a los grupos acilo.

MATERIALES Y MÉTODOS

MUESTRA: La muestra objeto de estudio fue una mezcla comercial de ésteres de esteroles vegetales.

PROCESO DE TERMOOXIDACIÓN: Porciones de 4 g fueron sometidas a un proceso de termooxidación a 180 °C en una estufa con aireación durante 6 h, tomándose alícuotas cada hora para su análisis.

ESTUDIO: Las alícuotas tomadas antes y a lo largo del calentamiento fueron estudiadas por RESONANCIA MAGNÉTICA NUCLEAR DE PROTÓN como en estudios previos [2].

RESULTADOS Y DISCUSIÓN

EVOLUCIÓN DE GRUPOS ESTERILO Y DE GRUPOS ACILO

La Figura 1 muestra la evolución lo largo del tiempo de las concentraciones, en mmol/kg, de los grupos esterilo más abundantes en la muestra objeto de estudio y de los grupos acilo insaturados. Puede observarse que:

- Los grupos esterilo se degradan con el calentamiento, sobre todo los grupos β-sitosteril+campesteril, que son los que se encuentran en concentración más alta.
- Los grupos acilo, entre los cuales predominan las cadenas insaturadas, también sufren un proceso de termooxidación, que da lugar a un descenso en sus concentraciones a lo largo del tiempo.

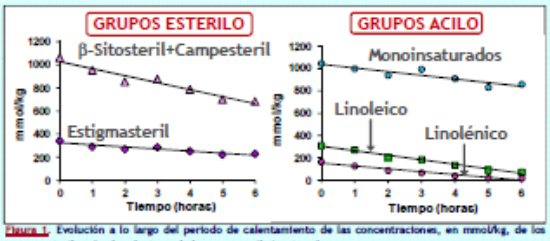

Figura 1. Evolución a lo largo del período de calentamiento de las concentraciones, en mmol/kg, de los grupos esterilo más abundantes y de los grupos acilo insaturados.

GENERACIÓN DE COMPUESTOS DE OXIDACIÓN

Como consecuencia de la degradación de grupos esterilo y de grupos acilo, se originan compuestos de oxidación derivados de ambos tipos de componentes. Éstos se muestran en la Figura 2. En ella se observa que:

- Entre los productos de oxidación de los grupos esterilo predominan los 7-hidroxiderivados.
- Entre los compuestos de oxidación de los grupos acilo se han encontrado aldehídos de diferente naturaleza, siendo los (*E*)-2-alquenales los más abundantes durante prácticamente todo el período de calentamiento. Es de destacar la presencia de algunos aldehídos oxigenados $\alpha\beta$–insaturados tóxicos tales como 4,5-epoxi- y 4-hidroxi-alquenales [3].

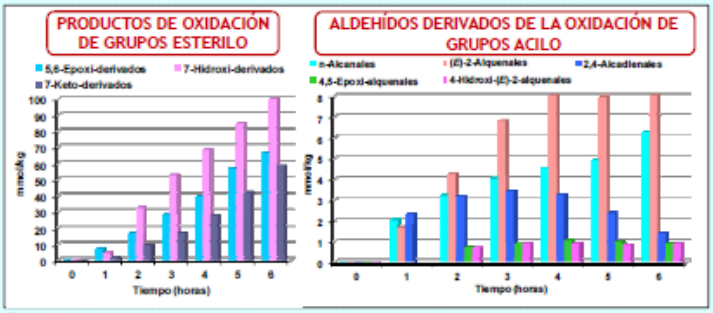

Figura 2. Evolución de las concentraciones de algunos productos de oxidación de grupos esterilo y de grupos acilo a lo largo del período de calentamiento, expresadas en mmol/kg.

CONCLUSIÓN

Los ésteres de esteroles incorporados a alimentos se pueden oxidar en las dos partes que conforman su molécula por efecto del calentamiento a alta temperatura. Esto puede repercutir en la seguridad de estos alimentos, ya que entre los productos de oxidación generados hay compuestos tóxicos.

REFERENCIAS
[1] García-Llatas, G.; Rodríguez-Estrada, M.T. (2011) Chem. Phys. Lipids, 164, 607-624.
[2] Sopelana, P.; Arizabaleta, I.; Ibargoitia, M.L.; Guillén, M.D. (2013) Food Chem., 141, 3357-3364.
[3] Guillén, M.D.; Goicoechea, E. (2008) Crit. Rev. Food Sci., 48, 119-136.

AGRADECIMIENTOS
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CONTRIBUTION VII

Advance of oxidation during *in vitro* digestion of edible oils of different unsaturation degree containing hydroperoxides. A study by SPME-GC/MS and by ^{1}H NMR

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Several works have shown that *in vitro* digestion promotes lipid oxidation (1,2), and that oxidation can be enhanced when the lipids subjected to digestion are slightly degraded (3). This is an important issue, since some oxidation products are toxic and, if they are formed in the gastrointestinal tract, they can be directly available to be absorbed. In this work, three slightly oxidized vegetable oils were subjected to a static *in vitro* digestion process; two of them were rich in polyunsaturated acyl groups and the third one in monounsaturated groups. The oils and the extracts of the corresponding digestates were studied by ^{1}H Nuclear Magnetic Resonance in order to examine the occurrence of oxidation reactions. With the same aim, the headspace of each of the oils and of their digestates was analyzed by Solid-Phase Microextraction followed by Gas Chromatography/Mass Spectrometry. The results obtained prove that, in line with previous findings (3), in all cases, irrespective of the oil composition, the oxidation process just initiated evolve during digestion, giving rise to the formation of different types of oxidation products, both volatile and non volatile; among these, aldehydes and compounds with conjugated hydroxy-dienes can be cited. Therefore, the ingestion of partially degraded lipids could give rise to the formation of directly absorbable oxidation products, so their consumption should be avoided.

Acknowledgements

This work has been supported by the Spanish Ministry of Economy and Competitiveness (MINECO AGL 2015-65450-R), by the Basque Government (IT916-16) and by UPV/EHU (UFI-11/21). A.S. Martin thanks the MINECO for a predoctoral contract

(1) Larsson, K.; Cavonius, L.; Alminger, M.; Undeland, I. (2012) J. Agric. Food Chem. 60, 7556-7564.
(2) Kenmogne-Domguia, H.B.; Moisan, S.; Viau, M.; Genot, C.; Meynier, A. (2014) Food Chem. 152, 146-154.
(3) Nieva-Echevarría, B.; Goicoechea, E.; Manzanos, M.J.; Guillén, M.D. (2017) Food Res. Int. 91, 171-182..

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International Conference INFOGEST Rennes, France April 4-6, 2017

ADVANCE OF OXIDATION DURING *IN VITRO* DIGESTION OF EDIBLE OILS OF DIFFERENT UNSATURATION DEGREE CONTAINING HYDROPEROXIDES. A STUDY BY SPME-GC/MS AND BY ^{1}H NMR

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INTRODUCTION

It has been shown that *in vitro* digestion promotes lipid oxidation [1], and that oxidation can be enhanced when the lipids subjected to digestion are slightly degraded [2]. This is an important issue, since some oxidation products are toxic and, if they are formed in the gastrointestinal tract, they can be directly available to be absorbed.

OBJECTIVE

To examine the occurrence of oxidation reactions and their extent during the *in vitro* digestion process of slightly oxidized vegetable oils of different unsaturation degree.

MATERIALS AND METHODS

STARTING OILS

Three slightly oxidized vegetable oils containing hydroperoxides: corn oil (COx) and virgin soybean oil (VSOx), rich in polyunsaturated acyl groups, and olive oil (OOx), rich in monounsaturated groups.

Molar % of the different types of acyl groups

	Linolenic	Linoleic	Oleic	Saturated
COx	0.3	49.5	34.2	16.0
VSOx	5.3	40.4	29.6	24.7
OOx	0.5	4.3	77.3	17.9

IN VITRO DIGESTION

This was performed following the model described by Versantwoort et al. [3] with some modifications [4]. A blank consisting of the reagents used for digestion without oil was also subjected to the whole procedure.

STUDY OF THE SAMPLES

The starting oils, the corresponding digestates (DCOx, DVSOx and DOOx) and the blank (B) were studied by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and by Headspace-Solid Phase Microextraction (HS-SPME) coupled to Gas Chromatography/Mass Spectrometry (GC/MS), as in previous studies [2].

RESULTS AND DISCUSSION

In line with previous findings [2], in all cases, the oxidation process just initiated evolves during digestion.

➢ *STUDY BY ^{1}H NMR → LIPIDIC FRACTION*

Figure 1 shows the spectral region where the signals of conjugated dienes associated to hydroperoxides (CD-OOH) appear, both in the starting oils and in the lipidic extracts obtained from their digestates. The evolution of the oxidation process is evidenced by:

- ❑ The diminution of the intensity of the signals of CD-OOH, especially of those with (*E,E*)-isomerism.
- ❑ The generation of conjugated hydroxy-dienes (CD-OH) in the case of the most unsaturated oils (COx and VSOx).

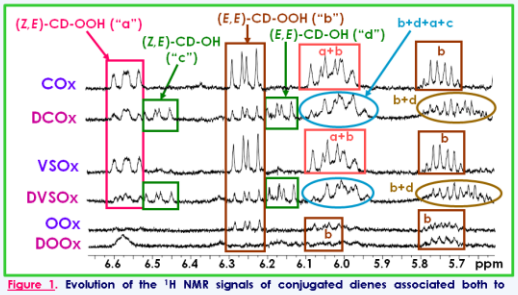

Figure 1. Evolution of the ^{1}H NMR signals of conjugated dienes associated both to hydroperoxides (CD-OOH) and to hydroxides (CD-OH) after the *in vitro* digestion process of the different oils studied.

➢ *STUDY BY HS-SPME-GC/MS → VOLATILE COMPOUNDS*

As a consequence of the oxidation progress, different types of aldehydes are generated and the concentrations of others already found in the starting oils increase. Some of these are shown in Figure 2. The nature of the aldehydes formed is in line with the composition in acyl groups of each studied oil [5].

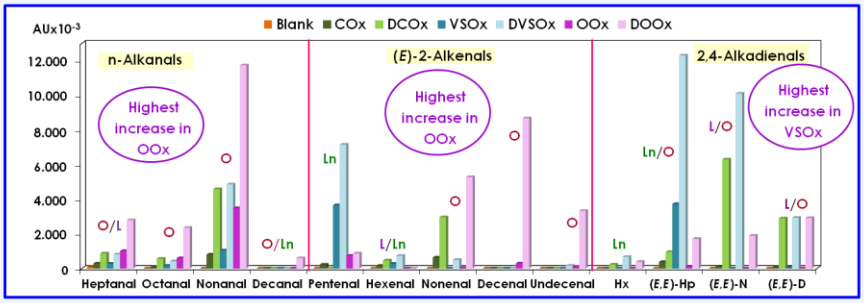

Figure 2. Abundances of aldehydes before and after the *in vitro* digestion process, expressed in arbitrary area units (AU), divided by 10^3. Main precursor acyl groups of each aldehyde, according to Guillén and Uriarte [5]: O-oleic; L-linoleic; Ln-linolenic. Hx: 2,4-Hexadienal; (*E,E*)-Hp: (*E,E*)-2,4-Heptadienal; (*E,E*)-N: (*E,E*)-2,4-Nonadienal; (*E,E*)-D: (*E,E*)-2,4-Decadienal.

CONCLUSIONS

- ❖ Irrespective of the oil composition, the oxidation process just initiated evolves during digestion, giving rise to the formation of different types of oxidation products, which can be directly absorbable.
- ❖ As it would be expected, the composition of the starting oil clearly influences the nature of the compounds generated.
- ❖ The ingestion of partially degraded lipids should be avoided, since in all cases reactive and/or toxic aldehydes are formed.

REFERENCES
[1] Larsson, K.; Cavonius, L.; Alminger, M.; Undeland, I. (2012) J. Agric. Food Chem. 60, 7556-7564.
[2] Nieva-Echevarría, B.; Goicoechea, E.; Manzanos, M.J.; Guillén, M.D. (2017) Food Res. Int. 91, 171-182.
[3] Versantwoort, C.H.M., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J.M., Sips, A.J.A.M. (2005) Food Chem. Toxicol., 43, 31-40.
[4] Nieva-Echevarría, B.; Goicoechea, E.; Manzanos, M.J.; Guillén, M.D. (2016) Food Chem. 211, 17-26.
[5] Guillén, M.D.; Uriarte, P.S. (2012) Food Chem. 131, 915-926.

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CONTRIBUTION VIII

Study by ^{1}H Nuclear Magnetic Resonance of the evolution of refined soybean oil with different oxidation status under *in vitro* digestion conditions

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Previous studies have shown that a slight level of lipids degradation in sunflower oil promotes their oxidation under *in vitro* digestion conditions (1). However, little is known about the influence of the oxidation status of food lipids on their evolution throughout the digestion process. Taking this into account, in this work, refined soybean oil with different degrees of oxidation has been subjected to a static *in vitro* digestion process, and its evolution has been followed by ^{1}H Nuclear Magnetic Resonance; the samples subject of study include the unoxidized oil, the oil with an incipient stage of lipids degradation containing hydroperoxides, and the oil with a more advanced oxidation status containing both hydroperoxides and different types of secondary oxidation products such as aldehydes. The results show that, during the digestion process, not only oxidation takes place but also the concentrations of the different types of oxidation products change when compared with the undigested samples, in a different way depending on their nature. In addition, the oxidation status of the oil has an influence on the lipolysis degree reached in the different samples, this being lower in the case of the most oxidized oil; this points to a loss of efficiency of the digestive enzymes presumably due to their interaction with certain oxidation products.

Acknowledgements

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References

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International Conference INFOGEST Rennes, France April 4-6, 2017

STUDY BY ^{1}H NUCLEAR MAGNETIC RESONANCE OF THE EVOLUTION OF REFINED SOYBEAN OIL WITH DIFFERENT OXIDATION STATUS UNDER *IN VITRO* DIGESTION CONDITIONS

Martin-Rubio, A.S., Sopelana, P., Ibargoitia, M.L., Guillén, M.D.*
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INTRODUCTION

Previous studies have shown that a slight level of lipids degradation in sunflower oil promotes their oxidation under *in vitro* digestion conditions [1]. However, little is known about the influence of the oxidation status of food lipids on their evolution throughout the digestion process.

OBJECTIVE

Studying by ^{1}H Nuclear Magnetic Resonance the influence of the oxidation status of refined soybean oil on its evolution under *in vitro* digestion conditions.

MATERIALS AND METHODS

STARTING OILS: Fresh refined soybean oil (RSO) and RSO with different degrees of oxidation: with hydroperoxides (RSOx1), and with hydroperoxides plus secondary oxidation products such as aldehydes (RSOx2).

IN VITRO DIGESTION: This was carried out following the model described by Versantwoort *et al.* [2] with some modifications [3].

ANALYSIS OF THE SAMPLES: The starting oils and the lipidic extracts of their corresponding digestates (D-RSO, D-RSOx1 and D-RSOx2) were studied by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR), as in previous works [1].

RESULTS AND DISCUSSION

➢ OCCURRENCE OF OXIDATION REACTIONS AND EVOLUTION OF THE OXIDATION PRODUCTS ALREADY PRESENT IN THE STARTING OILS

FRESH OIL

Figure 1 reveals that, after the *in vitro* digestion process of the fresh oil (RSO), small ^{1}H NMR spectral signals of conjugated dienes associated to hydroperoxides (CD-OOH) appear, indicating that oxidation has taken place to a certain extent.

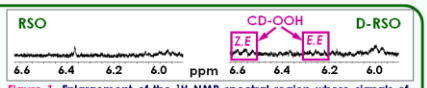

Figure 1. Enlargement of the ^{1}H NMR spectral region where signals of CD-OOH appear, taken from the spectra of RSO and D-RSO samples.

OXIDIZED OILS

Evolution of hydroperoxides (primary oxidation products)

Figure 2 shows that, when oxidized oils are subjected to *in vitro* digestion, changes in CD-OOH are also observed.

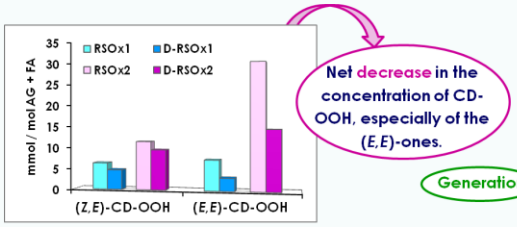

Figure 2. Evolution of the concentration of CD-OOH after the *in vitro* digestion process. AG: acyl groups; FA: fatty acids.

Evolution of aldehydes (secondary oxidation products)

Figure 3 evidences that not all aldehydes evolve in the same way during the *in vitro* digestion process.

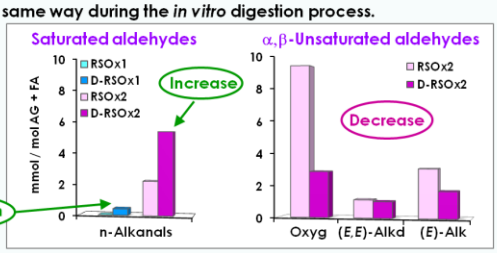

Figure 3. Evolution of the concentration of different types of aldehydes after the *in vitro* digestion process. AG: acyl groups; FA: fatty acids; Oxyg: oxygenated α,β-unsaturated aldehydes; (*E,E*)-Alkd: (*E,E*)-2,4-alkadienals; (*E*)-Alk: (*E*)-2-alkenals.

➢ INFLUENCE OF THE OIL OXIDATION STATUS ON THE LIPOLYSIS DEGREE

The oxidation status of the oil has an influence on the lipolysis degree reached in the different samples, this being lower in the case of the most oxidized oil.

	D-RSO	D-RSOx1	D-RSOx2
% TG*	22.1	27.7	35.7

*Molar percentage of intact triglycerides (TG)

CONCLUSIONS

- The *in vitro* digestion process provokes a certain oxidation of the fresh oil and causes a decrease in the concentration of hydroperoxides together with the generation of saturated aldehydes in the oxidized oils.
- The digestion of the most oxidized oil also entails a reduction in the concentration of unsaturated aldehydes, presumably due to their reaction with other components present in the digestive fluids, like enzymes.
- The considerable diminution observed in the amount of oxygenated α,β-unsaturated aldehydes after digestion evidences their high reactivity and their potential to interact with biological components.
- The lipolysis degree diminishes as the oxidation extent of the starting oil gets higher, suggesting a loss of efficiency of the digestive enzymes as the lipids degradation level increases.

REFERENCES
[1] Nieva-Echevarría, B.; Goicoechea, E.; Manzanos, M.J.; Guillén, M.D. (2017) Food Res. Int. 91, 171-182.
[2] Versantwoort, C.H.M., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J.M., Sips, A.J.A.M. (2005) Food Chem. Toxicol. 43, 31-40.
[3] Nieva-Echevarría, B.; Goicoechea, E.; Manzanos, M.J.; Guillén, M.D. (2016) Food Chem. 211, 17-26.

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This work has been supported by the Spanish Ministry of Economy and Competitiveness (MINECO AGL 2015-65450-R), by the Basque Government (IT916-16) and by UPV/EHU (UFI-11/21). A.S. Martin-Rubio thanks the MINECO for a predoctoral contract.

CONTRIBUTION IX

EFECTO DEL ENRIQUECIMIENTO DE ACEITE DE SOJA CON γ-TOCOFEROL SOBRE SU PROCESO DE OXIDACIÓN BAJO CONDICIONES DE ALMACENAJE ACELERADO. COMPARACIÓN CON α-TOCOFEROL

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– El estudio mediante Resonancia Magnética Nuclear de Protón del efecto del enriquecimiento de aceite de soja refinado con γ-tocoferol muestra que este compuesto acelera ligeramente la degradación del aceite bajo condiciones de almacenaje acelerado, más cuanto mayor es su concentración. Sin embargo, retrasa la formación de aldehídos.

INTRODUCCIÓN

Según diversos estudios, el γ-tocoferol (γ-T) puede ejercer un efecto antioxidante mayor que el α-tocoferol (α-T) dependiendo de su concentración. Además, la capacidad del γ-T para exhibir una acción prooxidante a concentraciones altas es menor que la del α-T [1]. Teniendo esto en cuenta, y dado que se conoce poco acerca del mecanismo de actuación del γ-T, en este trabajo se estudia, mediante Resonancia Magnética Nuclear de Protón (RMN de ^{1}H), el efecto de la adición de diferentes proporciones de γ-T en el proceso de termoxidación de aceite de soja refinado, considerando la formación de compuestos de oxidación primarios (hidroperóxidos) y secundarios (aldehídos). Este efecto se compara con el observado cuando el aceite se enriquece con α-T.

MATERIALES Y MÉTODOS

Las muestras objeto de estudio fueron aceite de soja refinado (ASR) sin enriquecer y enriquecido con un 0,2 y un 2% en peso de γ-T. Todas ellas se mantuvieron bajo condiciones de almacenaje acelerado en estufa a 70ºC, tomándose alícuotas periódicamente hasta su completa polimerización, y fueron estudiadas antes y a lo largo del proceso de termoxidación mediante RMN de ^{1}H.

RESULTADOS Y DISCUSIÓN

La adición de γ-T acelera, aunque en menor medida que el α-T, la degradación del aceite, provocando una generación más rápida de (*Z,E*)-hidroperóxidos, sobre todo en la muestra con mayor concentración de γ-T. Sin embargo, en los aceites enriquecidos el tiempo necesario para

alcanzar la concentración máxima de hidroperóxidos es mayor, de manera que su descomposición, y en consecuencia la aparición de productos de oxidación secundarios como aldehídos, se producen más tarde que en el aceite sin enriquecer. Este efecto es especialmente notable en la muestra con mayor cantidad de γ-T, en la que también la polimerización total se ralentiza. El retraso en la generación de aldehídos debido a la adición de γ-T contrasta con lo observado en muestras de aceite de soja refinado enriquecido con las mismas proporciones de α-T, en las cuales la formación de aldehídos se acelera [2].

CONCLUSIONES

El enriquecimiento de ASR con γ-T en las proporciones ensayadas reduce ligeramente su estabilidad oxidativa, pero una vez iniciado el proceso de oxidación, éste transcurre más lentamente cuanto mayor es la concentración de γ-T.

El γ-T y el α–T afectan muy diferentemente a la generación de aldehídos.

Es necesario considerar el proceso de oxidación globalmente para poder valorar adecuadamente el efecto de cualquier compuesto con potencial capacidad antioxidante, pues de lo contrario se pueden obtener conclusiones erróneas.

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REFERENCIAS

[1] Seppanen, C M. et al. (2010). J. Am. Oil Chem.Soc. 87: 469-481.

[2] Martin, A. S. et al. (2015). En Actas del VIII Congreso CYTA/CESIA (p. 70), Badajoz, España.

EFECTO DEL ENRIQUECIMIENTO DE ACEITE DE SOJA CON γ-TOCOFEROL SOBRE SU PROCESO DE OXIDACIÓN BAJO CONDICIONES DE ALMACENAJE ACELERADO. COMPARACIÓN CON α-TOCOFEROL

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INTRODUCCIÓN

Según algunos estudios, el γ-tocoferol (γT) puede ejercer un efecto antioxidante mayor que el α-tocoferol (αT) dependiendo de su concentración. También se ha descrito que la capacidad del γT para exhibir una acción prooxidante a concentraciones altas es menor que la del αT [1]. Por todo ello, y dado que se conoce poco acerca del mecanismo de actuación del γT, se considera de interés estudiar su efecto sobre el proceso de oxidación de aceites vegetales enriquecidos en este compuesto.

OBJETIVOS

- Estudiar mediante Resonancia Magnética Nuclear de Protón (RMN de ^{1}H) el efecto de la adición de diferentes proporciones de γT en el proceso de almacenaje acelerado de aceite de soja, considerando la formación de compuestos de oxidación primarios (hidroperóxidos) y secundarios (aldehídos).
- Comparar este efecto con el observado cuando el aceite se enriquece con αT.

MATERIALES Y MÉTODOS

ACEITES OBJETO DE ESTUDIO
Aceite de soja refinado (ASR)
ASR + 0,2% en peso de γT (ASR+0,2 γT)
ASR + 2% en peso de γT (ASR+2 γT)
ASR + 0,2% en peso de αT (ASR+0,2αT)
ASR + 2% en peso de γT (ASR+2αT)

ALMACENAJE ACELERADO
En una estufa a 70ºC con aireación, hasta la completa polimerización del aceite.

ANÁLISIS DE LAS MUESTRAS
Los aceites de partida y las muestras tomadas a lo largo del proceso de almacenaje acelerado fueron estudiadas por RMN de ^{1}H, como en estudios previos [2].

RESULTADOS Y DISCUSIÓN

EFECTO SOBRE LA GENERACIÓN Y EVOLUCIÓN DE LOS PRODUCTOS DE OXIDACIÓN PRIMARIOS: HIDROPERÓXIDOS

- La adición de γT acelera en una primera etapa la degradación del aceite, provocando una generación más rápida de (Z,E)-hidroperóxidos ((Z,E)-CD-OOH), sobre todo en la muestra con la concentración más alta de γT (ASR+2γT) (ver Figura 1).

Sin embargo, cuanto mayor es la cantidad de γT, más tiempo se necesita para alcanzar la concentración máxima de hidroperóxidos; en consecuencia, la polimerización total de la muestra ASR+2γT se produce más tarde.

- La adición de αT también acelera la formación de (Z,E)-CD-OOH, más cuanto mayor es el nivel de enriquecimiento. Sin embargo, a diferencia de lo observado en los aceites con γT añadido, este efecto se mantiene en el tiempo, de modo que los hidroperóxidos alcanzan su concentración máxima antes que en el aceite sin enriquecer.

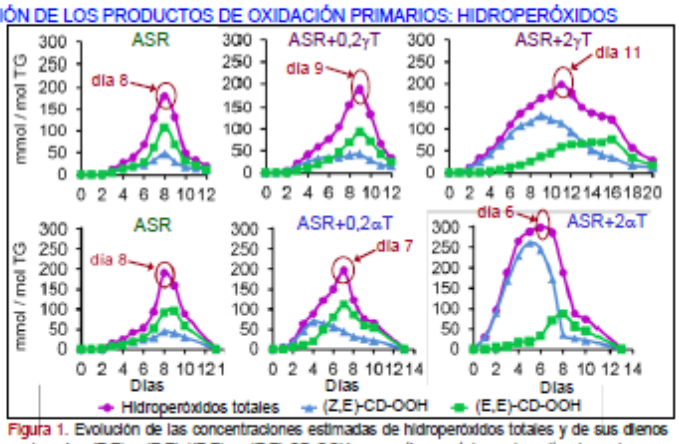

Figura 1. Evolución de las concentraciones estimadas de hidroperóxidos totales y de sus dienos conjugados (Z,E) y (E,E) ((Z,E)- y (E,E)-CD-OOH, respectivamente) en el aceite sin enriquecer (ASR) y en los aceites con γT y αT añadido a lo largo del proceso de almacenaje acelerado, en mmol/mol TG.

EFECTO SOBRE LA GENERACIÓN Y EVOLUCIÓN DE PRODUCTOS DE OXIDACIÓN SECUNDARIOS: ALDEHÍDOS

- El enriquecimiento de ASR con γT provoca un retraso en la aparición de productos de oxidación secundarios tales como aldehídos. Éste es mayor cuanto más alta es la concentración de γT (ver Figura 2).
- Este efecto es opuesto al observado en los aceites enriquecidos con αT. En estos últimos, en consonancia con la evolución de hidroperóxidos (ver Figura 1), la formación de aldehídos se acelera [3].

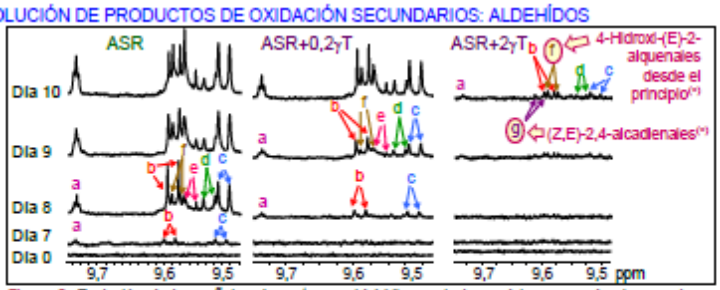

Figura 2. Evolución de las señales de protones aldehídicos a lo largo del proceso de almacenaje acelerado de ASR y de los aceites enriquecidos con γT. a: n-alcanales; b: 4-hidroperoxi-(E)-2-alquenales; c: (E)-2-alquenales; d: (E,E)-2,4-alcadienales; e: 4,5-epoxi-alquenales; f: 4-hidroxi-(E)-2-alquenales; g: (Z,E)-2,4-alcadienales. (*) Coincide con lo observado en el caso del αT [3].

CONCLUSIONES

- El enriquecimiento de ASR tanto con γT como con αT en las proporciones ensayadas reduce su estabilidad oxidativa.
- El γT y el αT afectan de forma muy diferente al proceso de oxidación de ASR. La adición de γT acelera la degradación del aceite en un principio, pero luego ésta se ralentiza en relación con el aceite sin enriquecer y se retrasa la generación de productos de oxidación secundarios, más cuanto mayor es la concentración de γT. Sin embargo, cuando se añade αT, el proceso de oxidación transcurre más rápidamente durante todo el periodo de almacenaje acelerado.
- Es necesario considerar el proceso de oxidación globalmente para poder valorar adecuadamente el efecto de cualquier compuesto considerado como antioxidante, pues de lo contrario se pueden obtener conclusiones erróneas.

REFERENCIAS
[1] Seppanen, C. M. et al. (2010). J. Am. Oil Chem.Soc. 87: 469-481.
[2] Guillén, M. D.; Uriarte, P .S. (2012). Food Chem. 134:162-172.
[3] Martín, A. S. et al. (2015). En Actas del VIII Congreso CYTA/CESIA (p. 70), Badajoz, España.

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CONTRIBUTION X

Antioxidant effect of lysine on soybean oil evidenced by 1H Nuclear Magnetic Resonance

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The antioxidant effect that proteins and amino acids may have in oils and emulsions is a very interesting topic due to the implications that lipid oxidation processes have in food nutritional, quality and safety parameters (1). Actually, some authors have shown that under certain oxidative conditions, amino acid residues are capable to slowdown lipid oxidation processes (2). In spite of all the multiple works dealing with this issue, there is not a single study which monitoring lipid peroxidation in a global way in a food model system.

Thus, in this work the effect of L-lysine on the evolution of refined soybean oil submitted to oxidative conditions has been studied. The degradation of the oil acyl groups of the oil, and the subsequent generation of oxidation compounds the oil have been studied. With this purpose refined soybean oil (RSO) and this same oil containing a 2% in weight of this amino acid have been heated under continuous stirring in magnetic stirrer at 70ºC simulating accelerate storage conditions. The evolution of the was monitored by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR).

LYS meaningfully slowed down the degradation of acyl groups of the oil as well as the generation of primary, and in turn, secondary oxidation compounds. It must be mentioned that the amount of α, β-unsaturated aldehydes detected was also lower in the oil with LYS added. This work shows that lysine addition to RSO slows down lipid oxidation processes under these oxidative conditions. (POSTER PRESENTATION)

REFERENCES:

(1) Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α, β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition*, *48*, 119-136

(2) Hidalgo, F. J., León, M. M., & Zamora, R. (2006). Antioxidative activity of amino phospholipids and phospholipid/amino acid mixtures in edible oils as determined by the Rancimat method. *Journal of agricultural and food chemistry*, *54*(15), 5461-5467.

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Antioxidant effect of lysine on soybean oil evidenced by ^{1}H Nuclear Magnetic Resonance

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INTRODUCTION

The antioxidant effect of proteins and peptides, which is a well-known issue (1), constitutes a very interesting topic in food systems due to the implications that lipid oxidation processes have in food nutritional, organoleptic and safety parameters. In this context, although many studies have been conducted to assess the antioxidant effect of a great variety of proteins and protein hydrolysates (1,2), much less is known about the action that specific amino acids can have in food lipids. In addition, the assessment of the antioxidant effect of protein, peptides and amino acids is usually carried out by using unspecific methodologies such as peroxide value or TBARS assay, which provide scarce information about the oxidation course (2,3).

OBJECTIVE

Studying by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) the effect of L-lysine on the oxidation process of refined soybean oil, paying attention to both acyl groups degradation and oxidation products generation

MATERIALS AND METHODS

Samples: Refined soybean oil (RSO) and this same oil containing a 2% in weight of lysine (RSO+LYS).

Oxidative conditions: Continuous stirring in 5 cm diameter beakers at 70 °C, simulating accelerate storage conditions.

Study of the samples: Aliquots of the oils were taken throughout the accelerate storage process to monitor their evolution by ^{1}H NMR as in previous studies (4).

RESULTS AND DISCUSSION

Evolution of the several kinds of acyl groups

Figure 1 shows that both in RSO and in RSO+LYS the molar percentage of the unsaturated acyl groups decreases very slowly during a first longer stage, but very quickly afterwards. The addition of a 2% of lysine to the oil markedly delays this process, since it is not up to day 22 when a sudden decrease of the molar percentages of linolenic and linoleic groups occurs. Therefore, lysine notably makes the first oxidation phase longer compared to RSO, although a similar evolution through time is observed.

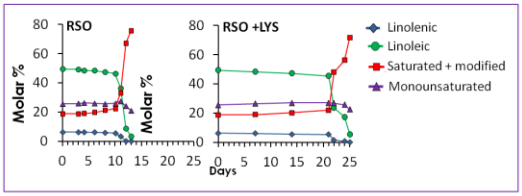

Figure 1: Molar percentage evolution of acyl groups in RSO and RSO+LYS throughout the oxidative process

Oxidation products generation

As Figure 2 reveals, the generation of hydroperoxides (primary oxidation products) is also considerably retarded in the lysine-enriched oil; this goes in line with the evolution of acyl groups and causes a delay in the generation of secondary oxidation products such as aldehydes (see Figure 3). It must be highlighted the lower concentration of unsaturated aldehydes detected in the oil with lysine added.

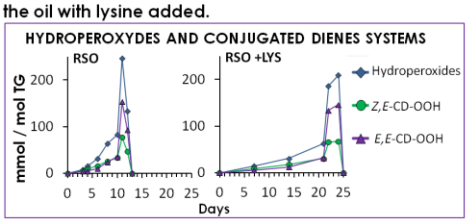

Figure 2: Generation and evolution of hydroperoxides and their associated conjugated diene systems, expressed in mmol/mol TG, throughout the accelerated storage process.

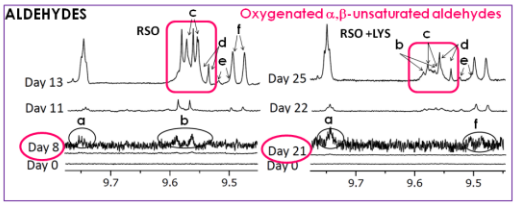

Figure 3: Evolution of the aldehydic proton signals. Aldehydes identified: n-alkanals (a), 4-hydroperoxy-(*E*)-2-alkenals (b), 4-hydroxy-(*E*)-2-alkenals (c), 4,5-epoxy-alkenals (d), (*E,E*)-2,4-alkadienals (e) and (*E*)-2-alkenals (f).

CONCLUSIONS

- The addition of a 2% of lysine to refined soybean oil exerts a clear antioxidant effect under accelerated storage conditions by retarding noticeably the oil acyl groups degradation and the generation of primary oxidation compounds
- The presence of lysine not only causes a delay in the generation of aldehydes, but also a marked reduction in the concentration of toxic oxygenated α,β-unsaturated ones.
- These findings not only prove the feasibility of lysine as a potential antioxidant ¿to be used? in lipidic food systems but also could help in adjusting the addition of exogenous antioxidants to foodstuffs containing this type of amino acid.

REFERENCES

[1] Elias, R.J.; Kellerby, S.S.; Decker, E.A. (2008). *Crit Rev Food Sci Nutr*, 48, 430-441.
[2] Al-Shamsi, K.A.; Mudgil, P.; Hassan, H.M.; Maqsood, S. (2018). *J Dairy Sci*, 101, 47-60
[3] Cheetangdee, N.; Benjakul, S. (2015). *J Sci Food Agric*, 95, 1461-1468.

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CONTRIBUTION XI

La adición de L-cisteina permite alargar la vida útil del aceite de soja

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Se estudió el proceso de oxidación del aceite de soja en presencia o ausencia de un 2% de L-cisteína a 70ºC con agitación mediante resonancia magnética nuclear de protón (^{1}H RMN). Se observó que este aminoácido retrasa la oxidación del aceite y modifica la ruta de oxidación de algunos epóxidos.

Key words – L-cisteína, aceite de soja, oxidación de lípidos, ^{1}H NMR.

INTRODUCCIÓN

Los procesos de autoxidación lipídica en alimentos es un proceso que conlleva consecuencias negativas en términos de calidad y seguridad alimentaria [1]. La industria recurre al uso de compuestos clásicamente considerados como antioxidantes [2] con el fin de minimizar el avance de la oxidación de lípidos. En este sentido y según la literatura, algunos aminoácidos también podrían ejercer actividad antioxidante como se ha demostrado en diferentes sistemas de modelo [3]. En esta comunicación se propone el estudio de un aminoácido, la L-cisteína (CYS) como alternativa al uso de los antioxidantes clásicos comúnmente empleados. De hecho, entre las tendencias más recientes que la industria alimentaria desea implementar se contempla el uso de ingredientes de fuentes naturales que sean capaces de actuar como antioxidantes en sistemas alimentarios reales. Dentro de este contexto el uso de este aminoácido como antioxidante podría encajar.

Teniendo en cuenta lo anterior, con objeto de valorar el efecto de la presencia de un aminoácido, como la CYS en el proceso de oxidación del aceite de soja, se propuso el estudió de la oxidación del aceite de soja presencia o ausencia de un 2% de CYS bajo condiciones de almacenamiento acelerado a 70ºC con agitación empleando ^{1}H RMN. Esta es una técnica muy interesante ya que permite observar la tanto la degradación de los componentes principales de los aceites, así como la generación de una amplia variedad de compuestos de oxidación, ofreciendo así visión global de estos procesos de oxidación lipídica [1].

MATERIALES Y METODOS

Las muestras de estudio fueron aceite de soja o aceite de soja enriquecido un 2% en peso con CYS. Muestras de 10 g (en vasos de precipitados) se colocaron en un agitador magnético múltiple con calefacción y se calentaron a 70ºC. Se tomaron alícuotas periódicamente durante todo el proceso de oxidación del aceite para su estudio por ^{1}H RMN.

RESULTADOS Y DISCUSIÓN

Mediante ^{1}H RMN se observó que la presencia de CYS en el aceite retrasa notablemente la degradación de los grupos acilo linoleico y linolénico.

La presencia de CYS, también retrasa la aparición de los primeros hidroperóxidos. Además, la velocidad de generación de estos compuestos de oxidación primarios también es menor que la observada en el aceite calentado solo, la cual se produce en todos los casos en paralelo a la degradación de los grupos acilo insaturados del aceite ya mencionada.

Con respecto a epóxidos y aldehídos, su generación también fue retrasada por la adición de CYS al aceite.

Debe señalarse que la presencia de este aminoácido azufrado no solo retrasó la degradación grupos acilo insaturados del aceite y los productos de oxidación, sino que también modificó la ruta de oxidación de algunos epóxidos generados durante el proceso de oxidación y, en consecuencia, el tipo de compuestos de oxidación generados a partir de ellos.

CONCLUSIONES

Bajo las condiciones de este estudio se ha observado que la presencia de CYS en el aceite de soja puede mejorar su estabilidad oxidativa y, por lo tanto, actuar como antioxidante.

Por medio de ^{1}H NMR se demuestra que la presencia de este aminoácido retrasa la degradación de los grupos acilo poliinsaturados en aceite y, en consecuencia, de los productos de oxidación originados a partir de ellos.

Además, también fue posible observar que la presencia de CYS modifica la ruta de oxidación de algunos epóxidos generados a través de este proceso, así como el tipo de productos de oxidación que se generan a partir de ellos. Este es un hecho que cabe destacar, ya que los epóxidos generados durante la autoxidación de lípidos constituyen uno de los grupos de compuestos tóxicos que se forman en mayor proporción durante este proceso [1].

AGRADECIMIENTOS

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REFERENCIAS

[1] Food Chem. *245*: 312-323 (2018).
[2] Food Chem Toxicol, 51: 15-25 (2013).
[3] Food Chem. *54*: 5461-5467 (2006).

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LA ADICIÓN DE L-CISTEÍNA PERMITE ALARGAR LA VIDA ÚTIL DE ACEITE DE SOJA

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INTRODUCCIÓN

Los procesos de oxidación lipídica repercuten negativamente en la calidad de los alimentos e incluso en su seguridad [1]. Con el fin de evitar, o al menos reducir este tipo de reacciones, la industria alimentaria recurre al uso de compuestos con capacidad antioxidante. Sin embargo, algunos de los más comúnmente utilizados, tales como el alfa-tocoferol, pueden llegar a exhibir un efecto prooxidante bajo ciertas condiciones [2]. En este contexto, otro tipo de componentes que forman parte de la dieta y que pueden presentar potencial antioxidante son los aminoácidos [3]. Sin embargo, se conoce poco acerca del mecanismo de acción de este tipo de compuestos en sistemas alimentarios.

OBJETIVO

Estudiar mediante **Resonancia Magnética Nuclear de Protón** (RMN de ^{1}H) el **efecto de L-cisteína (CYS)** sobre el proceso de **oxidación** de **aceite de soja**, atendiendo tanto a la degradación de grupos acilo como a la generación de compuestos de oxidación primarios y secundarios.

MATERIALES Y MÉTODOS

MUESTRAS: Aceite de soja refinado (**ASR**) y este mismo aceite con un 2% en peso de L-cisteína (**ASR+CYS**).
PROCESO DE OXIDACIÓN: A 70 °C en placa calefactora con agitación hasta la polimerización total del aceite.
ANÁLISIS DE LAS MUESTRAS: Toma de alícuotas periódicamente durante el proceso de oxidación para su estudio por RMN de ^{1}H.

RESULTADOS Y DISCUSIÓN

❑ **Reducción de la velocidad de degradación de los grupos acilo insaturados del aceite en presencia de CYS**

Tal como se muestra en la Figura 1, la presencia de CYS en aceite de soja en una proporción del 2% en peso, ralentiza el proceso de degradación de todos los tipos de grupos acilo insaturados, de tal manera que éstos permanecen casi sin modificar el doble de tiempo que en el aceite sin CYS. En consecuencia, el tiempo necesario para alcanzar la polimerización total del aceite se extiende de 7 a 11 días. Además, el nivel de degradación de grupos linoleico y oleico al final del proceso de oxidación es algo menor en el aceite enriquecido con CYS.

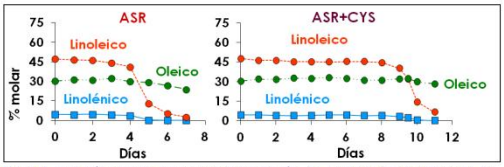

Figura 1. Evolución, durante el proceso de oxidación, de los porcentajes molares de los distintos tipos de grupos acilo insaturados presentes en los aceites estudiados.

❑ **Ralentización en la generación de hidroperóxidos**

La Figura 2 pone de manifiesto que la evolución de la concentración de hidroperóxidos está en concordancia con la de grupos acilo en ambos aceites. Así, aun cuando los hidroperóxidos se detectan solo un día más tarde en **ASR+CYS** que en **ASR**, la concentración de éstos crece a una velocidad menor en presencia de CYS.

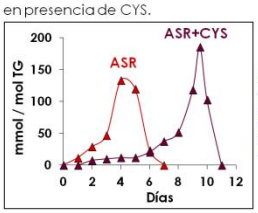

Figura 2. Evolución, durante el proceso de oxidación, de la concentración de hidroperóxidos, expresada en mmol/mol triglicérido (TG), en los dos aceites estudiados

❑ **Retraso en la aparición de aldehídos (compuestos secundarios)**

La ralentización en el proceso de generación de hidroperóxidos por la presencia de CYS, hace que la descomposición de éstos se produzca más tarde, lo que provoca un retraso en la generación de productos de oxidación secundarios tales como aldehídos (ver Figura 3). Además, cabe destacar que, **en presencia de CYS**, se observan **concentraciones más bajas de aldehídos**, especialmente de los aldehídos oxigenados α,β-insaturados, conocidos tóxicos [4].

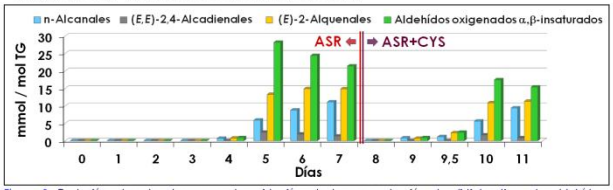

Figura 3. Evolución, durante el proceso de oxidación, de la concentración de distintos tipos de aldehídos, expresada en mmol/mol triglicérido (TG), en los dos aceites estudiados.

CONCLUSIONES

- ❑ En las condiciones de este estudio, la presencia de **CYS en una proporción del 2%** ejerce un **efecto antioxidante sobre el aceite de soja**, **alargando su vida útil**, preservando durante más tiempo nutrientes esenciales como ciertos ácidos grasos poliinsaturados y provocando una **reducción** en la concentración de **aldehídos tóxicos**.
- ❑ La **cisteína** podría constituir una **alternativa a otro tipo de antioxidantes**, bien sintéticos o bien naturales, en **alimentos lipídicos**.

REFERENCIAS
[1] Esterbauer, H. (1993). Am. J. Clin. Nutr. 57: 779S-785S.
[2] Martín-Rubio, A. S. et al. (2018) Food Chem. 245: 312-323.
[3] Ahmad, M. M. et al. (1983). J. Am. Oil Chem. Soc. 60: 837-840.
[4] Guillén, M. D. and Goicoechea, E. (2008). Crit. Rev. Food Sci. Nutr. 48: 119-136.

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